# THE DEVELOPMENT AND EVALUATION OF MOLECULAR BIOLOGICAL TECHNIQUES TO DETECT SOLID TUMOUR CELLS IN PERIPHERAL BLOOD.

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## ABSTRACT

Sensitive methods to detect solid tumour cells in peripheral blood are developed to facilitate clinical identification of micrometastases.

Current understanding of metastatic processes and the clinical significance of these processes is discussed. The study of circulating solid tumour cells in peripheral blood is reviewed from an historical perspective. Newer molecular and cell biological techniques which may facilitate more reliable tumour evaluation are reviewed with special reference given to polymerase chain reaction (PCR).

By combining the techniques of reverse transcription (RT) and PCR, it may be possible to utilise tissue specific gene expression as a means of detecting solid tumour cells in blood. This is demonstrated by the development of a sensitive RT/PCR technique to detect tyrosinase mRNA to identify melanoma cells in blood. Whilst a pilot study of detected tyrosinase mRNA expression in the blood of 4 of 9 advanced melanoma patients, a follow-up study was only able to demonstrate tyrosinase detection in 3 of 24 patients. This study is compared to other published series which have emulated this technique.

Cytokeratin expression to detect circulating carcinoma cells in blood is evaluated by two techniques. Firstly, a technique of cytospinning and double antibody labelling is developed and clinically evaluated with positive results in 2 of 20 breast cancer patients, 1 of 19 colorectal cancer patients, and 0 of 14 patients with other epithelial cancers. Secondly, a sensitive RT/PCR technique to detect cytokeratin 8 mRNA is developed. However cytokeratin 8 mRNA is shown to be expressed at low levels in normal blood. This result is compared with

other published RT/PCR techniques which have used other cytokeratin filaments as tumour markers.

CD44 variant expression has been raised as a possible discriminator of normal haemopoietic tissue and malignant tissue. The "epithelial" variant and the "metastatic" variant of CD44 are evaluated as RT/PCR targets. The large number of variants of CD44 detected by this technique demonstrates the complexity of the CD44 molecule. CD44 variant expression as a reliable tumour marker is discussed in light of reported intermittent variant CD44 expression by haemopoietic cells under non-malignant immunogenic stimulation.

A model to critically examine RT/PCR tumour cell detection techniques is proposed. RT/PCR techniques eventually may become part of routine cancer evaluation, and may redefine the terms "remission" and "cure", but only if such techniques are robust and reliable.

## STATEMENT BY THE AUTHOR

This thesis contains no material which has previously been accepted for the award of any other degree or diploma in any University and to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to the thesis being made available for photocopying and loan if applicable if accepted for the award of the degree.

Kenneth Brian Pittman

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# 1. INTRODUCTION

## 1.1 THE THESIS

### 1.1.1 The limitations of cancer diagnosis

The majority of cancer related death is due to the effects of metastatic disease [Sugarbaker *et al.* 1982, Fidler and Balch 1987]. In the majority of cases this is due to haematogenous or bloodborne metastases [Schirrmacher 1985]. Tumour cells frequently spread from the primary tumour via lymphatics or the bloodstream and eventually develop metastatic colonies in secondary organ sites. The new colonies (or*metastases*) grow and eventually compromise the local anatomy leading to organ failure and death. Metastasis formation is therefore the most life-threatening aspect of cancer [Frost and Levin 1992].

The accurate diagnosis of small volume malignant disease has become one of the great challenges of modern oncology. Standard or classical methods of diagnosing the presence of tumours are limited by the lack of means to detect very small numbers of tumour cells. By the time a tumour reaches 1 $cm^3$ in size (the lower limit of detection by routine evaluation) it may contain greater than $10^9$ tumour cells [DeVita 1993], and it has been calculated that metastatic disease may develop from tumours such as breast cancer when the primary tumour is less than 0.125 $cm^3$ [Koscielny *et al.* 1985]. As the vast majority of adult malignancy is incurable once gross metastases have developed, there is clearly a need for more sensitive tools to detect very small numbers of tumour cells, so that early treatment of micrometastatic disease in the form of adjuvant therapy may be properly evaluated.

### 1.1.2 Implications of early cancer diagnosis

The early diagnosis of metastatic disease has critical implications for the management of cancer generally. In all solid tumours, prognosis is greatly influenced by the stage of the disease at the time of diagnosis, and the presence of distant metastatic disease invariably carries a worse prognosis. Using breast cancer as an example, when the disease is small and confined to the breast (stage 1), following adequate local treatment with either mastectomy or lumpectomy and local radiotherapy, the 5 year disease free survival rate is about 80 %, whereas for disease which has spread to axillary lymph nodes (stage 2), the overall 5 year disease free survival rate falls to < 50 % with prognosis inversely proportional to number of nodes involved. For more advanced stage disease, the 5 year disease free survival rates are <20 % [Danforth 1992, Antman 1994, Livingston 1994] .

Treatment decisions such as the use of surgery, radiotherapy or chemotherapy for early stage breast cancer patients, usually rely on the accurate assessment of disease stage. Presently, decisions regarding the use of systemic treatments such as cytotoxic chemotherapy and hormonal manipulation is based upon assessment of risk of micrometastatic disease, which is closely related to disease stage [Todd *et al.* 1987]. Hence, the greater the likelihood of micrometastases being present, the worse the prognosis, and the greater the likelihood that systemic treatment will be instigated. Like the majority of cancers, few patients die because of the primary tumour in the breast, but die due to the effects of metastatic breast cancer [Harris *et al.* 1993]. The management of metastatic disease (either gross or micrometastatic) is therefore paramount to the management of cancer overall.

### 1.1.3 Bloodborne metastases

That tumour cells may spread to other parts of the body via the blood stream has been acknowledged for some 150 years [Langenbeck 1841]. The more basic understanding as to how these cells manage to lose their attachment from the primary tumour, invade and survive in the vascular compartment, evade the host's immunological defense mechanisms, exit the vascular compartment, lodge in a secondary site and finally grow as a secondary deposit and possibly metastasise once again has taken a considerable time to even begin to be unravelled. It is believed that a specialised sub-population of tumour cells from the primary tumour evolves quite early in the lifespan of the tumour. This sub-population of tumour cells is believed to possess particular characteristics required for metastatic spread [Fidler 1990].

At some stage in the history of most instances of metastatic cancer, tumour cells enter and spend some time circulating in the vascular compartment. In the clinical setting, whilst primary tumour cells, metastatic tumour cells in local lymph node groups, and even metastatic tumour cells in distant metastatic sites are, with the aid of the surgeon, relatively easy to access and therefore study, viable tumour cells which are circulating in the blood have proven difficult to reliably identify and therefore study. There is a relative paucity of information regarding the genetic and phenotypic changes associated with this important part of the metastatic process. Hence, the relationship between circulating tumour cells and the development of secondary disease is not yet completely understood.

### 1.1.4 Detection of bloodborne metastases

The possibility of being able to reliably detect small numbers of metastatic tumour cells as they circulate in the peripheral blood is particularly appealing to confirm the presence of tumour tissue outside the primary site, possibly predicting future establishment of gross metastatic disease and therefore influencing prognosis. Such information may therefore have significant therapeutic implications. The approach also has the advantage of screening a readily accessible body fluid, which can be examined in conjunction with other blood tests performed on a routine basis.

This work attempts to develop and evaluate novel molecular/cell biological methods of detecting circulating solid tumour cells in the perpiheral blood. These methods are based on the assumption that cells of non-haematopoietic origin may express certain genes that are not usually expressed by normal blood cells, and these differences in gene expression may allow tumour cells to be readily detected in a background of normal blood cells.

### 1.1.5 Aim of the thesis

The aim of the thesis is to develop and clinically evaluate molecular biological methods, which rely upon differences in tumour cell gene expression, to reliably detect solid tumour cells in peripheral blood.

To put this work into context, it is necessary to review the curent understanding of the process of metastasis, especially that work which deals specifically with metastatic tumour cells circulating in the blood, and also review the present applications of molecular and cell biological techniques which may be used to evaluate patients with

cancer. It is also necessary to review previous works that have evaluated circulating solid tumour cells in peripheral blood.

## 1.2 CLINICAL ASPECTS OF METASTATIC CANCER

### 1.2.1 Scope of the problem

With the exclusion of non-melanotic skin tumours, approximately 30 % of all patients with newly diagnosed malignancy already have clinically apparent metastatic disease at the time of diagnosis [Sugarbaker 1979a]. Of the remaining 70 % of patients with apparently localised disease, only about half of these may be cured by treatment of the primary tumour alone, with the remainder subsequently developing metastases. Therefore about 60 - 65 % of all cancer patients have either clinically detectable or clinically undetectable metastatic disease (micrometastases) at the time of initial diagnosis [Liotta and Stetler-Stevenson 1991].

In adult oncological practice, there are very few types of malignancy that are curable once overt metastatic disease has developed [Frost and Levin1992]. However, there is good clinical data that supports the premise that some patients with micrometastatic disease may be cured with chemotherapy or radiotherapy. Examples of this include the significant survival advantage demonstrated with the use of adjuvant cytotoxic chemotherapy in locally advanced breast cancer which has been demonstrated by the EBCTCG overview meta-analyasis [1992] which showed that there was a 25 - 30 % reduction in relapse rate with an absolute benefit in terms of disease free survival and overall survival at 10 years of 8.7 % and 6.8 % respectively when adjuvant chemotherapy was used in patients with positive axillary nodes. Several studies involving patients with locally advanced colorectal cancer have showed that adjuvant

chemotherapy with fluorouracil based regimens improve disease free and overall survival by about 30 % at 4 years [Laurie*et al.* 1989, Moertel *et al.* 1995, Wolmark *et al.* 1993] for patients with Dukes' stage C colonic cancer. Horwich *et al.* [1992] demonstrated that compared to standard routine nodal radiotherapy, a policy of surveillance only following orchidectomy for stage I seminoma was associated with an increased risk of recurrence at 5 years of 15 %. Also Riethmuller *et al* [1994] demonstrated that monoclonal antibody therapy employed as adjuvant treatment in Dukes C colon cancer was associated with a decreased recurrence rate.

In each case mentioned, the addition of chemotherapy, radiotherapy or specific biological treatment to standard treatment of the primary tumour has statistically improved the relapse rate for each group as a whole. The implication being that the addition of adjuvant treatment has destroyed micrometastatic disease that may otherwise have developed into gross metastatic disease. But this strategy also means that all patients in "at risk" groups are treated, and therefore a significant proportion of those patients treated are likely to be cured by the primary therapy and for these patients individually, the adjuvant therapy is unnecessary.

Even for patients with cancers for which micrometastatic disease is not chemosensitive and therefore not cured by the addition of adjuvant chemotherapy, the diagnosis of metastatic disease may be important to ensure that only those patients without metastases receive radical "curative" treatments. Patients with metastatic disease may avoid such procedures and either be treated with novel/experimental therapies or be managed in a purely palliative fashion. In any case, the accurate diagnosis of metastatic disease is a quality of life issue.

### 1.2.2 Presentation and diagnostic evaluation

The presentation of metastatic disease is generally dependent upon the site of the metastasis and the primary tumour type. Frequently, metastatic disease is multi-focal [Weiss 1992]. For patients whose initial presention is with gross metastatic disease, the diagnostic evaluation is dependent upon whether the tumour type is potentially curable with systemic treatment. With the exclusion of haematological solid tumours, in adults the only grossly metastatic solid tumour types that are presently considered to be curable are germ cell tumours of testis and ovary, and trophoblastic tumours [Kelly and Meyer 1983]. Hence for patients who present with metastatic malignancy, the initial evaluation must exclude haematological malignancy and the above mentioned curable solid tumours [Gatter *et al.* 1985]. Once these have been excluded, non-curable but chemo/hormone highly responsive malignancy need to be excluded. Examples include breast cancer, small cell lung cancer, ovarian cancer and prostate cancer. Papillary carcinoma of the thyroid also requires exclusion, as metastases frequently regress significantly following radioactive iodine therapy. For the remaining tumour types, investigations should be directed purely at symptomatic disease so that appropriate palliative treatments can be undertaken [Kennedy and Luedke 1979].

Certainly, extensive work up of patients who present with tumours of unknown primary site with upper/lower GI evaluation, urinary tract evaluation, and pulmonary tree evaluation is unwarrented as diagnosis of a primary tumour arising from these sites will not influence patient survival and therefore will have little influence upon therapy decisions [Leonard and Nystrom 1993]. Also, retrospective studies of such patients have shown that when such extensive investigations are undertaken in patients without any symptoms suggestive of a primary site, the likelihood of discovering the primary site of

malignancy is < 50 %. Even if autopsy is included as a diagnostic test, prospective series of patients presenting with tumours of unknown primary site have demonstrated that the combined antemortem and postmortem diagnosis of the primary site is 50 - 60 % [Leonard and Nystrom 1993]. Hence not only is such extensive work up not clinically warrented, it is frequently unsuccessful.

### 1.2.3 Management of micrometastatic disease

As has been previously discussed, patients with a number of tumour types receive adjuvant chemotherapy following therapy of the primary tumour if the tumour is already locally advanced and therefore at greater risk of systemic relapse [1.2.1]. Although significant survival benefits have been demonstrated for adjuvant chemotherapy in the setting of large groups of patients treated with locally advanced breast and colorectal cancer, clearly the majority of individual patients who undergo adjuvant chemotherapy do not benefit from the treatment which in many instances may be quite toxic. Based upon numerous randomised trials, it is estimated the addition of adjuvant cytotoxic chemotherapy for early stage breast cancer patients reduces the rate of recurrence at 5 years by 25 - 30 % [Henderson 1994]. In absolute terms, the percentage of stage I breast cancer patients (for whom the recurrence rate at 5 years is estimated as 14 - 30 % [Fentiman 1990] who will benefit from conventional adjuvant cytotoxic chemotherapy is only about 3.5 - 9 %. The remainder either are never going to relapse or the treatment is ineffective and disease recurs anyway. However each stage I breast cancer patient who choses to undergo such adjuvant treatment, whether or not she finally benefits from treatment, is exposed to the same potential toxicities of cytotoxic chemotherapy [McGuire and Clark 1992].

Certain prognostic features regarding the primary tumour other than size, extent of local invasion etc. may also predict for likelihood of relapse. Examples of this include oestrogen receptor status [Osborne 1991], degree of differentiation of the tumour cells [Hensen *et al.* 1991], ploidy [Maguire and Clark 1992], expression of *c-erb-B2* (*HER2* or *neu*) oncogene [Perren 1991, Hynes 1993], and *p53* mutation [Thor *et al.* 1992]. Each new test described is aimed to categorise tumours further into degrees of risk of relapse, and therefore aid in treatment decisions. Despite the advances made in such diagnostic methods, within each risk category, considerable prognostic variability still exists.

Clearly, more accurate detection of micrometastatic disease may allow more effective use of adjuvant therapies, so that only those patients who have the greatest chance of benefiting from adjuvant therapy actually receive it, whilst those patients not at risk of relapse can avoid such treatment. Such detection strategies may also identify patients who are certain to relapse and for whom standard adjuvant therapies will be ineffective. This may be a group for whom standard adjuvant therapy should be avoided and novel adjuvant treatment strategies evaluated.

## 1.3 BIOLOGY OF THE METASTATIC PROCESS

### 1.3.1 Clinical correlations

For the common solid epithelial tumours, the probability of metastasis is linked to the size of the primary tumour. For example, for patients with primary breast cancer, tumours less than 10 mm in diameter are associated with a 20 - 25 % incidence of axillary lymph node metastases, whereas tumours greater than 30 mm in diameter are associated with a 50 % incidence of axillary lymph node metastases [McGuire and Clark

1992]. A similar association with tumour involvement of draining lymph nodes with primary tumour size is observed in the setting of colorectal cancer and with primary tumour thickness in the setting of malignant melanoma [Sugarbaker*et al.* 1982]..

A number of theories exist as to why such a corrdation occurs. Firstly, larger tumours tend to have a greater degree of tumour vascularisation, and rate of tumour cell release into draining veins may be directly related to the degree of tumour vascularity [Liotta *et al.* 1974]. Alternatively it may be that more aggressive sub-populations of tumour cells are able to evolve in older larger tumours. Another possibility is that larger tumours are able to bestow a greater antigenic burdon, which may favour the survival of disseminated tumour cells [Fidler and Hart 1982].

Whilst the clinical patterns of haematogenous metastatic spread of tumour cells often follows an anatomical pattern, such as liver metastases being common with colorectal carcinoma [Brown and Warren 1938, Dionne 1965], or axillary nodes frequently involved in breast carcinoma [Danforth 1992], several clinical aspects of the metastatic process cannot be adequately explained with simple anatomical correlations. For example, some organs that receive a relatively high proportion of blood flow such as the heart, kidney and spleen seem to be under-represented as sites of metastatic spread [Weiss 1992]. It would therefore appear that individual tumour cell biology, and tumour cell - stroma interactions have a significant bearing on the manifestation of metastatic disease [Mareel 1992].

This may have significant clinical implications in that the overall management of a particular tumour type may depend largely upon the tumour being controlled at a particular site. A simple example of this is with primary brain malignancies which

virtually never spread outside the central nervous system. [Levin *et al.* 1993] Clearly, control of local spread of the brain tumour is the key to successful treatment. An alternative example may be ovarian cancer which often spreads via surface implantation or lymphatics to remain confined within the abdominal cavity [Janovski and Paramandandha 1973, Fuks 1980], so that novel treatments directed at inhibiting the local growth of disease within the abdominal cavity with metalloprotease inhibitors [Davies *et al.* 1993], rather than conventional sytemic cytotoxic chemotherapy may eventually become a vital component of management.

### 1.3.2 Metastatic inefficiency

Fidler [1970] demonstrated in an animal model, using tumour cells labelled with $^{125}$I-5-iododeoxyuridine, and injected directly into the circulation, that the vast majority of tumour cells released into the circulation die before gaining the opportunity to form secondary colonies. It is estimated that less than 1 % of cells released into the circulation survive greater than 24 hours and less than 0.1 % of tumour cells released into the circulation become viable metastases. Proposed causes of the high rate of cell death of circulating solid tumour cells include mechanical shearing forces, loss of attachment substrate, oxygen toxicity, and destruction by host-derived natural killer cells [Fidler 1973]. Subsequent in vivo studies have confirmed these observations and also suggest that whilst the number of final metastases tends to be in proportion to the number of circulating cells, the best correlation of higher rates of successful metastasis occurs with clumps of tumour cells in circulation[Liotta *et al.* 1974, Manzotti *et al.* 1993].

### 1.3.3 Development of cancer metastasis theory

Models of metastasis have developed over a one hundred year period. Paget, in 1889, first postulated the "seed and soil" hypothesis [Paget 1889]. This postulated that for metastasis to occur, the correct "seed" (tumour cell type) would only flourish in the appropriate "soil" (common metastatic site). So that whilst 50 - 60 % of metastasis distribution can be attributed purely to circulatory/lymphatic anatomy, preferential sites of metastasis are common for certain tumour types. The original example used by Paget [1889] was that breast cancer metastases frequently occur in bone, without evidence of metastases to lung (the first capillary bed likely to be encountered). However, subsequent investigators postulate that early bone metastases from breast cancer is due to retrograde paravertebral venous spread [Schirrmacher 1985]. A more contemporary clinical model is that women with recurrent ascites secondary to ovarian cancer who underwent placement of peritoneal-venous shunts did not develop widespread metastatic disease despite the large number of viable tumour cells in circulation [Tarin *et al.* 1984].

In 1928, Ewing proposed the concept that metastases were the product of mechanical entrapment of tumour cells in circulation. Arrested cells would then form their own tumour microenvironment producing a metastatic deposit [Ewing 1928]. Theories encompassing both the "soil and seed" theory and the "mechanical entrapment" theory were proposed by Proctor [1976] and Sugerbaker [1979b]. The concepts of "specific adhesive interactions" between tumour cells and substrate were introduced by Nicolson and Winkelhake [1975], Schirrmacher *et al.* [1980], and Kieran and Longnecker [1983]. Tarin and Price [1981] and Fidler and Hart [1982] proposed the concept that a particular organ site modulated tumour growth within it, and that only

tumour cells with the ability to adapt to the new site would successfully develop into viable secondary tumour colonies.

Hence the concept of specific tumour cell - metastatic site interaction, as orignially suggested by Paget [1889] over one hundred years ago has very basically stood the test of time, although the mechanisms involved in metastasis are now somewhat better understood. The modern hypothesis of invasion and metastasis is therefore based on 3 major premises:-

(i) primary tumours are heterogenous with regard to biologic and metastatic properties;

(ii) the process of metastasis is non-random consisting of a series of limited, sequential steps, each requiring completion before each subsequent step may take place; metastasis then favours the survival and growth of a few sub-populations of cells within a primary tumour;

(iii) the outcome of the metastatic process is dependent upon the interaction of metastasising cells with different organ environments [Fidler 1990].

### 1.3.4 Cascade theory of metastasis

The pathogenesis of metastasis as described by Fidler and Hart [1982] consists of a series of non-random sequential interrelated steps. Each step must be completed before any subsequent steps occur. Thus each step is potentially rate limiting. The major steps therefore are (i) progressive tumour growth at the primary site following malignant transformation; (ii) extensive neo-vascularisation (this is essential if tumour mass is to exceed 2 mm) following synthesis and secretion of several angiogenesis factors [Folkman 1986, Bicknell and Harris 1991, Mahadevan and Hart 1991]; (iii) local

invasion of host stroma and subsequent invasion of thin walled venules or lymphatics [Liotta 1986, Duffy 1992]; (iv) detachment and embolisation of small aggregates of tumour cells into the circulation, transport within the circulation and evasion of potentially lethal events in circulation; (v) arrest in the capillary beds of metastatic organ site; (vi) extravasation from the capillaries into the distant organ tissue; (vii) proliferation within the distant organ tissue with further neo-vascularisation, and immunological events to allow evasion of host immune mechanisms [Fidler *et al*1978]. Once the "cascade" has reached this stage, further detachment, invasion, embolisation etc. may occur producing "metastases from metastases" [Poste and Fidler 1979, Sugarbaker 1979b, Killion and Fidler 1989, Kohn and Liotta 1993].

### 1.3.5 Identification of tumour cells with high metastatic potential

There is considerable evidence that tumour dissemination occurs very soon after primary tumour vascularisation. Koscielny *et al.* [1985] showed that in a breast cancer model, metastases may be initiated when the primary tumour is less than 0.125 $cm^2$ in size. Fidler and Hart [1982] have proposed that a sub-population of highly metastatic cells exist within a primary tumour at a very early stage of development. The size of the proposed sub-population of highly metastatic cells is not known, but it appears that the metastatic sub-population dominates the primary tumour which is destined to eventually metastasise, quite early in growth. [Fidler and Kripke 1977, Kerbel 1990]. Kerbel [1990] has suggested that selective growth of the metastatic sub-population may be due to altered response of these cells to local cytokines. Presumably the altered response to local stimuli is a function of oncogene up-regulation with subsequent greater oncogene product production [Cairns 1981, Fidler and Radinsky 1990]. This certainly concurs

with the general observation that the average level of oncogene (or specific protein marker) amplification measured within a primary tumour tends to correlate with the clinical parameters of metastasis and recurrence [Field and Spandidos 1990]. Additionally, reduced expression of the *nm23* (tumour suppressor) gene [Hirayama *et al.* 1991, Radinsky *et al.* 1992] or mutations of *p53* (tumour suppressor gene) in primary malignancy may also correlate with increased propensity to early metastases and worse prognosis [Hart and Easty 1991, Hennessy *et al.* 1991, Harris and Hollstein 1992].

### 1.3.6 Metastatic organ distribution / preference

There are numerous theories that attempt to clarify why certain tumour types have a tendancy to metastasise to particular organs (once direct anatomical lympho-vascular connections are taken into acount). Presently, the most attractive of these theories are :-

(i) Tumour cells disseminate equally throughout the body, but preferentially grow in specific organ sites due to response to local growth factors or hormones.

(ii) Circulating tumour cells adhere preferentially to endothelial luminal surfaces only within target organ sites, due to organ specific endothelial determinants.

(iii) Tumour cells respond to soluble factors diffusing from a target organ. Such factors may be chemotactic, thus attracting tumour cells from the circulation.

As several mechanisms may occur for different tumour types, these theories are not mutually exclusive. [Schirrmacher 1985, Nicolson *et al.* 1985, Naito *et al.* 1987, Juacaba *et al.* 1989, Nicolson 1988].

### 1.3.7 Cell/matrix interactions in the metastatic process

Discussion of the process of metastasis must include reference to the important cell adhesion molecules (CAMs) which appear to play a major role in the metastatic process. *E-cadherin* is one of the *cadherin* group of cell surface glycoproteins that mediate intercellular adhesion [Takeichi 1991]. Experimental studies suggest that the activity of *E-cadherin* has a major role in tumour behaviour. Tumours with low levels of *E-cadherin* activity also displayed greater invasive/migratory behaviour. Whereas insertion of DNA encoding *E-cadherin* into highly invasive tumour cell lines resulted in reduction in invasive capacity suggesting that not only is *E-cadherin* important in normal cell/cell interactions, but that it may function as a tumour invasion suppressor [Vleminckx *et al.* 1991, Shimoyana and Hirohashi 1991].

Numerous cell/extracellular matrix protein interactions are mediated through *integrins,* which are a group of transmembrane glycoproteins which are complexes of $\alpha$ and $\beta$ subunit proteins. *Integrins* may align adhesion proteins on the cell surface thus altering cell shape. It is this mechanism which is believed to affect platelet/tumour cell interaction, lymphoid cell/endothelium binding and circulating tumour cell/endothelium interaction. *Integrin* function may be inhibited by peptides containing the Arg-Gly-Asp (RGD) sequence [Hynes 1987]. Experiental models suggest that high levels of RGD peptides may inhibit metastasis formation potential in tumour cells [Humphries *et al.* 1986]. *Integrins* may not only mediate adhesion to the extracellular matrix and associate with cytoskeletal proteins, providing a continuous path for mechanical force between cells and their immediate environment, but may also function as receptors that may activate intracellular signalling pathways [Schwartz and Ingber 1994].

*Laminin* is a basement membrane glycoprotein that is likely to play an important part in tumour cell adhesion to the basement membrane. Many tumour types contain high levels of *laminin* receptors, and the number of *laminin* receptors may be amplified and the distribution may be significantly altered in tumour cells displaying increased invasiveness [Barsky *et al.* 1984, Wewer *et al.* 1986].

Evasion of normal host immune surveillance is vital to successful metastasis. As cytotoxic T cells recognise antigens only when they are presented in association with major histocompatibility (MHC) class I molecules, reduced expression of MHC class I molecules on malignant cells may be one effective mechanism of evading host immune mechanisms [Bernards *et al.* 1983, Kindt and Robinson 1984, Cordon-Cardo *et al.* 1991]. Also excessive production of the intercellular adhesion molecule 1 (*ICAM-1*), a surface protein which normally facilitates cytotoxic T cell / tumour cell interaction, by tumour cells may actually block the corresponding receptor on the cytotoxic T cell, thereby inhibiting tumour cell destruction by lymphocytes [Marlin and Springer 1987, Hart 1989, Johnson *et al.* 1989].

Much of the cell interaction in the metastatic process resembles immune cell/matrix interaction that occurs during inflammatory processes [Liotta and Stetler-Stevenson 1991]. Numerous endothelial adhesion molecules are up-regulated during inflammation facilitating migration of inflammatory cells from the circulation [Springer 1990]. The *CD44* molecule which has been demonstrated to play a role in lymphocyte homing [Gunthert *et al.* 1991] has also been shown to be an important molecule in the capacity of tumours to metastasise. The overexpression of the many variants of this molecule in tumour cells has become an important area of the molecular study of metastatic behaviour as will be discussed later.

## 1.4 THE MOLECULAR APPROACH TO CANCER DIAGNOSIS

### 1.4.1 Limitations of cancer diagnosis

As has been previously discussed, the management of cancer is paramount to the management of metastatic disease. Therefore the management of cancer is also very dependent upon the accurate diagnosis of advanced or metastatic disease. Presently conventional cancer diagnosis is dependant upon histopathological evaluation, and is inherently observer dependent. Depending on particular tumour types, interpretation of histopathological parameters is influenced by observer variability. Presently, histopathological and simple cytological methods are not able to detect tumour cells at a sensitivity of better than 1 in 100 - 1000 cells. Molecular methods of diagnosis allow considerably greater sensitivity (1 in $10^5$ - $10^6$ cells).

The purposes of such sensitive tests are :-

(I) for accurate primary tumour diagnosis,

(ii) determination of dissemination as part of initial staging,

(iii) prognostication at time of initial diagnosis,

(iv) detection of minimal residual disease after primary treatment,

(v) screening purposes.

### 1.4.2 Types of tumour markers

Many molecular markers are closely related to the basic biology of the malignancy. Malignant progenitors arise from mutations (within oncogenes, or tumour suppressor genes) to produce a malignant clone. Additional mutations may produce a subclonal cell population within the tumour. Molecular tumour markers may reflect the

clonality of the malignant phenotype progenitor or the clonality of the proliferative subclonal population [Bishop 1991]. Molecular markers include :- cytogenetic markers (microscopic chromosomal changes), DNA rearrangements, DNA deletions, DNA amplification, DNA content, viral nucleic acids or proteins, RNA or protein products (such as tissue specific gene products or markers of clonality acquired prior to neoplastic transformation).

Proteins are used quite extensively in clinical oncology practice. Serum markers in common usage include α-feto protein (αFP) and β-human chorionic gonadotropin (β-HCG) which are used to monitor non-seminomatous germ cell tumours of the testis, protate specific antigen (PSA) as a marker for prostate cancer, Ca-125 which may be used to follow patients with ovarian cancer, and serum monoclonal paraprotein which is frequently used in the follow up of patients with multiple myeloma. In each case the measured product is a protein associated with the tumour. The measured protein product is not exclusively associated with the tumour however, and must therefore be evaluated against a "normal, background" amount of the protein. There also may be non-malignant reasons for these markers being markedly elevated (the simplest example being pregnancy producing elevations of β-HCG or αFP).

### 1.4.3 Molecular detection techniques

(i) In-situ hybridisation of DNA probes [Langer-Safer *et al.* 1982, Lichter *et al.* 1988, Kallioniemi *et al.* 1993], in which DNA localisation is performed directly on cells or tissue stimulated into metaphase allows regionalisation of tumour phenotype to be demonstrated. A variation of this technique may be used to detect RNA within cells [Angerer and Angerer 1991]. Due to the potential wide variation in regionalisation of

markers within an heterogenous population, wide variations in results may be obtained purely due to the particular regions examined.

(ii) Flow cytometry, in which single columns of cells are passed by a laser beam to produce refraction differences relating to DNA content may be used to characterise ploidy and quantify S-phase fraction DNA. Such parameters have been shown to correlate with prognosis in several tumour types such as breast cancer [O'Reilly *et al.* 1990] and colonic cancer [Hood *et al.* 1990, Meling *et al.* 1991].

(iii) Southern blot hybridisation [Southern 1975] in which DNA fragments are separated by gel electropheresis and more permanently hybridised to a nitrocellulose membrane (and subsequently probed for specific DNA sequences) allows quite specific confirmation of target DNA presence, but tends to be limited by a sensitivity of only 1 in 100 sampled cells.

(iv) Pulse-field gel electrophoresis in Southern blot hybridisation [Schwartz and Cantor 1984] permits scanning of large DNA sequences with a single hybridisation probe, but is only applicable to fresh tissue, and reproducible digestion is often difficult.

(v) Polymerase chain reaction (PCR) allows considerably greater sensitivity (picogram amounts of DNA can be detected) [Saiki *et al.* 1988]. However, it tends to be limited to sequences up to only a few kilobases in length. By being so exquisitely sensitive, the potential for outside contamination is great. It is also not directly quantifiable.

(vi) Immunohistochemistry/immunocytology is not often considered to be a molecular biological method of examination, but essentially it is simply assessing the protein products of genes [Gatter *et al.* 1985]. Examination of antigenic proteins and

polysaccharides are often performed with the aid of direct microscopy and may be quantifiable by flow cytometric techniques.

These molecular biological techniques allow direct evaluation of cell nucleic acid activity providing a more exact diagnosis compared with observation of simple morphological phenomena. Most tumours are believed to arise from several genetic alterations accumulating in a final malignant phenotype. If some of these genetic events occur sequentially, it is possible that the molecular detection of these phenotypic phenomena will be specific to a particular time in the development of the tumour. This may subsequently provide more information about the biological origins of the malignancy being examined [Kefford and Trent 1989]

### 1.4.4 Clinical examples of techniques that evaluate DNA changes in malignancy

#### 1.4.4.1 Haematological malignancies

*Myeloid leukaemias*

CML :- The t(9:22)(q34;q11) is shown to be present in 95 % of cases of CML [Ganesan 1986]. This has been determined by conventional cytogenetic techniques, Southern blot hybridisation, in-situ hybridisation, and polymerase chain reaction. The Philadelphia (Ph') gene is produced by the joining of the 5' portion of the *Bcl* gene on chromosome 22 and the 3' portion of the *ABL* gene on chromosome 9. This gene is transcribed into an 8.5 kb mRNA sequence which is subsequently translated into a 210 kd protein (believed to increase tyrosine kinase activity) [Konopka *et al.* 1984]. Precise breakpoints within DNA vary considerably, but if the RNA sequence produced is the same despite the variability in the DNA changes, then by performing PCR on cDNA

generated from RNA using the enzyme reverse transcriptase or RT, (a technique abbreviated to RT/PCR), one should be able to detect the product (where ever the DNA break) [Lee *et al.* 1989]. The detection level is as sensitive as 1: $10^6$ cells. However, as RNA is considerably less stable than DNA, there is considerable risk of false negatives due to RNA degradation. Despite these shortcomings, such a technique may allow very sensitive testing for minimal residual disease in patients following definitive treatment. Indeed, the detection of the translocation in blood of patients who have undergone bone marrow transplantation over one year before is associated with an adverse prognosis [Delage *et al.* 1991, Hughes *et al.* 1991, Cross *et al.* 1993].

*Lymphocytic tumours*

Lymphocytes express particular surface antigens or secrete particular proteins at certain stages of development so that gene products produced by lymphocytic tumours may be used to define the stage of developmental arrest of the particular tumour. Most lymphocytic tumours are now characterised by antigen receptor rearrangements. [Arnold *et al.* 1983, Sklar *et al.* 1987, Sklar and Weiss 1988]. Chromosomal translocations are common, but individual pathognomonic chromosomal translocations are quite rare. One of the more specific rearrangements is the t(14:18)(q32:q21) translocation found in about 90 % of follicular lymphomas [Cleary and Sklar 1985]. This translocation joins DNA of the Ig heavy chain gene on chromosome 14 to DNA of the proto-oncogene *Bcl2*. The product of this translocation has been experimentally implicated in the delay of apoptosis of B cells in follicular centres of activated lymph nodes. Southern blot hybridisation and subsequent probing may detect this product wherever the breakpoint is [Hockenberry *et al.* 1990]. However the t(14:18) translocation is certainly non-specific and may be found in 50 % of reactive nodes by

PCR [Limpens *et al.* 1991]. Its detection is still important in certain situations however, such as following bone marrow transplantation for follicular lymphomas when non-detection of t(14:18) in bone marrow appears to carry a better prognosis [Gribben*et al.* 1991].

DNA changes in haematological malignancies are relatively common and can therefore often be used as a monitor for micrometastatic disease, often by serial examinations of the blood or bone marrow by PCR. The technique is increasingly utilised to detect malignant contamination in autologous bone marrow or peripheral blood progenitor cells in the bone marrow "transplant" setting [Gribben *et al.* 1991, Potter *et al.*1993].

#### 1.4.4.2 Non-haematological malignancies (solid tumours)

*Neuroblastoma*

Various methods of detecting up-regulation of *n-myc* oncogene has been associated with poor prognosis for patients with neuroblastoma, independent of stage [Brodeur *et al.* 1984]. DNA content has also been shown to have prognostic significance [Brodeur 1990], with increased DNA content being associated with a poorer outcome

*Breast cancer*

Although stage 1 breast cancer carries the best prognosis for all stages, the absence of lymph node spread in breast cancer is still associated with a 30 % risk of relapse at 5 years. There appears to be prognostic subgroups (based upon molecular markers) within each stage of disease. Study of various gene products for prognostic significance has revealed that increased expression of epidermal growth factor and *cathepsin-D* is associated with an increased relapse rate independent of stage [Sainsbury

*et al.* 1987, Tandon *et al.* 1990], while decreased expression of *nm23* (which codes for an NDP kinase) is associated with increased relapse rate [Hennessey *et al.* 1991].

*c-erb-B2* (*Her 2, neu*) gene encodes for a surface protein that has homology with EGFR. About 25 - 30 % of breast cancers have amplification of *c-erb-B2*. This amplification closely correlates with increased *c-erb-B2* RNA and subsequent protein levels. Several studies have shown up-regulation of *c-erb-B2* (as measured by Northern Blot hybridisation, Western blotting, and immuohistochemistry) to be associated with poor prognosis [Wright *et al.* 1989, Gullick *et al.* 1991, Winstanley et al. 1991]. Another study interestingly showed that patients with axillary node positive breast cancer whose breast tumours display high expression of *c-erb-B2* had a better relapse free survival if treated with an adjuvant chemotherapy combination containing higher doses of doxorubicin, cyclophosphamide and fluorouracil [Muss *et al.* 1994]. However, there was no advantage to increased doses of cytotoxics for patients whose breast tumours expressed lower levels of *c-erb-B2* .

*BRCA 1* gene has recently been identified and mapped to chromosome 17. This "breast cancer susceptibility" gene, (one of three such suscepitibility genes) appears to be most significant in women with breast cancer who are below the age of 45 years. The finding of a germline mutation of the *BRCA 1* gene in an individual woman appears to have very significant risk implications, with as many as 85 % of such women likely to develop breast cancer during their lives. This important gene also appears to be associated with increased risk of ovarian cancer, colonic cancer and prostate cancer [Miki *et al.* 1994, Ford *et al.* 1994].

*Bladder cancer*

Tumour suppressor gene *p53* has many cell and nuclear regulatory functions. It is known to encode for a protein that binds viral oncoproteins, such as E7 protein of human papilloma virus, and thus may inhibit malignant progression by negatively regulating key events in the cell cycle by binding cellular proteins or by directly interacting with DNA [Levine *et al.*1991, Harris and Holstein 1992]. Mutations in *p53* appear to be associated with poor prognosis for bladder cancer. These mutations have been detected in urine specimens of patients with known bladder cancer. The DNA of cells recovered from the urine may be amplified by PCR, and analysis of the amplified products by Southern blot hybridisation is able to detect as few as 1 cell in 10,000 [Sidransky *et al.* 1991].

*Retinoblastoma*

*RB1* gene is a tumour suppressor gene that, like p53, encodes a gene product that binds several viral oncoproteins. In retinoblastoma cells, there is inactivation of *RB1* by mutations or deletions. Thus detection of down regulation of the *RB1* gene may be utilised to screen at risk families for increased risk of developing retinoblastoma [Lee *et al.* 1987]. Germline mutations of the *RB1* gene have also been demonstrated in patients with other malignancies such as breast cancer, lung cancer and osteosarcoma suggesting that this gene plays a wider role in cellular proliferation and may therefore be implicated in the development in a broader range of malignancy [Friend *et al.* 1986, Harbour *et al.* 1988, Lee *et al.* 1988].

*Ewing's sarcoma*

The t(11:22)(q24:12) gene rearrangement which juxtaposes the *FLI-1* and EWS genes has quite recently been characterised and shown to be present in 85 % of Ewing's

sarcomas [Zucman *et al.* 1992]. Studies to evaluate this gene rearrangement in relation to prognosis for Ewing's sarcoma patients in remission are underway.

Therefore there are presently very few DNA abnormalities which are consistantly observed in adult patients with solid tumours. Hence, the ability to use differences in DNA (of solid tumour cells as opposed to normal host cells) as a marker of residual disease appears markedly limited.

## 1.5 CIRCULATING SOLID TUMOUR CELLS

### 1.5.1 Critical evaluation of early techniques of detection

Engell [1959] reported results of a study of tumour cells in venous blood of patients with colorectal cancers. At the time of surgery, local blood vessels were sampled, and in over 50 % of patients, tumour cells were deemed to have been present in blood. The detection of tumour cells in the blood appeared to correlate well with primary tumour stage. However, follow up studies of surviving patients 5 - 9 years after surgery showed that 51 % of these had detectable tumour cells in the blood at the time of surgery. One of the main conclusions of this study was that in these patients, tumour cells must have perished in the blood stream.

In all likelihood, a significant proportion of tumour cells identified in the Engell [1959] paper, were not tumour cells but cytological artefacts. Subsequent examination of the techniques available at that time have suggested that the false positive rate was very high [Goldblatt and Nadel 1962, Frost 1965, Nadel 1965]. Even so, the final conclusion regarding the fate of tumour cells in the blood remains pertinent in the present context.

At the time of Engell's paper [1959], when technical advances had begun to allow greater concentrations of cells in blood preparations, numerous investigators published work relating to methods of detecting solid tumour cells in peripheral blood, mainly using basic cell morphology as criteria for positive identification of tumour cells [Nagy 1965, Pruitt *et al.* 1965]. Goldblatt and Nagel [1962] reviewed over 40 different investigations of tumour cells in the blood in which 20 different techniques were used. Review of 81 samples of "positive" tumour cells in blood, suggested that less than 5 % of positively identified cells fulfilled cytological criteria of cells being suspicious for malignancy. Reports of rates of detection of circulating tumour cells at that time varied from 1 - 95 % [Nedelkoff *et al.* 1962, Candar *et al.* 1962]. Using most strict cytological diagnostic criteria, the average positive detection rate fell to about 5 % [Griffiths and Salisbury 1963, Sellwood *et al.* 1964].

Of greater concern was the fact that the detection rate in patients with fulminant metastatic disease did not increase proportionately, and detection of tumour cells in the blood of patients with apparent good prognosis disease was disproportionately high. Even worse were reports of positively identified malignant cells in the blood of healthy volunteers [Moore *et al.* 1962]. Such reports probably prompted the conclusions reached by some commentators that the study of circulating tumour cells was essentially a waste of time [Annotation 1964].

### 1.5.2 The determinants of rates of detection

What were the reasons for such wide discrepancy in the rate of recording tumour cells in the blood?

(i) Number and size of blood samples.

Data suggest that increased number of blood samples taken per patient increases the likelihood of detecting tumour cells [Roberts *et al.* 1962]. The volume of blood examined also appeared to be a significant factor in tumour cell detection [West*et al.* 1964]. It therefore seems to follow that increasing the volume of blood examined would increase the tumour cell yield, as limits of detection at that time seemed to be at 10 cells per ml. of blood. By performing sequential analyses of blood samples, not only was an increased total volume of blood examined, but different times were also examined. Some evidence exists supporting the theory of tumour cells are shed into the blood in "showers" at different times. By examining several different times, the chance of performing the test during a period of "showering" is increased [Griffiths and Salisbury 1965].

(ii) Local venous blood vs. peripheral venous blood.

Many of the early studies involved the examination of draining venous blood in the peri-operative period. These studies tended to report a much higher incidence of tumour cell presence in blood. Also the total number of tumour cells observed in the blood tended to be significantly higher [Griffiths*et al.* 1973].

(iii) Method of concentration of blood for examination.

To facilitate the examination of blood, much of the normal constituents needed to be eliminated. This would leave only the cellular fraction of blood for examination. This concentration step varied widely with various mechanisms used in either centrifugation and/or red cell lysis [Pruitt *et al.* 1965]. Each of these processes potentially losing tumour cells and distorting normal blood cells, which may be subsequently be misinterpreted by a cytologist as being malignant.

(iv) Types of primary tumour studied.

A wide range of positive identification rates existed with different tumour types. Moore and Sandberg [1965] reported rates of tumour cells in peripheral blood of around 25 % for gastric cancer, melanoma and soft tissue sarcoma but less than 10 % for colorectal cancer, lung cancer, and gynaecological cancer.

(v) Standards of criteria for positive identification of tumour cells.

Strict standards of identification implies not only the positive identification of tumour cells, but the establishment of criteria to exclude cytologically bizarre blood cells (produced by varying concentration techniques) or blood cell precursors. The strictest criteria tends to decrease the positive identification rate, but also eliminates the unacceptable level of false positive results [Christopherson 1965, Stevenson and von Haam 1965, Romsdahl *et al.* 1965a, Salisbury 1975].

### 1.5.3 Prognostic value of detection of tumour cells in blood

From the very early studies, what can be said about prognosis detection of tumour cells in peripheral blood? Some evidence supported the assumption that the presence of tumour cells in the peripheral blood carries a worse prognosis, although the prognostic value was not particularly strong [Watne *et al.* 1960, Zeidman 1965, Romsdahl *et al.* 1965b, Kuper and Bignall 1966]. Confounding the issue was the observation that over 50 % of patients who were identified as being positive soon after initial "curative" surgery were longterm survivors [Engell 1959, Griffiths *et al* 1973]. In essence, the detection of tumour cells in the blood carried little useful prognostic information, especially when tumour cells in blood were evaluated in the peri-operative period.. With such a high rate of apparent tumour cell detection, several authors

speculated that the process of metastatic cells in blood colonising and creating secondary deposits was a very inefficient process [Clifton and Agostino 1962, Romsdahl *et al.* 1961, Fisher and Fisher 1965]. Such hypotheses appear to have been well founded, based upon later reports [Fidler 1970, Weiss1986].

### 1.5.4 Evaluation of bone marrow

Bone and bone marrow being one of the most common sites of metastatic disease [Weiss 1992] has become a common site to evaluate for micrometastatic disease. According to metastatic theory [Fidler and Hart 1982], bone marrow stroma may have a major influence upon the "homing" of circulating tumour cells to bone marrow. Bone marrow also is a relatively easy organ to sample. Using an immunocytochemical method that measured an epithelial surface membrane antigen (EMA), Redding *et al.* [1983] detected bone marrow micrometastatic disease in 28 % of patients examined. Long term follow-up of patients tested with this method revealed that the detection of bone marrow micrometastases was an independent predictor of overt metastasis development [Dearneley *et al.* 1991]. Cote *et al.* [1988] and Mansi *et al.* [1991] using similar methods found similar results in breast cancer patients. However, an earlier study suggested that EMA may be expressed in haemopoietic cells [Heydermann and McCartney 1985], making interpretation of these results difficult.

Stahel *et al.* [1985] using an antibody against a membrane antigen on small cell lung cancer (SCLC) cells, showed that the level of bone marrow infiltration at diagnosis of SCLC could be increased from 20 % (using standard morphological methods) to 69 %. These results were confirmed by Berensden*et al.* [1988], using a different antibody and detecting micrometastases in 63 % of bone marrow aspirates examined. Pantel*et*

*al.* [1993] used a cytokeratin 18 antibody to detect bone marrow micrometastases in 22 % of "operable" non-small cell lung cancer patients. Positive identification proved to be of prognostic significance. Other tumour types, including neuroblastoma, prostate cancer and colorectal cancer have also been examined in the same way with significant results [Beck *et al.* 1986, Mansi *et al.* 1988, Schlimok *et al.* 1990].

Increased sensitivity of detection of bone marrow micrometastases could be achieved with the used a combination of the molecular techniques of reverse transcription (RT) and polymerase chain chain reaction (PCR). This was demonstrated by Naito *et al.* [1991] for neuroblastoma, and Lindemann *et al.* [1992] for colorectal cancer.

As a significant proportion of bone marrow aspirate is whole blood, it may be postulated that if the bone marrow is just part of the normal circulation of whole blood, then examination of whole blood alone may yield equally positive results if the very sensitive method of RT/ PCR is utilised.

## 1.6 PCR BASIC PRINCIPLES AND AUTOMATION

### 1.6.1 PCR principles and history

The principles behind the polymerase chain reaction (repeated denaturation, annealing and extension to exponentially increase nucleic acid sequences) were first described by Kleppe *et al.* [1971]. The term polymerase chain reaction, or PCR as it is abbreviated, and it's present basic protocol was devised in the late 1980s by Mullis and colleagues at the Cetus corporation [Mullis and Faloona 1987, Saiki *et al.* 1988]. The process now allows for rapid in vitro enzymatic amplification of a specific segment of DNA (or target DNA). The number of PCR applications appears to be growing and

presently include direct cloning from genomic DNA or cDNA, in vitro mutagenesis and DNA engineering, genetic fingerprinting, assays for presence of infectious agents, prenatal diagnosis of genetic disease, analysis of allelic sequence variation, genomic footprinting, direct nucleotide sequencing of genomic DNA and cDNA and analysis of RNA transcripts [Coen 1991, Liu *et al.* 1993].

Variations on the basic PCR technique that rapidly amplify DNA have been reported, but as yet, they remain unproven with regard to being as robust or reliable as PCR [Fahy *et al.* 1991, Weiss 1991].

### 1.6.2 Thermostable DNA polymerases

The initial use of the PCR was limited until the availablity of thermostable DNA polymerases. Initially, before the availability of thermostable DNA polymerases, *Klenow* polymerase (of Escherichia coli origin) was used. As this polymerase is not thermostable and denatured during the 90 - 95° part of the PCR, fresh DNA polymerase needed to be added to the reaction after each cycle [Mullis and Faloona 1987]. This time consuming step also increased the risk of contamination with each cycle. This drawback was able to be overcome by the use of a thermostable DNA polymerase purified from the thermophilic bacterium *Thermus Aquaticus* (Taq) instead of *Klenow* polymerase. Taq polymerase was able to survive extended incubation at 95° and did not need to be replenished after each cycle. As no direct handling of product was necessary with each cycle, the process became amenable to automation [Saiki *et al.* 1988].

### 1.6.3 The PCR process

DNA polymerases are able to promote the synthesis of a complementary strand of DNA in the 5' -> 3' direction using a single stranded DNA template, but starting from a double stranded region. PCR employs the same principle, but uses two sets of oligonucleotide primers, each complementary to opposite strands of a region of DNA (the target sequence of DNA) which have been denatured by heating. Each primer therefore promotes an exact copy of the target sequence of DNA (but in opposite directions), so that two copies of target sequence DNA are synthesised, both of which are flanked by the two oligonucleotide primers. By denaturing the double stranded DNA produced in this synthesis reaction, further single stranded DNA is made available for subsequent primer annealing and extension, again producing further copies of the target sequence of double stranded DNA [Taylor 1991, Finney 1991]. This process is depicted in Figure 1.

### 1.6.4 PCR automation

Each of the reactions of denaturation, annealing, and extension occur preferentially at certain temperatures, so that if the deoxynucleotides (building blocks) and the thermostable DNA polymerase are present in the reaction in great excess, repeated rapid cycling of temperature allows the synthesis of target DNA to progress virtually continuously. A PCR machine will repeatedly, accurately, and rapidly cycle the temperature of the reaction vessels for a set number of counts [Taylor 1991, Finney 1991]. The most preferable of these machines is a programmable incubation block. Most

PCR machines now are able to store several PCR cycling programmes, and many now have several heating blocks which are programmed independently allowing different PCR cycling conditions to run concurrently.

### 1.6.5 Visualisation of PCR products

If each cycle of PCR (double stranded DNA denaturation, oligonucleotide primer annealing, and DNA extension) doubles the amount of target sequence DNA, then 30 cycles of PCR will increase the target sequence DNA by $2^{30}$ (or $10^{9}$ ) copies. Even if the amount of target DNA present at the start of the reaction is single figure molecule amounts, PCR may be able to synthesise nanogram -> microgram of target DNA; enough to be able to be visualised using either radio-labelled probes and autoradiography or on an ethidium bromide stained gel [Sklar 1993].

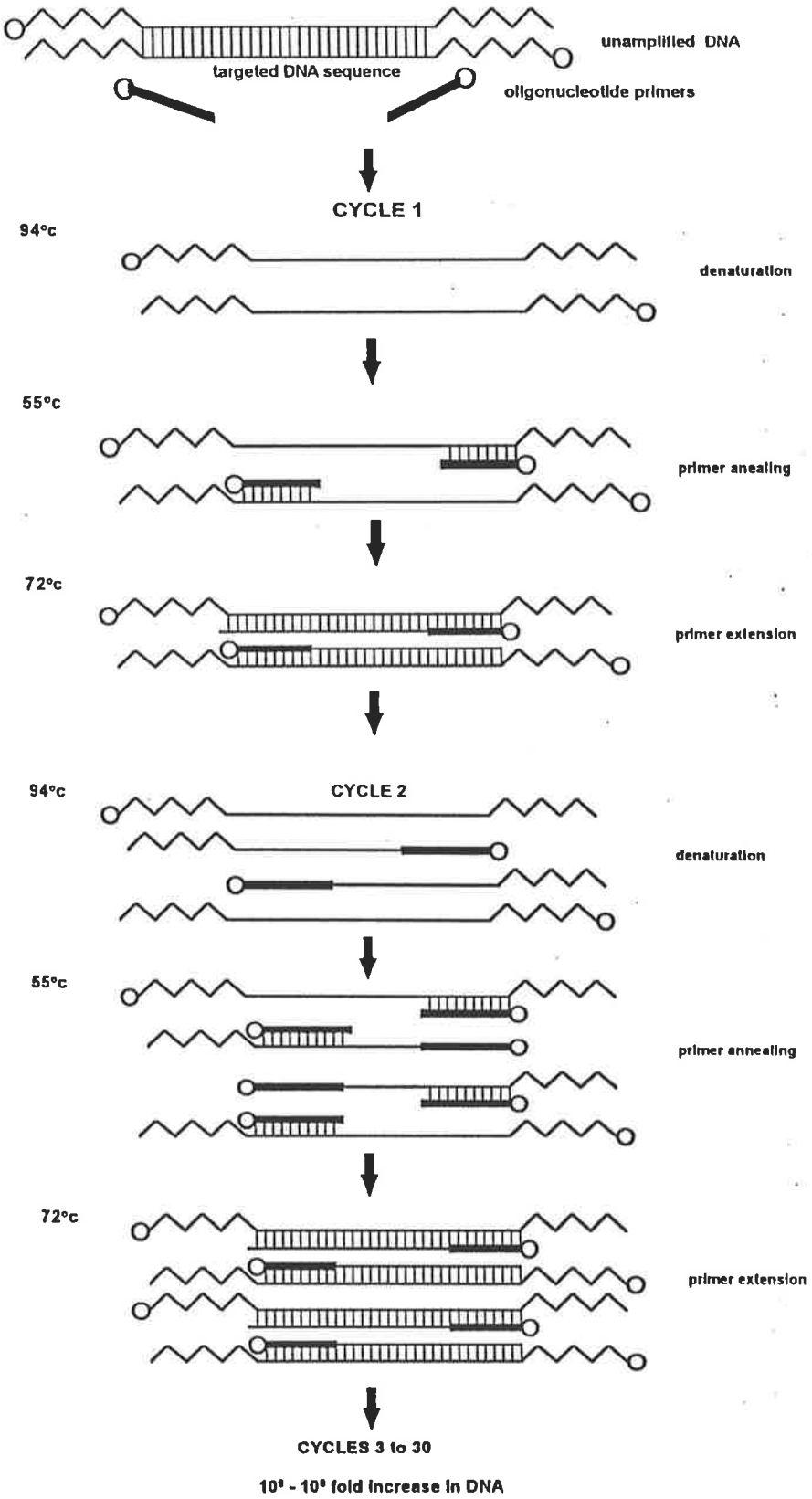



Figure 1. Schematic representation of the polymerase chain reaction (PCR), showing doubling of DNA content with each cycle

### 1.6.6 Basic components

#### 1.6.6.1 DNA polymerase

The most commonly used thermostable DNA polymerase is Taq polymerase isolated from *T. aquaticus* [Gelfand 1989]. The advantages of thermostable DNA polymerase are that no time consuming DNA polymerase replenishment steps are required, it remains active at high temperature which allows more specific annealing of primers and more rapid DNA synthesis, and as manipulations are kept to a minimum, there is decreased risk of contamination [Saiki *et al.* 1988]. Occasionally subtances that act as natural inhibitors of DNA polymerases within DNA samples are not removed by the usual DNA preparation methods. In these circumstances, DNA needs to be further purified to allow optimal DNA polymerase function.

#### 1.6.6.2 Deoxynucleoside triphosphates (dNTPs)

These are the "building blocks" of the DNA, and therefore are also the"building blocks" of PCR products. The concentration of dNTPs in PCR is usually kept at about 200 mM. Higher levels of dNTP concentration may be associated with chelation of magnesium, thereby altering the effective optimal magnesium concentration [Finney 1991]. Higher dNTP concentrations may also be associated with an increase in the error rate of the polymerase and very high concentrations of dNTP may actually inhibit taq polymerase [Gelfand 1989].

#### 1.6.6.3 Reaction buffer

Currently, reaction buffer is often tailored for synthesis of particular PCR products, although most are based upon a formula described by Saiki*et al.* [1988]. This

formulation contains Tris at a final concentration of 10 mM (pH 7.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin, 0.01 % NP40, and 0.01 % Tween 20. Often the gelatin and Tween 20 may be replaced by Triton X-100. Also, some sets of oligonucleotide primers work best at significantly different magnesium concentrations. It is usual to therefore optimise magnesium concentration for each set of PCR primers [Finney 1991].

#### 1.6.6.4 Oligonucleotide primers

These are generally synthesised in the range of 20-30 bases. They are conveniently stored in ammonia elluent which stays liquid at -20°C. This enables primers to be dispensed without repeated freeze-thawing. Primer sequences should have a similar guanine and cytosine (G + C) content, minimal secondary structure (ie. low degree of self-complementarity which may produce folding of the primer upon itself), and low complementarity to the other primer (preventing significant direct annealing between primers) especially at the 3' end. Concentrations of primers varies with individual PCRs, but usually 50 - 100 pmol of each primer is required for each reaction [Taylor 1991, Finney 1991].

#### 1.6.6.5 Reaction times

Incubation times are best kept as short as possible to minimise degradation of enzyme and product and also to minimise non-specific amplification. Denaturing and annealing times of about 30 seconds are usually adequate, and extension time may be calculated 1 minute per kilobase of target DNA, with a final extension of 2 minutes per kilobase of target DNA. The fewer the number of cycles, the more specific the PCR product, and the less non-specific product formation [Taylor 1991]. For very low levels

of target DNA, more cycles may increase sensitivity, but with a possible loss of specificity. The use of "nested primers" may improve both sensitivity and specificity of the PCR. This means that the product produced after PCR undergoes a second PCR with different oligonucleotide primers which lie within the target DNA sequence. However, this process requires a further manipulation of the product before further amplification, which may increase the risk of contamination, and amplification of contaminating DNA [Jackson *et al.*1991].

#### 1.6.6.6 Detection of the PCR product

The simplest way of detection of PCR fragment is to load the product onto an agarose gel with an appropriate molecular weight marker, and following electrophoresis, staining the gel with ethidium bromide. The product should be readily visible under ultraviolet transillumination [Taylor 1991]. Further confirmation may be made by restriction digestion of the PCR product, and running this product on an ethidium bromide stained agarose gel, the bands visualised under UV transillumination corresponding to expected sizes of the restriction digested product. If a PCR gel is blotted onto nitrocellulose paper, specific radiolabelled probes to detect sequences within the PCR product may be utilised and the desired product visualised by autoradiographic techniques [Southern *et al.* 1975, Thomas 1980]. Alternatively, the radiolabelled product may be cut out of the gel, purified and run on a DNA sequencing gel [Sklar 1993].

# 2. MATERIALS AND METHODS

## 2.1 CELL CULTURE

### 2.1.1 Cell lines

Human melanoma cell lines used were:- SK-19 (melanotic), SK-23 (melanotic), SK-28 (amelanotic) and M5 (amelanotic).

Human epithelial tumour cell lines used were:- RT112 (bladder transitional cell carcinoma), EJ (bladder transitional cell carcinoma), A431 (vulval squamous cell carcinoma) and HT29 (colon carcinoma)

Human neuroblastoma cell lines used were SKNSH and IMR-32.

Human T-lymphocyte cell line used was Jurkat.

Human macrophage cell line used was THP1.

### 2.1.2 Culture conditions

Aliquots of cells which had been frozen in liquid nitrogen were rapidly thawed in a 37°c bath. As soon as thawing took place the cells were transferred into culture medium containing Dulbecco's Modified Eagle's Medium (DMEM) : RPMI 1640 (Gibco)(1 : 1), 5 % Fetal Calf Serum (FCS)(Sera-lab), 1 % L-Glutamine and 1 % Phosphate Buffered Saline (PBS). After brief mixing, the cells were quickly spun down to remove the Dimethyl-sulphoxide (DMSO) and the pellet was resuspended in 20-25 ml of culture medium. The cells were incubated at 37°c for 3 - 5 days by which time the cell layer (for those lines producing confluent tissue layers) was confluent.

### 2.1.3 Cell transfer and cell lifting

Cultures were lifted and transferred by means of trypsin (0.25%) and 5 mmol/l EDTA.

### 2.1.4 Individual cell extraction / pipetting

1:10 dilutions of "lifted" cell suspensions in culture medium were layered onto 5cm petrie dish and, using a fine, heat-drawn capillary pipette, individual cells were micromanipulated under an inverted microscope.

## 2.2 RNA EXTRACTION AND ANALYSIS

### 2.2.1 Whole blood sample preparations.

RNA was extracted from 2 ml. of whole blood by an adaptation of the RNAzol™ (Biotecx)/ UltraSpec™ (Biotecx) technique [Chomczynski and Sacchi 1987, Chomczynski 1989]. These kit preparations contain high molar solutions of guanidinium isothiocyanate, phenol, and either caesium chloride (RNAzol™) or urea (UltraSpec™).

Eight ml. of Ultraspec™ was added to the 2 ml. frozen blood sample, and homogenised by vortexing vigorously until the blood had thawed completely. The cell lysate was then forcefully passed through a pipette several times. The homogenate was then incubated at $4^0$c for 5 minutes to allow complete dissociation of nucleoprotein complexes. The homogenate was then added to 1.2 ml of chloroform, the samples were sealed and shaken or vortexed vigorously for 20 seconds. The mixture was then treated as per 2.2.3.

### 2.2.2 Cell culture samples preparations.

Two ml. of Ultraspec™ was added to pelletted PBS washed cultured cells and homogenised by vortexing vigorously. The cell lysate was then forcefully passed through a pipette several times and then allowed to incubate at 4°c for 5 minutes to allow complete dissociation of nucleoprotein complexes. 0.2 ml. of chloroform was added, the samples were sealed and either shaken vigorously or vortexed for 20 secs. The mixture was then treated as per 2.2.3.

### 2.2.3 RNA dissociation, precipitation and extraction.

The mixture was incubated at 4°c for 5 minutes, and then centrifuged at 12,000 g (at 4°c) for 15 minutes. After this step, the homogenate formed two phases; the lower organic phase containing DNA and protein; the upper aqueous phase containing RNA.

The aqueous phase was transferred to a fresh tube. Care was taken not to disturb the interphase and organic phase. An equal volume of isopropanol was added to the transferred aqueous phase, and the mixture was incubated at 4°c for 15 minutes. The sample then underwent centrifugation at 12,000 g at 4°c for 15 minutes.

A white coloured pellet of RNA was visible at the bottom of the tube following centrifugation. The supernatant was removed and the pellet was washed twice by adding 75 % ethanol, vortexing and centrifugation for 5 minutes at 7,500 g at 4°c for 5 minutes. The pellet was then dried and dissolved in DEPC treated TE (with SDS), and an equal volume of ethanol was added. The sample was stored at -20°c in a 500 ul Eppendorf tube. The final preparation of RNA has an $A_{260}/A_{280}$ ratio of 1.8 - 2.0 [Jackson *et al.*1991, Perry-O'Keefe and Kissenger 1991, Yamguchi *et al.* 1993, Macfarlane and Dahle 1993].

### 2.2.4 RNA analysis

A small known quantity of the RNA preparation was ethanol acetate precipitated and washed twice in 75 % ethanol. After air drying it was dissolved in 15 ul of RNA loading buffer and loaded onto a 0.75 % agarose/MOPS/formaldehyde gel and electropheresed in a 60 volt constant-voltage field [Greenberg and Bender 1991]. The sample was then viewed and photographed under UV light.

#### 2.2.4.1 Northern blot analysis

The ethidium bromide stained gel was destained by washing for 15 minutes in DEPC treated Distilled $H_2O$. The RNA was transferred onto nitrocellulose paper by placing the gel on top of several chromatography papers which were blotting 20 x SSC. The nitrocellulose paper was placed directly on top of the gel and on top of this was laid 20 layers of chromatography paper, 5 cm of paper towels, a flat glass sheet and a weight. This was left to transfer overnight. The nitrocellulose paper was then allowed to air dry and then exposed to ultraviolet light for 5 minutes (RNA side down) to cross link the RNA to the nitrocellulose membrane [Thomas 1980].

#### 2.2.4.2 The 18s $^{32}$P Probe

The 18s probe [Raynal *et al.* 1984] was incubated for 30 minutes with $^{32}P$ labelled ATP and T7 kinase (Pharmacia) before washing and freeze precipitation. The nitrocellulose membrane was then pre-hybridised for 2 hours at 42°c in 30 ml of prehybridisation buffer consisting of 15 ml. formamide, 0.3 ml. ssDNA (10 mg/ml.), 3 ml. Denhardts solution (50 x), 1.5 ml. 10 % SDS, and 1.2 ml. DEPC treated distilled

$H_2O$. It was then hybridised overnight at 42°c in 15 ml. hybridisation solution consisting of 6 ml. formamide, 100 ul. ss DNS (10 mg/ml.), 1.2 ml. Denhardts solution (50 x), 0.6 ml. 10 % SDS, 0.3 ml. 18s $^{32}P$ probe, 0.2 ml. DEPC treated distilled $H_2O$, and 3 ml. Dextran sulphate. The membrane was then washed in 2 x SSC; 0.1 % SDS, twice at room temperature for 15 minutes, and twice at 68°c for 30 minutes. The membrane was then wrapped in plastic film and exposed to radiographic film at - 70°c for 24 hours (see Figure 2).

## 2.3 RNA RECOVERY AND DNA ELIMINATION

### 2.3.1 RNA recovery

An equal volume of ethanol and 5ul 3M Na Ac was added to the sample. The sample was incubated at -20°c and then centrifuged in a Microcentaur™ centrifuge for 15 minutes. The RNA pellet was visible at the bottom of the tube. The supernatant was removed and the RNA was washed twice in 75 % ethanol before vacuum drying.

### 2.3.2 DNA elimination

The pellet was dissolved in 20 ul of DEPC treated distilled water containing 2 %. RNAse inhibitor (RNAGuard™ - Pharmacia). Each sample was incubated at 37°c for 15 minutes with 4ul 10x PCR buffer (100mM Tris pH 8.4, 500mM KCl, 1mg/ml gelatin (or Triton X)), 3.2ul 100mM $MgCl_2$, 4 ul 10 mM dATP, dCTP, dGTP, dTTP, 1 ul (20 u) RNAse Inhibitor (RNAGuard™ - Pharmacia), 1 ul (5 U) FPLC pure DNAse I™ (Pharmacia) and 13.2 ul DEPC treated distilled water. (Total volume 36.4 ul). The DNA digestion step was stopped by heating to 95°c for 5 minutes.

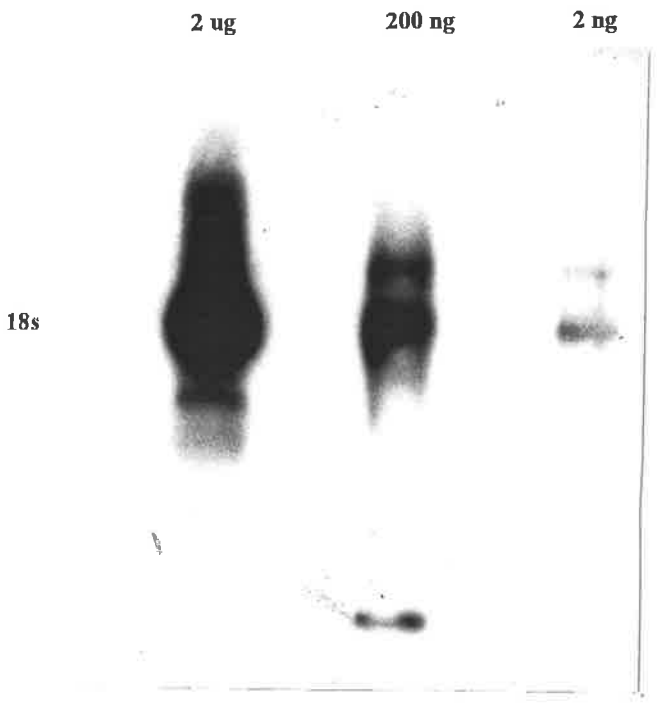



Figure 2. 18s $^{32}P$ probe demonstrating RNA integrity of varying quantities of whole blood RNA extracted by the Ultraspec (TM) technique.

## 2.4 RT/PCR

### 2.4.1 Reverse transcription

Each sample was divided into 2 aliquots of 18.2 ul. 0.5 ul 100um oligodT and 0.5 ul RNAse Inhibitor (RNAGuard™ Pharmacia) was added to each aliquot and 0.8 ul Moloney murine virus Reverse Transcriptase (RT) (Pharmacia), was added to one aliquot only. (A corresponding volume of DEPC treated distilled water was added to the other aliquot.) The total volume for each aliquot was 20 ul. The DNAse treated RNA samples were incubated at 37°c for 60 minutes before the reaction was stopped by heating to 95°c for 5 minutes. A diagramatic representation of the RT step is depicted in Figure 3. This final heating step, to completely inactivate RT, is important because active RT has been shown to partially inhibit the activity of Taq polymerase [Sellner*et al.* 1992].

### 2.4.2 Polymerase chain reaction

10 ul of RT product was mixed with 4 ul PCR buffer, 1 ul of 3' end 20 base oligonucleotide primer, 1 ul of 5' 20 base oligonucleotide primer, 0.5 ul Taq DNA polymerase and 33.5 ul DEPC treated distilled water. The 50 ul mixture was overlaid with 50 ul mineral oil. The mixture underwent 30 cycles of PCR. The cycling parameters were 95°c for 5 minutes (non-cycle), 95°c for 1 minute - 58°c for 1 minute - 72°c for one minute (30 cycles), 72°c for 10 minutes.

### 2.4.3 Gel electrophoresis

5 ul of a mixture of Brilliant Orange™ (Gibco) dye and 15 % Ficoll (Gibco) in TBE buffer was added to the sample. After mixing and centifugation about half the

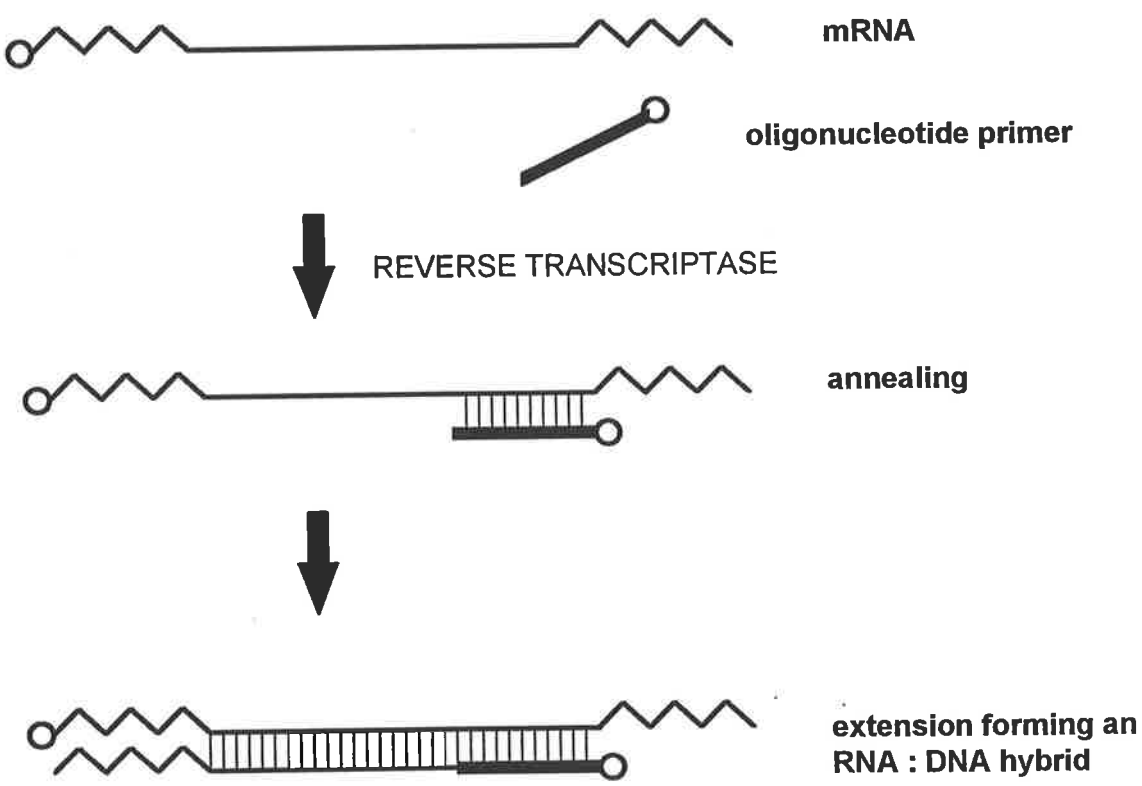



Figure 3. Diagramatic representation of the formation of cDNA via the process of reverse transcription catalysed by the enzyme *reverse transcriptase.*

sample was loaded onto a 2 % agarose in 1 x TBE gel and electrophoresed in an 80 volt constant voltage field.

## 2.5 PCR PRODUCT EVALUATION

### 2.5.1 Ethidium bromide staining and UV illumination

Following electrophoresis, gels were washed in 500 ml 1 x TBE containing 20 ul 100 mmol/l ethidium bromide for 10 minutes and then viewed and photographed under UV light.

### 2.5.2 Southern blot analysis

The gel was destained by washing for 30 minutes at room temperature in denaturing solution. It was then washed twice for 15 minutes at room temperature in neutralising solution. The gel to nitrocellulose transfer was performed as previously described [2.2.4.1, Southern 1975]. Following blotting overnight, the membrane was air dried and then the DNA was crosslinked to the nitrocellulose paper by exposure to ultraviolet light for 2 minutes. It was then possible for radiolabelled or digoxigenin labelled probes to locate specific gene sequences within the blotted sample.

### 2.5.3 Digoxigenin labelled probes and chemiluminescant detection

The membrane was pre-hybridised for 60 minutes at 68°c with 20 ml./100 $cm^2$ hybridisation solution consisting of 50 % v/v formamide, 5 x SSC, 2 % w/v blocking agent (added from a 10 % stock solution), 0.1 % (w/v) N-laurylsarcosine, and 0.02 % (w/v) SDS. The membrane was then hybridised for 60 minutes at 50°c with 2.5 ml/100 $cm^2$ hybridisation solution containing 10 ul digoxigenin labeled DNA probe. The

membrane was then washed at 50°c for 5 minutes in 100 ml 2 x SSC; 0.1 % SDS, then twice at 50°c for 5 minutes in 100 ml 1 x SSC; 0.1 % SDS, and then once at 37°c for 5 minutes in 100 ml 0.1 x SSC; 0.1 % SDS.

The membrane was washed at room temperature for 5 minutes in 100 ml. washing buffer consisting of 0.3 % w/v Tween 20 in Buffer 1 (maleic acid 0.1 mmol/l, NaCl 0.15 mmol/l, pH 7.0). It was then incubated at room temperaure for 30 minutes in 100 ml. of Buffer 2 consisting of Blocking stock solution ( blocking reagent 10 % w/v, in Buffer 1) diluted 1:10 in Buffer 1. It was then incubated for a further 30 minutes at room temperature in anti-digoxigenin-AP Fab fragments diluted 1:10,000 in buffer 2. Unbound conjugate was removed by twice washing with 100 ml. washing buffer for 15 minutes at room temperature. The membrane was equilibrated in Buffer 3 (Tris HCl 0.1 mmol/l, NaCl 0.1 mmol/l, $MgCl_2$ 50 mmol/l, pH 9.5) for 5 minutes and then incubated for 5 minutes at room temperature in AMPPD$^{(R)}$ diluted 1:100 in Buffer 3. Excess liquid was removed from the membrane which is then sealed in plastic. The membrane was then exposed to radiographic film at 37°c for 10 - 60 minutes [Beck *et al.* 1989, Boehringer Mannheim Biochemica 1992].

### 2.5.4 PCR product chemical sequencing

#### 2.5.4.1 Oligonucleotide labelling

100 picomols of 20 base oligonucleotide primer was mixed with 1 ul of 10 x T7 DNA kinase buffer, 1 ul 30 mmol DTT, 1 ul of 20 mmol spermidine, 3 ul of $^{33}P$ ATP, 1 ul T7 ATP kinase (Pharmacia), and 2 ul distilled $H_2O$. This was incubated at 37°c for 30 minutes. The kinase reaction was stopped by heating to 95°c for 5 minutes, and then cooled on ice. 50 ul 4M ammonium acetate was added and then 250 ul of chilled

absolute ethanol was added. The mixture was incubated at - 20°c for 20 minutes and then centrifuged on a microcentaur centrifuge at 13000 rpm for 15 minutes. The supernatant was withdrawn. Confirmation of $^{32}P$ ATP labelled oligonucleotide primer remaining in the tube was made by checking radioactivity with a Coulter Counter. The DNA pellet was washed twice with 75 % ethanol and then vaccuum dried.

#### 2.5.4.2 PCR

The DNA pellet was then dissolved in 5 ul PCR buffer, 2.5 ul 10 mmol $MgCl_2$, 1.25 ul 2 mmol dNTPs, 1 ul oligonucleotide primer corresponding to the DNA strand opposite the DNA strand to which the $^{32}P$ ATP labelled corresponds, 0.25 ul Taq DNA polymerase, and 40 ul distilled $H_2O$. The mixture was covered by 50 ul light mineral oil, and then the mixture underwent 30 cycles of PCR. Brilliant Orange$^{(TM)}$ (Gibco) dye was added to the PCR product which was then electropheresed at a constant voltage of 80 v on a 2 % agarose in 1 x TAE buffer gel until the dye had run 10 cm. The gel was ethidium bromide stained and viewed und ultraviolet light. The presence of radioactivity in any PCR band was confirmed either by checking with a Coulter counter, or by briefly exposing the gel to radiographic film and checking UV ethidium bromide visualised band size against radioactive band size.

#### 2.5.4.3 DNA purification

If the visualised band corresponded to a radioactive band, the band wass cut out of the gel and placed in a 15 ml. conical tube. 1 ml low salt buffer was added and the mixture was heated to 65°c for 1 hour. The product was run through an Elutip-D$^{(TM)}$ (Schleicher and Schewell) minicolumn. Briefly, the column was flushed with low salt

buffer, then high salt buffer. The product now dissolved in 1 ml low salt buffer was loaded onto the minicolumn. 1 ml of low salt buffer was passed through. 600 ul high salt buffer was then pushed through and the product collected was found to contain by far the majority of radioactivity. The product was ammonium acetate/ethanol freeze precipitated and after drying, was dissolved in TE buffer.

#### 2.5.4.4 Maxam-Gilbert sequencing

Four reactions were set up.

(i). Guanine modification

5 ul $^{32}P$ labelled PCR product added to 200 ul Guanine reaction buffer (from Sequenase(TM) kit). This was chilled to 4°c and 1 ul DMS was added. The mixture was incubated for 15 minutes at 20°c and finally the reaction was stopped by the addition of 50 ul 5M ammonium acetate.

(ii). Adenine + Guanine modification

10 ul $^{32}P$ labelled PCR product was added to 10 ml distilled $H_2O$. This was chilled to 4°c and 50 ul 88 % formic acid was added. The mixture was incubated for 15 minutes at 20°c and finally the reaction was stopped by the addition of 200 ul cold 0.5M ammonium acetate.

(iii). Thymidine + Cytosine modification

10 ul $^{32}P$ labelled PCR product was added to 10 ml distilled $H_2O$. This was chilled to 4°c and 30 ul hydrazine was added. The mixture was incubated for 15 minutes at 20°c and the reaction was finally stopped by the addition of 200 ul cold 0.5M ammonium acetate.

(iv). Cytosine reaction

5 ul $^{32}P$ labelled PCR product was added to 15 ul 5M NaCl. This was chilled to 4°c and 30 ul hydrazine was added. The mixture was incubated for 15 minutes at 20°c and the reaction was finally stopped by the addition of 200 ul cold 0.5 M ammonium acetate.

Each reaction was then dealt with in the same way. 1 ul tRNA was added to each mixture and then 900 ul ice cold 90 % ethanol was added. The mixture was incubated for 10 minutes at -20°c and then centrifuged for 15 minutes. The supernatant was removed and the pellet dissolved in 50 ul 0.5M ammonium acetate. 250 ul ice cold 90 % ethanol was added and the mixture was again incubated for 10 minutes at -20°c. Following centrifugation and removal of supernatant, the pellet was washed twice in 75 % ethanol. After the pellet had vacuum dried, it was dissolved in 100 ul 1M piperidine. It was then incubated at 95°c for 30 minutes and then immediately frozen to -20°c. The pellet was then vacuum freeze dried overnight. The pellet was then dissolved in 30 ul distilled $H_2O$, re-frozen and vacuum freeze dried again. This was repeated once more. The final pellet was then dissolved in 8 ul sequence loading buffer and frozen until time for loading onto an acrylamide sequencing gel [Maxam and Gilbert 1980, Cherry 1991].

#### 2.5.5.5 Sequencing gel

A 150 ml gel consisted of 8.55 g acrylamide (Gibco), 0.45 g bis-acrylamide (Gibco), 67.5 g urea (Gibco) and 15 ml 10 x TBE buffer made up to 150 ml by distilled $H_2O$. Just prior to pouring the gel 150 ul ammonium persulphate and 150 ml Temed$^{(TM)}$ was added. After the gel had set each sequence reaction product was run in a separate column of the acrylamide sequencing gel. The product was electropheresed at constant voltage for 150 minutes. Following this, the gel was transferred onto paper and vacuum

dried. The presence of radioactivity on the gel was confirmed with the Coulter counter. The gel was then exposed to radiographic film at - 70°c for 24 - 72 hours. After developing the film the banding sequence of the four columns enabled determination of the DNA base sequences of the PCR product [Cherry 1991].

## 2.6 BLOOD SPIKING

Tumour cell line cells were separated using trypsin [2.1.3]. The cells were then suspended in serum free medium. Under direct microscopy individual tumour cells were pipetted from the suspension into a defined aliquot of whole blood (collected into sodium heparin). The tumour cell spiked blood samples could then be immediately used for monoclonal antibody staining, or frozen and used later in RNA extraction.

## 2.7 WHOLE BLOOD CYTOSPINS

### 2.7.1 Direct red cell lysis technique

Ten ml. of 1 x lysing buffer was added to 1 ml. of whole blood in a 15 ml. tube. The tube was vortexed briefly and then allowed to stand at room temperature for 15 minutes. Red cell lysis was deemed to have occurred if after this time, the tube was translucent. The tube was then centrifuged at 1500 rpm for 7 minutes. A pellet of cells was then easily visible at the bottom of the tube. The red coloured supernatant was removed and discarded. The pellet was resuspended and washed in PBS and again centrifuged at 1500 rpm for 7 minutes. If there was any red discolouration of the pellet after the supernatant was removed, the pellet was again washed in PBS and centrifuged as before. The pellet was finally resuspended in 500 ul of PBS.

### 2.7.2 Ficoll cell separation

One ml of whole blood was carefully layered onto 3 ml of Ficoll Hypaque 1077(TM) in a 15 ml tube. The tube was then centrifuged at 400 g for 30 minutes. Following this a large red cell pellet had formed at the bottom of the tube, the "mononuclear" cell layer formed an opaque band midway up the upper transparent region. The supernatant above the mononuclear band was removed and discarded. The mononuclear band was then gently pipetted off and transferred to a new tube. The rest of the transparent fluid and the red cell pellet was discarded. The mononuclear cells were washed twice in PBS and finally centrifuged and resuspended in 500 ul of PBS.

### 2.7.3 Cytospin technique

Each 500 ul sample from either the direct red cell lysis or Ficoll separation techniques (suspended in PBS) was divided into 5 x 100 ul aliquots. Each 100 ul aliquot was loaded onto a cytospin chamber. Cytospin was performed at 500 rpm for 5 minutes. Each 1 ml of blood was now represented by 5 cytospin preparations on individual glass slides. Each slide was acetone fixed and air dried.

## 2.8 FLUORESCENT ANTIBODY LABELLING

### 2.8.1 Labelling technique

About 25 ul of primary antibody was dropped onto each cytospin preparation ensuring that each cytospin was completely covered. This was allowed to incubated at room temperature in an humidified container for 60 minutes. The cytospin was then washed 3 times with PBS and then washed twice with methanol:acetone and allowed to air dry. About 25 ul of secondary antibody completely covered the cytospin preparation

and the slide was allowed to incubate in an humidified container for 30 minutes. The cytospin was then washed 3 times with PBS and then washed twice with distilled water before air drying. Hoechst(TM) nuclear stain was added to the penultimate wash.

### 2.8.2 Cytological examination

A coverslip then covered the preparation which was then viewed using oil immersion under ultraviolet light. Genuine fluorescence secondary to antibody labelling was distinguished from auto-fluorescence by checking under FITC (red) light. Positive nuclear staining under blue light provided confirmation that the fluorescence was cellular and not artifact.

## 2.9 BLOOD COLLECTION

### 2.9.1 Samples for RNA analysis (RT/PCR)

Four ml. of whole blood was collected from each volunteer or patient. Each sample was divided into 2 ml. aliquots and aliquoted into EDTA and immediately frozen. It was then stored at -80°c until the time when it would undergo RNA extraction. Whole blood was stored rather than pelletted cells to minimise the risk of cell loss during handling.

### 2.9.2 Samples for immunofluorescent antibody staining

Four ml. of whole blood was collected in heparin (to prevent coagulation) from each volunteer or patient and immediately separated into 2 x 2 ml aliquots. Samples were prepared for analysis within 1 hour of venepuncture.

# 3. MALIGNANT MELANOMA

## 3.1 INTRODUCTION

### 3.1.1 Clinical scope of malignant melanoma

Malignant melanoma presently accounts for approximately 8 % of all new malignancy diagnosed in Australia, excluding early stage squamous or basal cell skin cancer [Sth. Aust. Cancer Registry 1994], with a crude incidence of 36 per 100,000 people. The overall death rate due to melanoma is 4.3 per 100,000 for males and 2.4 per 100,000 for females, with an overall 5 year survival of greater than 90 %. The crude incidence of melanoma in Australia has increased steadily over the past 20 years. However, the 5 year survival rate has improved during this time. This is believed to be due to the greater emphasis given to early detection to improve case survival [Sth. Aust. Cancer Registry 1989, 1994].

The incidence of melanoma in fair skinned populations increases with close proximity to the equator [Elwood and Lee 1975, Beardmore 1972], implicating sunlight exposure as an important risk factor. The disease is relatively uncommon amongst darker skinned populations whether or not they live in close proximity to the equator. The preponderance of disease in city dwellers in the at risk populations and the observation that melanoma patients have experienced a greater number of episodes of sunburn compared with case controls has implicated intermittent, brief, intense sun exposure as a significant risk factor [Urbach 1983, D'Arcy,*et al.* 1983].

The prognosis of early stage melanoma is tightly linked to the thickness of the primary melanoma. In multivariate analysis, primary melanoma thickness has been shown to be the best determinant of survival [Day *et al.* 1982]. For stage 1 (no evidence of nodal or distant metastases) disease, primary melanomas less than 0.75 mm thick

were associated with a 5 year survival approaching 100 %. With increased thickness of the primary melanoma, the 5 year survival declines, so that for tumours greater than 3 mm in thickness, the 5 year survival falls to less than 50 %. For patients with regional nodes involved at diagnosis, the prognosis is much poorer, with 5 year survival of 25 - 30 % [Tillman *et al.* 1991]. For patients with distant metastatic disease, median survival falls to about 6 months only [Amer *et al.* 1979, Koh 1991].

The key to treatment of melanoma therefore is early diagnosis and adequate surgical excision. The diagnosis is usually able to be confirmed histologically by either presence of melanin in cells or S100 positive staining with immunohistochemical techniques [Gatter *et al.* 1985]. Melanoma remains one of the generally chemo-insensitive malignancies, with most commonly used chemotherapy regimens producing responses in only 15 - 30% [Commis 1976, Cohen *et al.* 1986, York and Foltz 1988]. Individual studies of treating metastatic melanoma with either hormonal manipulation, biological response modifiers (interferons and interleukin 2), or high dose chemotherapy with bone marrow support have shown encouraging response rates [McClay *et al.* 1989, Lakhami *et al.* 1990, Dutcher *et al.* 1991]. However, no survival improvement has been demonstrated. The lack of effectiveness of such strategies is also demonstrated by the lack of improvement in survival in patients with high risk early stage disease treated with these modalities in the adjuvant setting. Bone/bone marrow, lung, liver, and brain are common distant metastatic sites for metastatic melanoma. Each of these metastatic sites is reached haematogenously. For this chemo-insensitive tumour, haematogenous spread of disease is its most life-threatening problem.

Malignant melanoma was chosen for the initial studies of RT/PCR gene expression because of the potential public health importance as well as the availability of a suitable target (tyrosinase) for RT/PCR.

### 3.1.2 Melanoma cell phenotypes

Melanoma usually evolves in a step-wise manner, ie:- melanocyte -- naevus -- dysplastic naevus -- superficial melanoma (radial growth phase) -- invasive melanoma (vertical growth phase) -- metastatic melanoma; with each step usually being clinically detectable [Clark *et al.* 1984, Clark 1991]. This evolution enables stage specific events to be investigated both in vivo and in vitro. In vitro characteristics of normal melanocytes and naevus cells tend to be markedly different from both primary and metastatic melanoma cells. Whereas normal melanocytes and benign naevus cells tend to have bipolar or tripolar morphology, slow growth rate with limited doublings, no chromosomal abnormalities, low colony forming efficiency in soft agar, and no tumourigenicity in athymic nude mice, primary melanoma and metastatic melanoma cells tend to have cuboidal or spindle morphology, rapid growth rate with infinite doublings, frequent chromosomal abnormalities, relatively high colony forming efficiency in soft agar and high tumourigenicity in athymic and immunosuppressed nude mice [Selby 1980, Herlyn M.*et al.* 1990, Herlyn 1990].

### 3.1.3 Melanocyte growth factor requirements

Melanocytes and benign naevus cells require at least four mitogens for continuous growth. These are insulin/insulin like growth factor (IGF), α-melanocyte stimulating hormone (α-MSH), basic fibroblast growth factor (bFGF), and 12-0-

tetradecanoyl-phorbol-13-acetate (TPA). This is in contrast to primary melanoma cells which require only insulin/IGF, and metastatic melanoma cells which do not require exogenous growth factors [Herlyn M 1990]. Metastatic melanoma cells produce a variety of growth factors, which are not produced by normal melanocytes and naevus cells. This phenomenon probably underlies the absence of growth factor requirements of the malignant cells. These growth factors not only have effects on the melanoma cells but also may have paracrine effects on normal cells in the surrounding stroma [Shih and Herlyn 1993]. These paracrine effects may include the enhancement of growth stimulation, angiogenesis, motility, and the production of certain growth factors by stromal cells [Herlyn D. *et al.* 1990, Kath *et al.* 1991].

Improved understanding of the function of the various growth factors in the control of cell phenotypes and growth will hopefully produce better insights into the pathogenesis of the evolution of melanoma and will also hopefully provide a framework for further basic scientific approaches to the diagnosis and treatment of malignant melanoma.

### 3.1.4 Proto-oncogene activation in melanoma

Several mechanisms have been shown to be involved in the activation of proto-oncogenes, thus enhancing oncogenic potential. Such mechanisms include mutation, amplification, promoter insertion, and deletion [Klein and Klein 1985]. The discovery of transforming genes within the DNA of melanoma cells was demonstrated by Sekiya*et al.* [1983], Albino *et al.* [1984] and Padua *et al.* [1985]. In each of these studies, the transforming genes were shown to be members of the human *ras* family.

### 3.1.5 *ras* oncogenes in melanoma

The majority of oncogenes discovered in melanoma cell lines were of the *ras* family [Lebowitz 1983, Bishop 1983, Cooper 1984, Albino *et al.* 1989, Dicker *et al.* 1990]. The frequency of oncogene activation was about 13 %, which is not dissimilar from the frequency observed in other adult solid tumours. The actual role of the *ras* oncogene in melanoma development is unclear, however there is some evidence based on experimentation of different cell lines from the same patient, that transforming *ras* gene activation occurs relatively late in the course of tumour progression and is not involved in tumour initiation, maintenance, or early metastasis development [Dracopoli *et al.* 1985]. Certainly there is evidence to show that *ras* oncogenes have a pronounced effect on melanocytes, producing effects on morphology, antigen expression and growth and chromosomal changes which may be necessary for progression to malignant phenotype [Albino 1988]. However, in light of the apparent low frequency of *ras* mutations in melanoma and evidence that suggests that their development is a late phenomenon, there seems little potential for the routine use of *ras* mutation detection in the analysis of patients with melanoma.

### 3.1.6 Non-*ras* oncogenes in melanoma

Other oncogenes have been extensively examined in relation to the pre-malignant - primary - metastatic melanoma pathway. Minor alterations have been shown with regard to the *c-myb*, proto-oncogene [Yakota *et al.* 1986, Linnenbach *et al.* 1988]. This may be significant as this oncogene lies on chromosome 6 [Dalla-Favera *et al.* 1982], which has been reported to be altered in a non-random manner in melanoma

[Trent *et al.* 1983, Meese *et al.* 1989]. The true significance of these abnormalities in relation to the development of melanoma is not yet defined.

### 3.1.7 Chromosomal abnormalities in melanoma

Karyotypic abnormalities for human melanoma are very variable. Kacker *et al.* [1990] analysed a number of human melanoma cell lines which were passaged through nude mice and discovered multiple chromosomal abnormalities. The most frequently involved chromosomes were 1, 6, 7, and 9. Abnormalities, especially deletions involving the long arm of chromosome 6 have been reported most frequently.

The long arm of chromosome 6 is functionally important as it contains several important gene sequences including the proto-oncogenes *ros*, *myb*, and *mas1* [Trent *et al.* 1989]. To date, there has been no confirmation that abnormalities within these gene sequences predisposes to the malignant phenotype, although a role in tumour progression rather than initiation has been proposed [Kacker *et al.* 1990].

About 10 % of malignant melanoma cases are inherited in a familial setting. Individuals affected in this clinical setting, have an abundance of naevi widespread over the body. This clinical syndrome known as "dysplastic naevus syndrome" appears to be inherited as an autosomal dominant mode [Bale *et al.* 1986]. Gene linkage studies have implicated the 9p21 loccus as a potential candidate gene for familial malignant melanoma [Cannon Albright *et al.* 1992, Fountain *et al.*1994]. More recently, a multiple tumour suppressor gene (MTS 1) has been localised within the 9p21 locus, so that abnormalities within this locus may be significant in the progression from dysplastic naevus to malignant melanoma [Kamb *et al.* 1994]

The frequency of the non-random chromosomal abnormalities in clinical samples of human melanoma is probably less than 20 %, which corresponds to the relatively low frequency of oncogene activation observed in human melanoma cell lines by Albino *et al.* [1984], and Kefford [1992]. As such, whilst important progress is being made in the understanding of the basic biology of melanoma by continued study of these genetic changes, it appears that chromosomal abnormalities as detected by karyotyping presently cannot be utilised as reliable, clinically useful markers of disease.

### 3.1.8 Differentiation of melanoma and phenotype

Albino [1988] also showed no significant amplification or rearrangement of 17 known oncogenes in both cultured melanoma and non cultured primary and metastatic melanoma. Additionally there were no apparent increases in the level of transcribed mRNA of these oncogenes by northern blot analysis. However these experiments were able to detect degrees of differentiation based upon surface antigen phenotypes [Dippold *et al.* 1980, Old 1981]. Early or foetal type melanomas expressed "early" markers and showed epithelial morphology, lacked pigmentation and did not express tyrosinase. In contrast, melanomas expressing late or adult markers, had spindle shaped or dendritic morphology, were pigmented and expressed high levels of tyrosinase [Albino 1988].

### 3.1.9 Tyrosinase metabolism

Tyrosinase is an important rate limiting enzyme in the synthesis of melanin from tyrosine. The enzyme tyrosinase catalyses the oxidation of L-tyrosine to L-DOPA, and the dehydrogenation of DOPA to DOPAquinone. DOPAquinone is very reactive and quickly enters the melanin pathway [Mason 1948]. Melanin pigments may be

cutaneous or neuronal. The exact function of neuronal melanin is not fully understood. Cutaneous melanin, which is found in the skin, the hair follicles, and the retina is synthesised and secreted by melanocytes (specialist pigment cells) [Rawles 1948]. Cutaneous melanin is not known to be produced by any other cell type.

### 3.1.10 Tyrosinase expression in malignant melanoma

The majority of melanoma cell lines produce the melanin pigment which can be detected by routine histology and immunocytochemical techniques [Thomson *et al.* 1985]. Although some melanoma cell lines do not synthesise and secrete melanin pigment, they may still be high expressers of tyrosinase mRNA [Albino 1988]. The human tyrosinase cDNA sequence (pmel 34) was first reported to be located on the TYR locus of human chromosome 11 [Kwon *et al.* 1987, Barton *et al.* 1988]. The genomic organisation of the tyrosinase gene has been identified [Tomita *et al.* 1989, Kwon 1993], as has its transcriptional control by melanogenic inhibitors [Ando *et al* 1993]. This makes tyrosinase mRNA an ideal target for identification of melanotic cells.

### 3.1.11 Tyrosinase as a tumour associated antigen

Numerous studies have recently examined the autologous CD8+ T-cell mediated response to melanoma, with a picture emerging of melanoma expresssing multiple T-cell defined epitopes. Several of these peptide epitopes have now been identified using cDNA exprerssion cloning and protein biochemical approaches [Van den Eynde *et al.* 1989, Bakker *et al.* 1994, Coulie *et al.*1994, Kawakami *et al.* 1994, Wolfel *et al.* 1994]. Whilst the majority of anti-melanoma cytolytic T cell clones derived from HLA-A2+ tumour infiltrating lymphocytes seem to react against Melan-A

derived epitopes [Kawakami *et al.* 1994], and a significant minority react against gp 100 derived sequences [Coulie *et al.* 1994], a smaller proportion of clones appear to react against tyrosinase derived sequences [Wolfel *et al.* 1990]. The tyrosinase antigens, like Melan-A and gp 100 antigens, show restricted expression in melanosomes present in pigmented cells such as normal melanocytes, retinal tissue and melanoma cells. Melanoma expression of these gene products seems to be notably increased in comparison to homologous tissue controls [Bakker *et al.* 1994]. This suggests that cytolytic T cells may preferentially react with melanoma cells in comparison with normal melanocytic cells, minimising the degree of pathological autoimmunity.

Hypopigmentation (either generalised or localised) is not an uncommon occurrence in patients with melanoma, and the development of such hypopigmentation in untreated patients has been reported to be associated with improved survival when compared to patients without hypopigmentation of similar stage of disease [Kelly *et al.* 1982, Nordlund *et al.* 1983]. This may be the clinical manifestation of a spontaneously developing T cell response against tyrosinase peptides of both melanoma tissue and normal melanocytes.

The potential for using tyrosinase peptides to induce a cytolytic T cell response against melanoma is the subject of ongoing investigation and results of such studies are awaited with great interest.

### 3.1.12 Tyrosinase as a potential marker of blood borne melanoma metastases

Malignant melanoma is a typical example of a tumour which produces most of it's life threatening effects due to blood borne metastases. Melanoma has a large range of

metastatic sites, the majority of which are reached haematogenously. As previously discussed, there are no DNA changes which are regularly observed in clinical melanoma cases. Therefore, direct PCR upon DNA from melanoma cells (to detect differences between melanoma DNA and normal cell DNA) is not going to be a reliable, sensitive method of detecting minimal disease. As tyrosinase mRNA is believed to be expressed only by cells of melanocytic origin, and given that normal melanocytes are not believed to circulate in the blood, the detection of cells in peripheral blood expressing tyrosinase mRNA may indicate the presence of haematogenous melanoma metastases. As metastatic melanoma remains incurable, the detection of melanoma micrometastases in peripheral blood may provide significant prognostic information. It may also have critical implications with regard to extent of surgery in apparently early stage patients as well as the selection of patients for novel adjuvant therapies.

## 3.2 METHODS AND RESULTS : TYROSINASE RT/PCR

### 3.2.1 Tyrosinase primers

The primer sequences for the tyrosinase cDNA were derived from a previously published sequence for human tyrosinase cDNA [Kwon *et al.* 1987]. Four sets of primers chosen were :- HTyr 1 (outer sense) = TTGGCAGATTGTCTGTAGCC; HTyr 2 (outer antisense) = AGGCATTGTGCATGCTGCTT; HTyr 3 ("nested" sense) = GTCTTTATGCAATGGAACGC; HTyr 4 ("nested" antisense) = GCTATCCCAGTAAGTGGACT. The primers spanned at least one intron sequence (outer primers spanning 2 intron sequences) so that genomic DNA would not be amplified [Rappolee *et al.* 1989, Grillo and Margolis 1990]. The outer primers were calculated to produce a PCR product of 284 bases. The inner or nested primers were

calculated to produce a PCR product of 207 bases (see Figure 4). RNA preparation, the basic RT/PCR process, and evaluation of PCR products were carried out as per Chapter 2.

### 3.2.2 Tyrosinase detection in melanoma cell lines

The human cDNA clone Pmel34 (tyrosinase) was used to confirm that PCR with each set of primers gave product bands of expected sizes. Thirty cycles of PCR following RT was performed using outer primers initially, and a 1% dilution of this PCR product underwent a second 30 cycle round of PCR using inner primers. Both melanotic and amelanotic cell line RNA was evaluated for tyrosinase positivity. In the melanotic cell lines SK-mel-19 and SK-mel-23 and the amelanotic cell line SK-mel-28, tyrosinase mRNA was readily detectable, confirming that melanin production per se was not necessary for tyrosinase mRNA detection. One amelanotic cell line (M5) did not appear to express tyrosinase mRNA. (see Figure 5)

Maxam - Gilbert chemical sequencing of the 207 base product was performed and the following sequence was readily identifiable :-

GAC AAA TCC AGA ACC CCA AGG CAC CCC TCT TCA GCT GAT GTA GAA TTT TGC CTG AGT TTG ACC CAA TAT GAA TCT GGT TCC ATG GAT AAA GCT GCC AAT TCC AGC TTT AGA AAT ACA CTG G (see Figure 6) This 121 base sequence corresponds directly with part of the expected sequence of tyrosinase cDNA, 54 bases from the nested sense primer.

The sensitivity of detection of the RNA as determined by serial dilution experiments. SK19 RNA was dissolved in a total of 1 ug tRNA with SK19 RNA levels ranging from 1 ug to 1 pg. The sensitivity was 10 nanograms of cellular mRNA for a

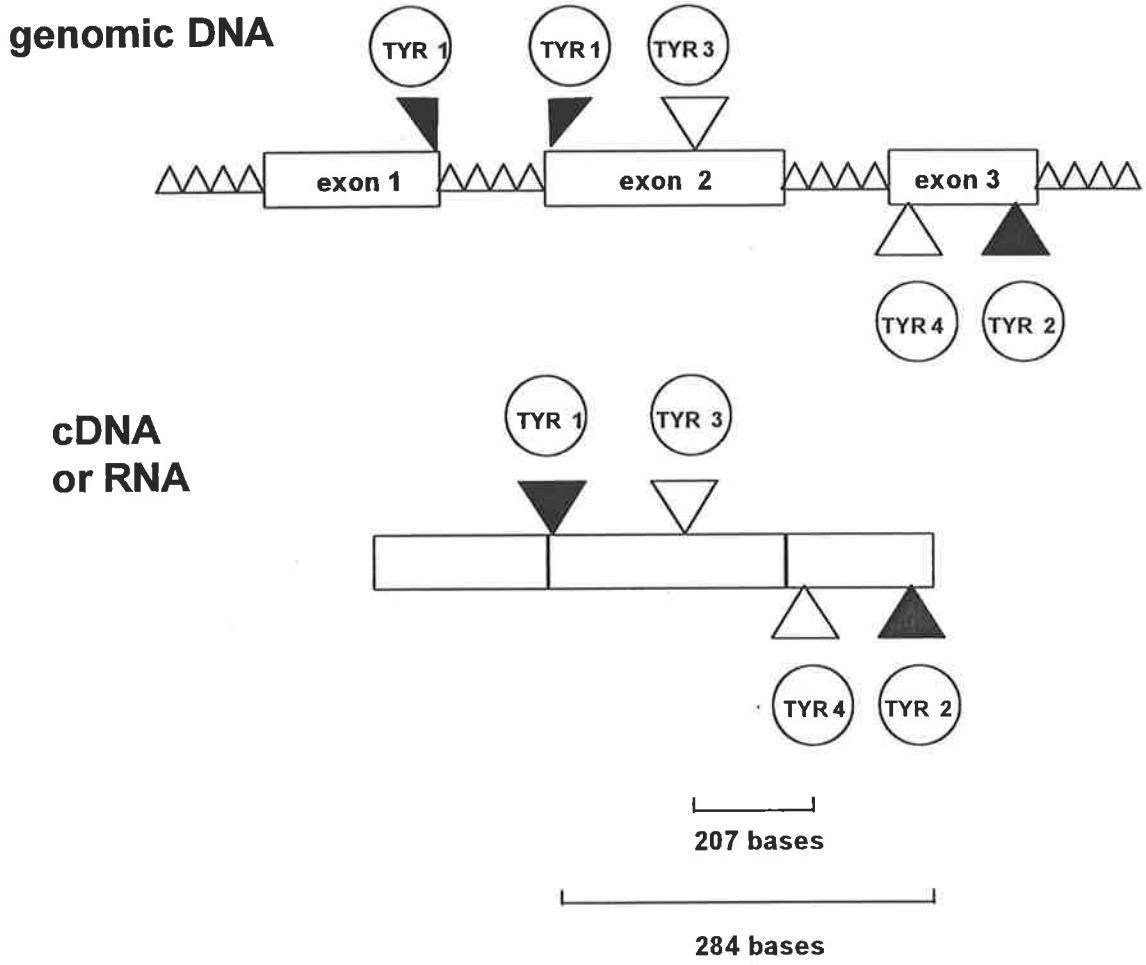



Figure 4. Depicting relative postions of human tyrosinase PCR primers 1, 2, 3 and 4 on both tyrosinase genomic DNA and the RNA or cDNA transcript. As each pairing of PCR primers spans an intron sequence any amplified genomic DNA will not produce a PCR product of expected sequence length.

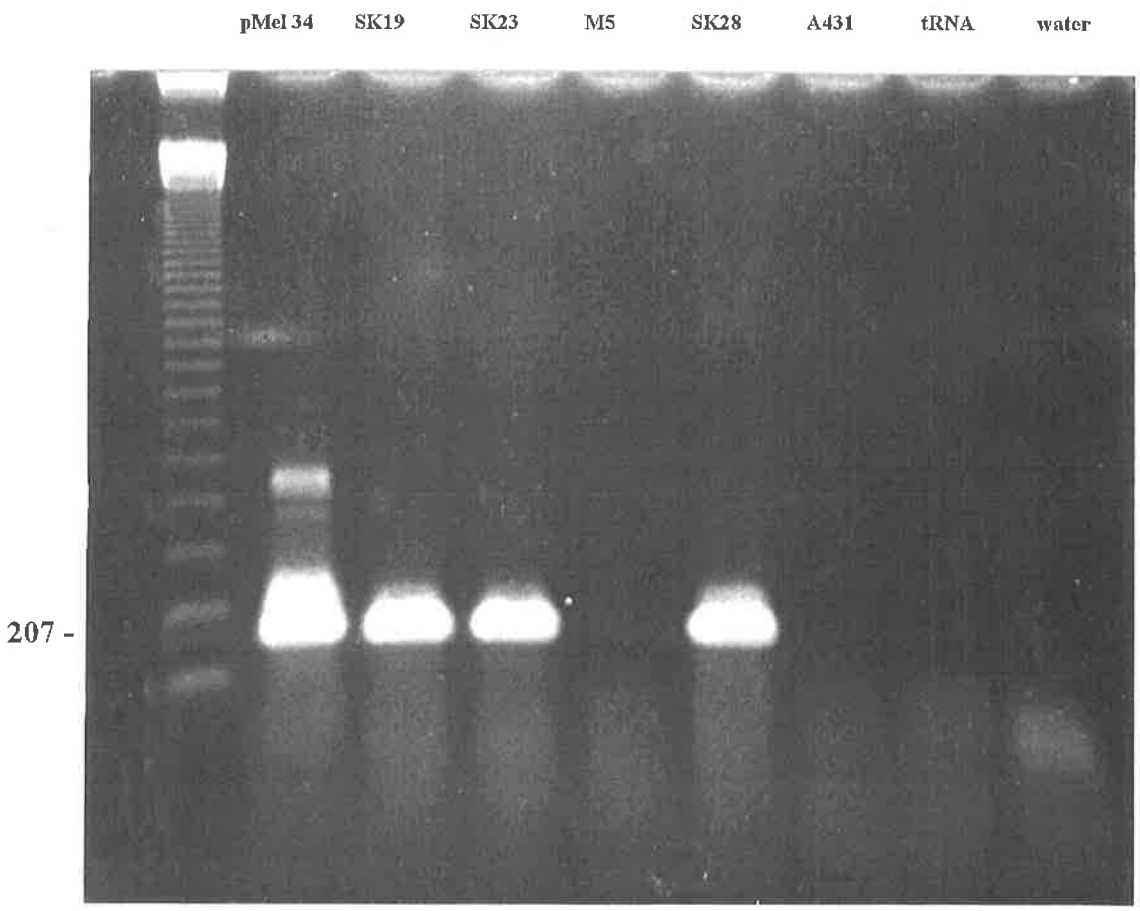



Figure 5. Tyrosinase expression in melanoma cell lines SK19, SK23, and SK28 (but not M5) demonstrated by 207 bp product using RT/PCR with nested primers. Vulval cancer cell line A431, tRNA and water used as negative controls .

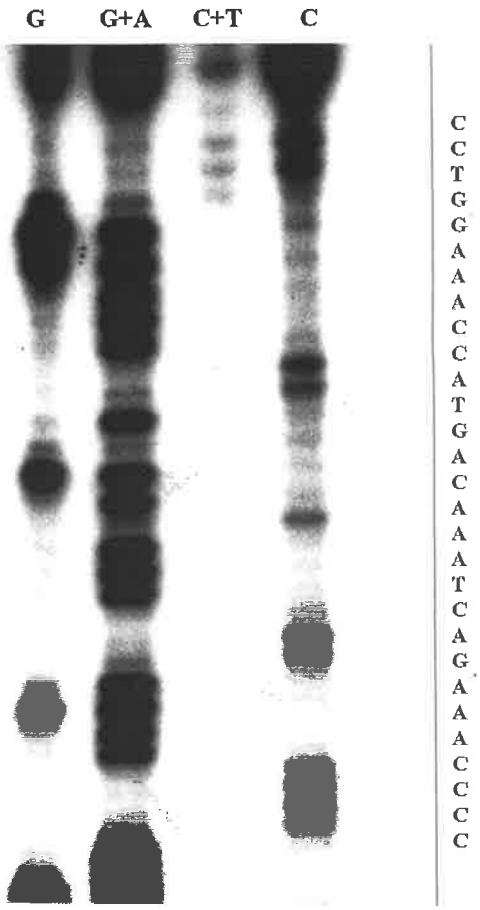



Figure 6. Part of the sequence of the Tyrosinase PCR product as determined by the Maxam-Gilbert method of chemical sequencing.

single round of RT/PCR of 30 cycles with outer primers only. Using the dual round RT/PCR technique in which 5 ul of a 1 % dilution of the first round PCR product underwent 30 cycles of PCR using nested primers, the sensitivity was between 1 and 10 picograms of cellular RNA (see Figure 7). (In this particular experiment, the PCR reaction was run concurrently with a PCR reaction for the globin protein using globin primers as an internal control of RNA patency). This level of sensitivity was confirmed with the detection tyrosinase mRNA of one spiked tumour cell in one ml. of peripheral blood (data not shown).

Initially, the dual round (with nested primers) method of RT/PCR was used for both preclinical and clinical evaluation, because of its sensitivity and lack of background noise seen on gels analysed. However, an unacceptable rate of false positivity became apparent in "negative control" samples (see Figures 8 and 9). It was not clear whether this problem was related to accumulation of carry-over of product from the first round of PCR or whether it was due to general laboratory contamination by cloned pMel34 cDNA which had taken place in an adjacent laboratory. Despite the most strict isolation techniques and anti-contamination procedure, intermittent false positivity occurred. By using the same strict isolation techniques and anti-contamination procedures, by DNAse treating all RNA samples as decribed in 2.3.2 (see Figure 10), and by changing to the single round (50 cycle) RT/PCR technique using the inner primers only, sensitivity of between 1 and 10 pg (or 1 cell) in 2 ml. whole blood was able to be maintained with no evidence of false positivity (see Figures 11a and 11b). Using the same tumour cell spiking techniques, a level of sensitivity between 2 and 5 cells per 2 ml. of whole blood was achieved (see Figures 12 and 13).

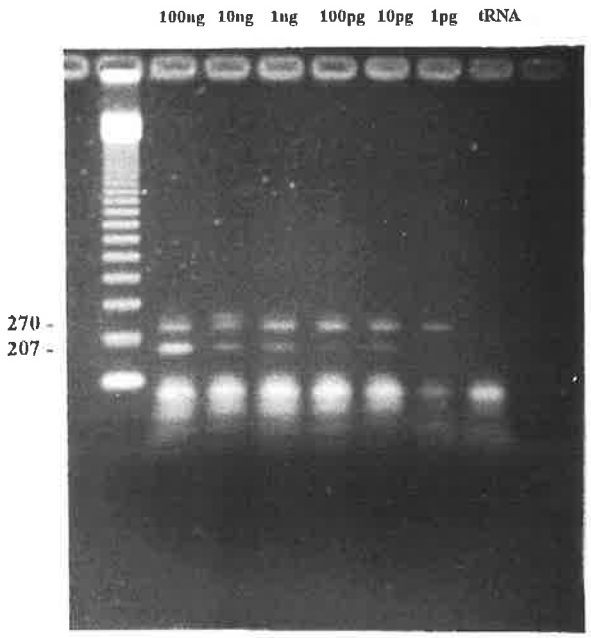



Figure 7. Serial dilutions of SK19 RNA dissolved in 1ug whole blood RNA following RT/PCR, dual rounds of 30 cycles, with nested tyrosinase primers and using globin primers (which produce a 270 bp product) as an internal positive control.

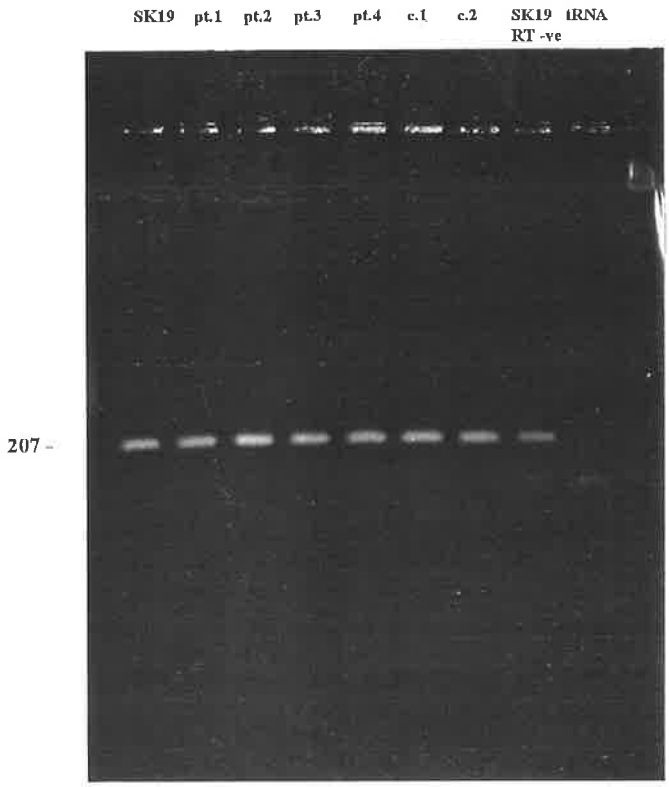



Figure 8. Several false positive results observed following RT/PCR using two rounds of 30 cycles with nested tyrosinase primers in whole blood RNA samples from 4 patients with metastatic melanoma (pts. 1 - 4), 2 healthy volunteers (c. 1, 2), normal blood spiked with 1ng SK 19 RNA (one sample in which reverse transcriptase was omitted as a negative control), and normal blood spiked with tRNA. All RNA samples except the sample of normal whole blood to which tRNA was added gave positive results

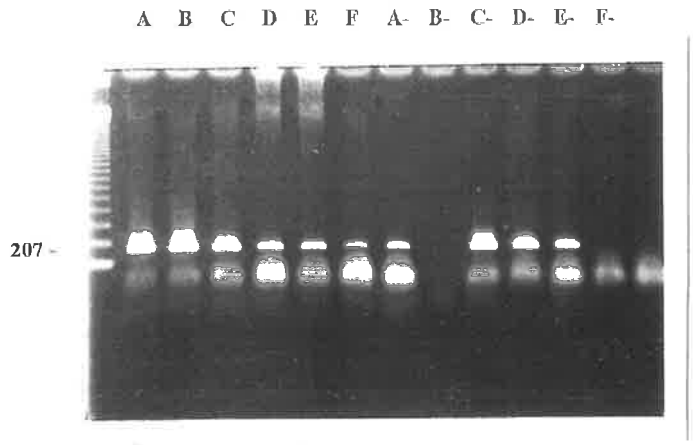



Figure 9. RT/PCR with dual round (30 cycles) of PCR with tyrosinase primers performed on RNA extracted from melanoma cell lines SK 28 (A and A-) and SK 19 (B and B-), neuroblastoma cell line NSH (C and C-), T lymphocyte cell line Jurkat (D and D-), vulval carcinoma cell line A431 (E and E-) and tRNA (F and F-). Each sample divided into two aliquots and only one underwent the reverse transcription step ("-" indicating RT negative). Positive results seen in all samples except SK 19 RT-ve and tRNA RT -ve, demonstrating widespread contamination with tyrosinase PCR product.

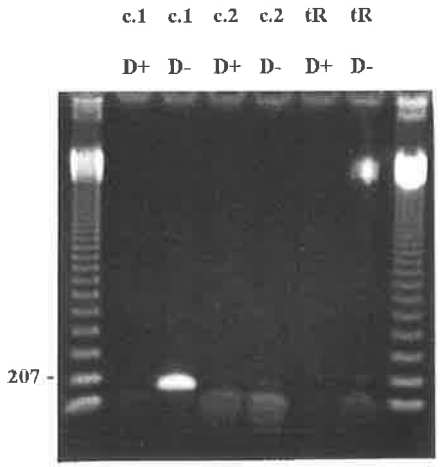
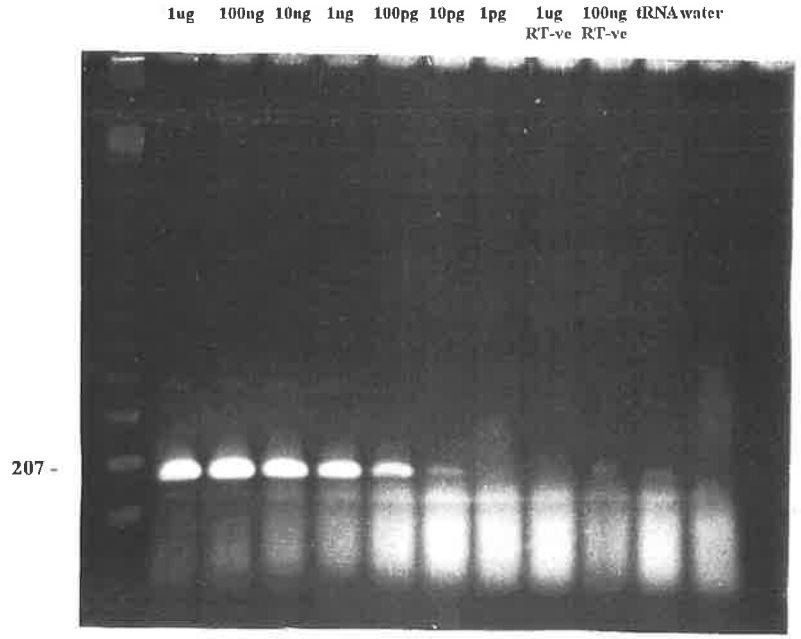
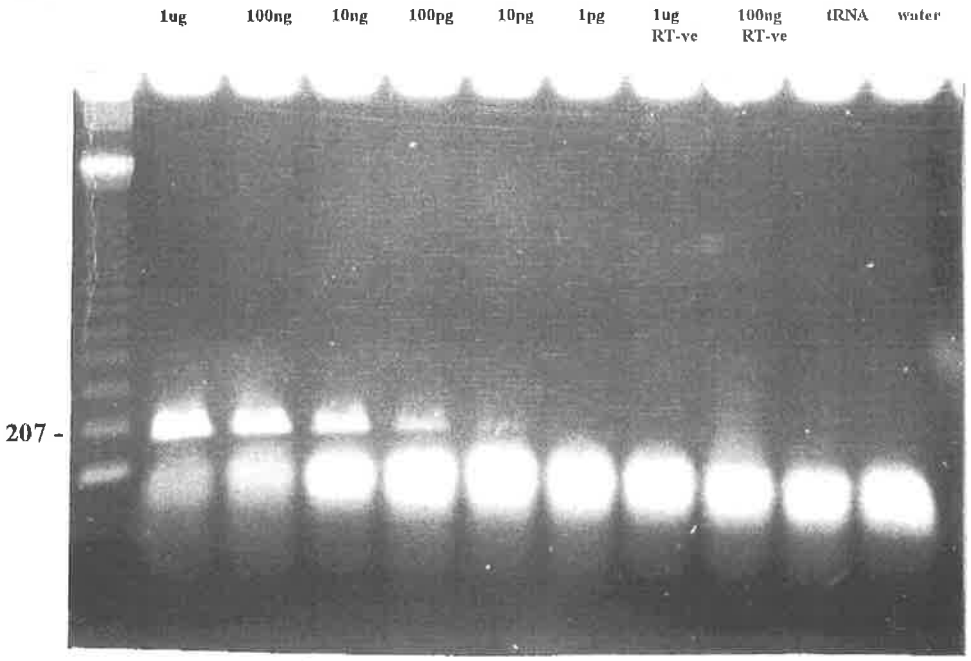



Figure 10. Single round RT/PCR (50 cycles) with tyrosinase primers on RNA extracted from whole blood samples from healthy volunteers (c. 1, 2), and tRNA (tR). Each sample was divided into two aliquots, only one of which underwent DNAse treatment. Positive results seen in both blood samples which did not undergo DNAse treatment demonstrate the presence of contaminating tyrosinase PCR product, and therefore the need for DNAse treatment of all samples.

A.




B.




Figures 11a and 11b. Serial dilutions of SK19 RNA in 1 ug whole blood RNA following RT/PCR with inner tyrosinase primers for a single round of 50 cycles. 100 ng tRNA dissolved in 1 ug whole blood RNA and water (alone) were used as negative controls. All samples were DNAse treated. Each sample was divided into 2 aliquots, only one of which underwent the RT step. The 207 bp PCR product was detected at a level between 1 and 10 pg of RNA. There were no false positive results in any of the RT negative samples.

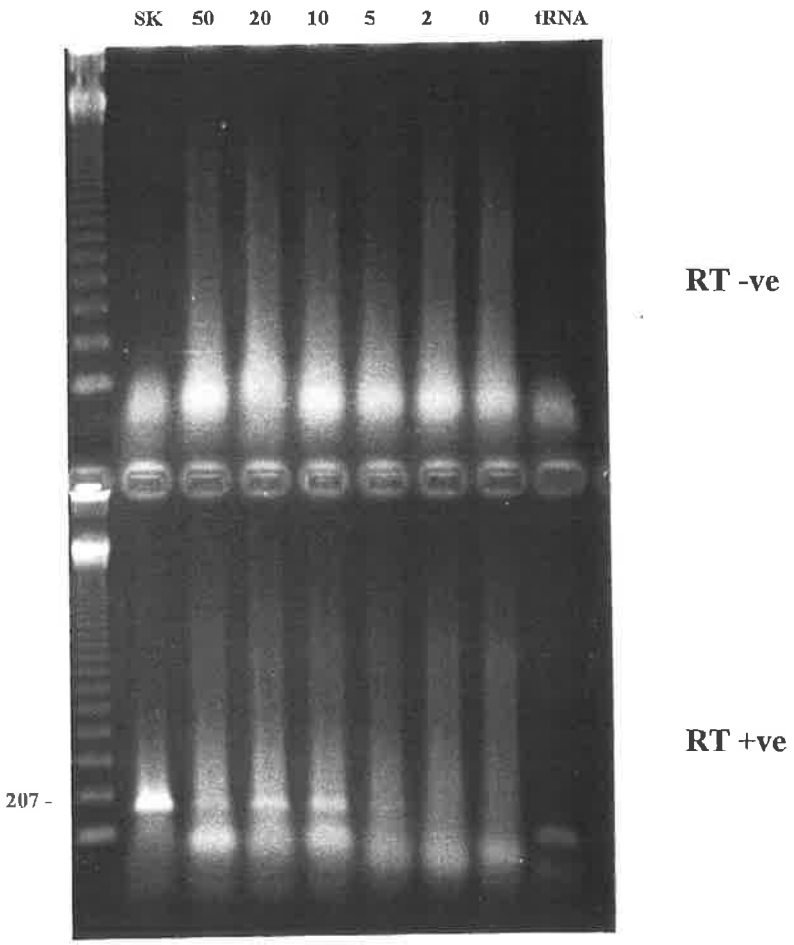



Figure 12. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. normal whole blood samples spiked with varying numbers of SK 19 cells. 1 ug of SK 19 RNA (SK 19) used as the positive control and 1 ug tRNA used as the negative control. Detection level of the 207 bp PCR product at 5 cells per 2 ml. whole blood. No false positive results were seen in any of the RT negative samples.

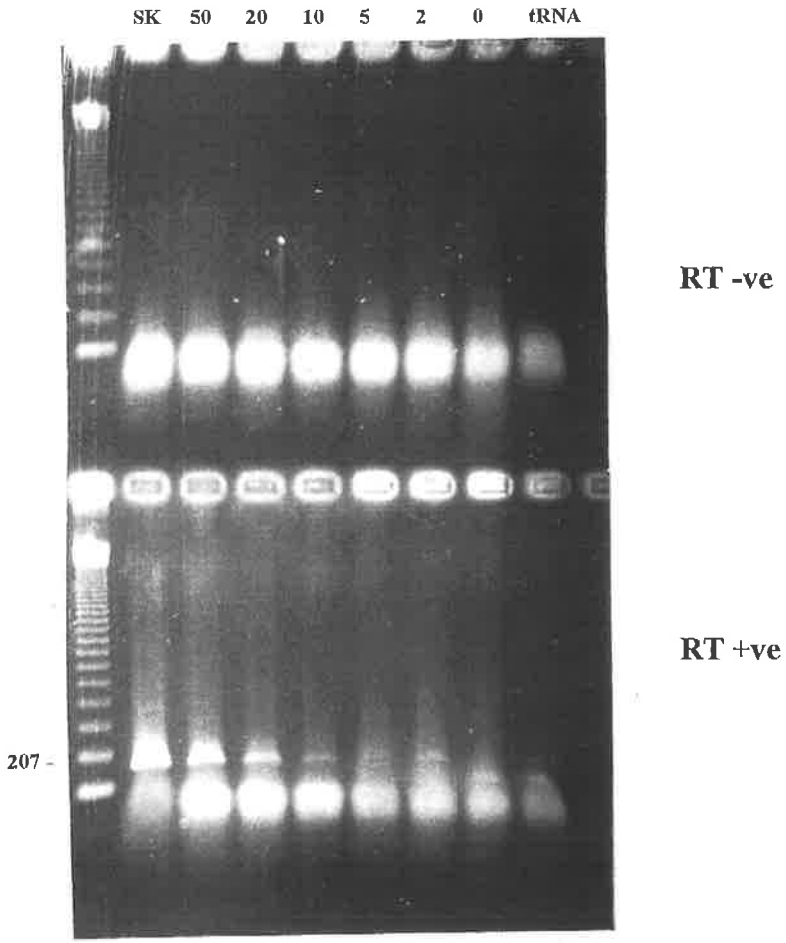



Figure13. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. normal whole blood samples spiked with varying numbers of SK 19 cells. 1 ug of SK 19 RNA (SK 19) used as the positive control and 1 ug tRNA use as the negative control. Detection level of the 207 bp PCR product at at 2 cells per 2 ml. whole blood. No false positive results were seen in any of the RT negative samples.

### 3.2.3 Tyrosinase expression in patients with metastatic melanoma

#### 3.2.3.1 Two rounds of 30 cycles of PCR with nested primers

In a pilot study using two 30 cycle PCR reactions, with nested primers in an initial cohort of clinical samples, 4 out of 7 positive results were obtained from patients with known metastatic melanoma [Smith *et al.* 1991]. There were no positive results in patients with other malignancies and there were no positive results in blood samples from healthy volunteers (see Table 1 and Figure 14). Subsequent analyses of further cohorts of patients were associated with the problem of contamination resulting in high rates of false positivity as previously described in 3.2.1 [see Figure 9]. Therefore, this method whilst being extremely sensitive, subsequently proved to be fallible in the context of working in a moderate sized laboratory or research establishment. The appearance of this high contamination rate coincided with a time when an associated worker cloned pmel 34 cDNA in an adjacent laboratory. It is not clear whether the contamination was directly due to this event or whether it was due to a gradual build-up of "carry-over" PCR product within the laboratory. This meant that despite using all known precautions for the reduction of risk of contamination, there remained an unacceptable level of false positive detection of tyrosinase cDNA.

#### 3.2.3.2 One round of 50 cycles of PCR

Within the limitations of working in the same laboratory, the sensitivity of the test needed to be adjusted so that false positive results would not occur. This was eventually achieved by removing the second round of PCR (nested PCR) from the process. By using the primers originally chosen as "nested" primers as the only primers to be used, and by increasing the number of cycles of PCR from 30 to 50, a zero rate of

**TABLE 1.**

## TYROSINASE mRNA DETECTION IN MELANOMA PATIENTS FOLLOWING 2 ROUNDS (30 CYCLES WITH NESTED PRIMERS) OF RT/PCR

| PATIENT NO. | AGE | SEX | SITES OF DISEASE | TYROSINASE mRNA |
|---|---|---|---|---|
| *Malignant Melanoma* | | | | |
| 1 | 34 | M | L GROIN NODES<br>R SUPRACLAV. NODE<br>STERNUM | NEGATIVE |
| 2 | 47 | M | ABDO. NODES,<br>STOMACH,<br>PERITONEUM | **POSITIVE** |
| 3 | 52 | F | L GROIN AND NODES<br>SUBCUT. TISSUES | **POSITIVE** |
| 4 | 34 | M | R AXILLA, R CHEST WALL<br>BRAIN | NEGATIVE |
| 5 | 65 | M | R FOOT, R GROIN<br>R LEG IN TRANSIT METS. | NEGATIVE |
| 6 | 61 | F | L THIGH, L GROIN,<br>LUNG | **POSITIVE** |
| 7 | 35 | M | LIVER | **POSITIVE** |
| *Other Cancer* | | | | |
| 8 | 64 | M | LIVER | NEGATIVE |
| 9 | 59 | M | LIVER | NEGATIVE |
| 10 | 48 | F | PELVIS | NEGATIVE |
| 11 | 53 | F | LIVER | NEGATIVE |
| *Healthy Volunteers* | | | | |
| 12 | 36 | M | | NEGATIVE |
| 13 | 30 | F | | NEGATIVE |
| 14 | 34 | M | | NEGATIVE |
| 15 | 39 | F | | NEGATIVE |

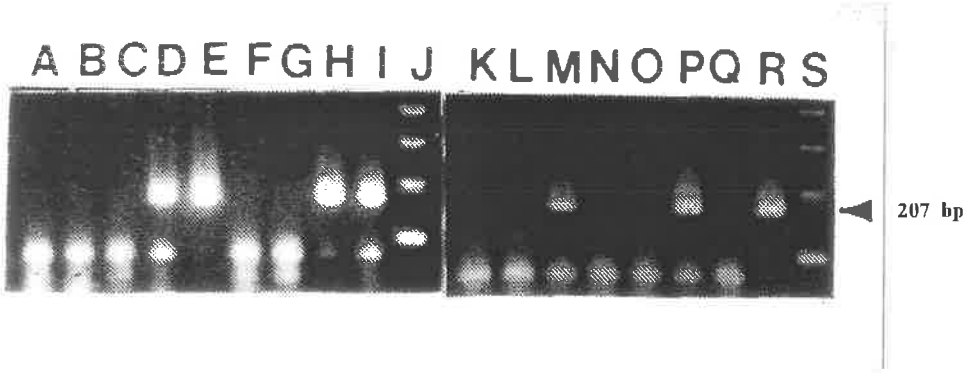



Figure 14. Detection of tyrosinase mRNA in blood by RT - dual PCR with nested primers. Melanoma patients blood samples in lanes C, D, E, F, G, M, N, O and P. Healthy volunteers (negative controls) in lanes A and K. Patients with other cancers (negative controls) in lanes B, L, O and Q. Blood samples spiked with SK28 cells (positive controls) in lanes H,I and R. All positive control samples gave positive results. There were no false positive results in any of the negative control samples. Of the 9 samples from patients with melanoma, there were 4 positive results (in samples D, E, M and P). See also Table 1.

false positivity was achieved without any significant decrease in sensitivity (less than 10 fold). Pre-clinical assessment of this method and sensitivity has been discussed in 3.2.2.

Single round (50 cycles) RT/PCR was used to test patients with known metastatic melanoma. 24 patient samples were examined in 6 runs of 4 patients per run. 1 ug RNA from each sample was initially run on a formaldehyde RNA gel and only those samples in which the 18s and 12s bands were visible on an ethidium bromide gel transilluminated by UV light were used in the RT/PCR experiments. Each run of 4 patient RT/PCR run included RNA from a 2 ml sample of normal blood spiked with 8 SK19 cells as a comparitive positive control, and 2 RNA samples of normal volunteer whole blood as a negative control and 1 ug of tRNA as a negative control. Each sample had it's own RT negative control. In only 3 out of 24 patient samples was the 207 base product detected. (see Table 2). RT negative samples did not give any positive results. Positive results were determined as 207 base pair bands after PCR, and these were confirmed with chemical sequencing of the G-A content within the bands. There were no false positive results in blood samples from healthy volunteers (see Figures 15 - 20).

#### 3.2.3.3 The longevity of free RNA in whole blood

When SK19 melanoma cell line RNA was incubated in freshly extracted whole blood (collected in EDTA) at 37°c for varying lengths of time (0 min, 15 min, 30 min 60 min and 90 min) and the blood samples subsequently underwent RNA extraction and then a single 50 cycle round of RT/PCR, only the sample in which the RNA was added immediately after the blood sample was homogenised with Ultraspec$^{(TM)}$ gave a positive result (see Figure 21). This experiment was consistent with the hypothesis that

## TABLE 2.

## TYROSINASE mRNA DETECTION IN MELANOMAPATIENTS FOLLOWING SINGLE ROUND 50 CYCLES OF RT/PCR

| PATIENT NO. | AGE | SEX | SITE OF DISEASE | TREATMENT WITHIN 3 MONTHS | TYROSINASE DETECTION |
|---|---|---|---|---|---|
| 1 | 26 | M | ABDOM NODES, BRAIN | IFN | NEGATIVE |
| 2 | 59 | M | SUBCUT NODULES | IFN | NEGATIVE |
| 3 | 44 | F | LIVER, LUNG | NONE | NEGATIVE |
| 4 | 61 | F | INTRA ABDOM NODES | NONE | NEGATIVE |
| 5 | 42 | F | SUBCUT NODULES, LUNG | DTIC, TAMOXIFEN | **POSITIVE** |
| 6 | 62 | M | SUBCUT NODULES, PELVIC NODES | NONE | NEGATIVE |
| 7 | 66 | F | SKIN, LUNG, BRAIN | DTIC, TAMOXIFEN | NEGATIVE |
| 8 | 43 | F | WIDESPREAD NODES, LUNG, BRAIN | NONE | NEGATIVE |
| 9 | 36 | M | CERVICAL NODES, LUNG | IFN | NEGATIVE |
| 10 | 73 | F | SKIN, LIVER | DTIC, TAMOXIFEN | NEGATIVE |
| 11 | 64 | F | SKIN, SUBCUT NODULES, PELVIC NODES | DTIC, TAMOXIFEN | NEGATIVE |
| 12 | 58 | F | LUNG | NONE | NEGATIVE |
| 13 | 38 | M | SKIN, PELVIC NODES, LIVER | IL-2/IFN/PLAT | NEGATIVE |
| 14 | 24 | M | LIVER | IL-2/IFN/PLAT | NEGATIVE |
| 15 | 66 | M | LUNG, STOMACH, BRAIN | FOTEMUSTINE/ DTIC | NEGATIVE |
| 16 | 47 | M | LIVER, LUNG | IL-2/IFN/PLAT | **POSITIVE** |
| 17 | 58 | M | SKIN, WIDESPREAD NODES | IL-2/IFN/PLAT | NEGATIVE |
| 18 | 47 | F | WIDESPREAD NODES | IL-2/IFN/PLAT | NEGATIVE |
| 19 | 68 | F | SKIN, SUB-CUT NODULES, WIDESPREAD NODES | IL-2/IFN/PLAT | NEGATIVE |
| 20 | 58 | M | SUBCUT NODULES, FEMORAL NODES, LIVER | IL-2/IFN/PLAT | NEGATIVE |
| 21 | 53 | M | ABDOM NODES, LUNG, KIDNEY, BRAIN | FOTEMUSTINE/ DTIC | **POSITIVE** |
| 22 | 65 | M | LUNG | IL-2/IFN/PLAT | NEGATIVE |
| 23 | 54 | F | LIVER, LUNG, SPLEEN, BONES | IL-2/IFN/PLAT | NEGATIVE |
| 24 | 24 | M | BRAIN | FOTEMUSTINE/ DTIC | NEGATIVE |

**IFN - α-INTERFERON** **IL-2 - INTERLEUKIN-2**
**DTIC - DACARBAZINE** **PLAT - CISPLATIN**

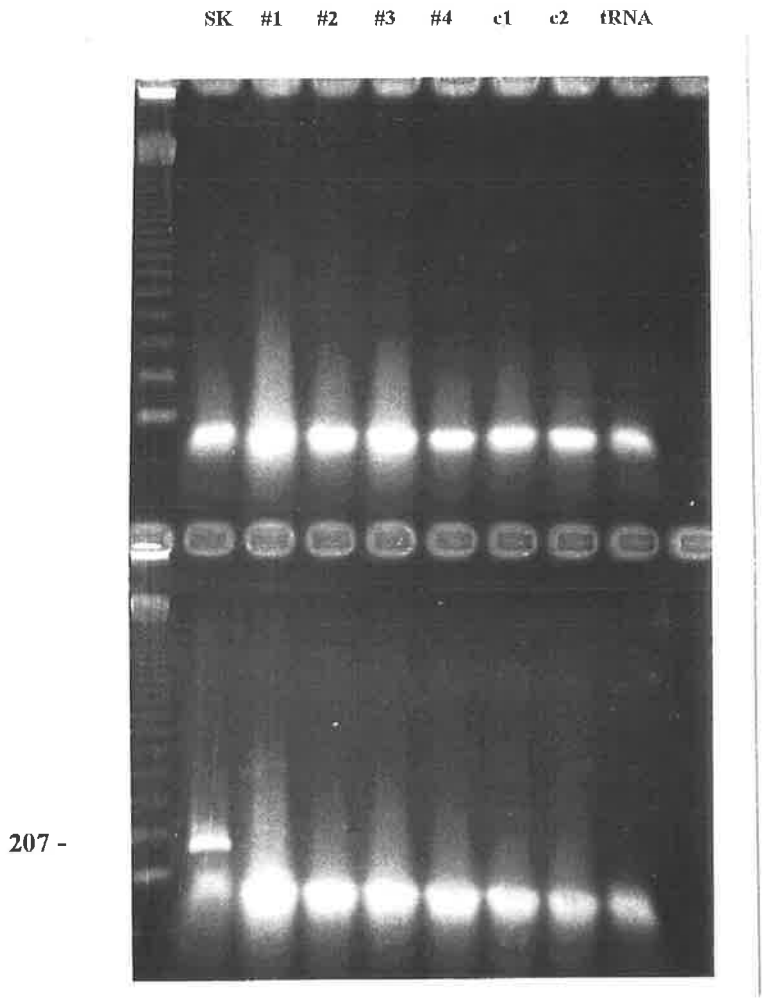



Figure 15. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#1 - #4). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control (SK), whilst 2 ml. samples of whole blood from healthy volunteers (c1, c2) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen only in the positive control sample. No false positive results were seen in any of the RT negative samples.

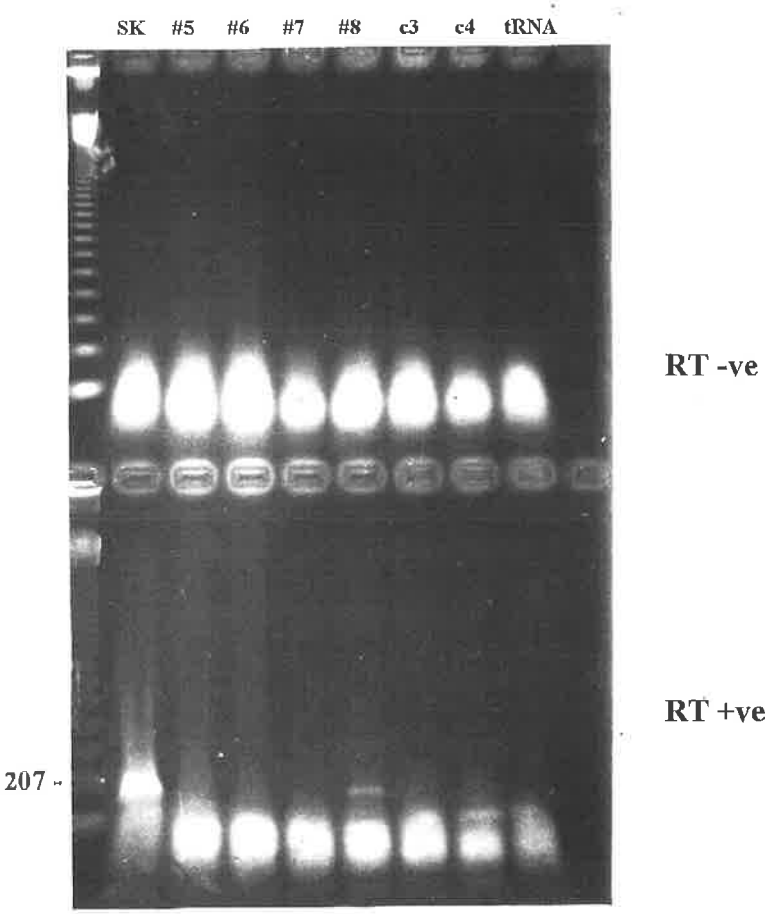



Figure 16. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#5 - #8). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control, whilst 2 ml. samples of whole blood from healthy volunteers (c3, c4) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen in both the positive control sample and the sample from patient #8. No false positive results were seen in any of the RT negative samples.

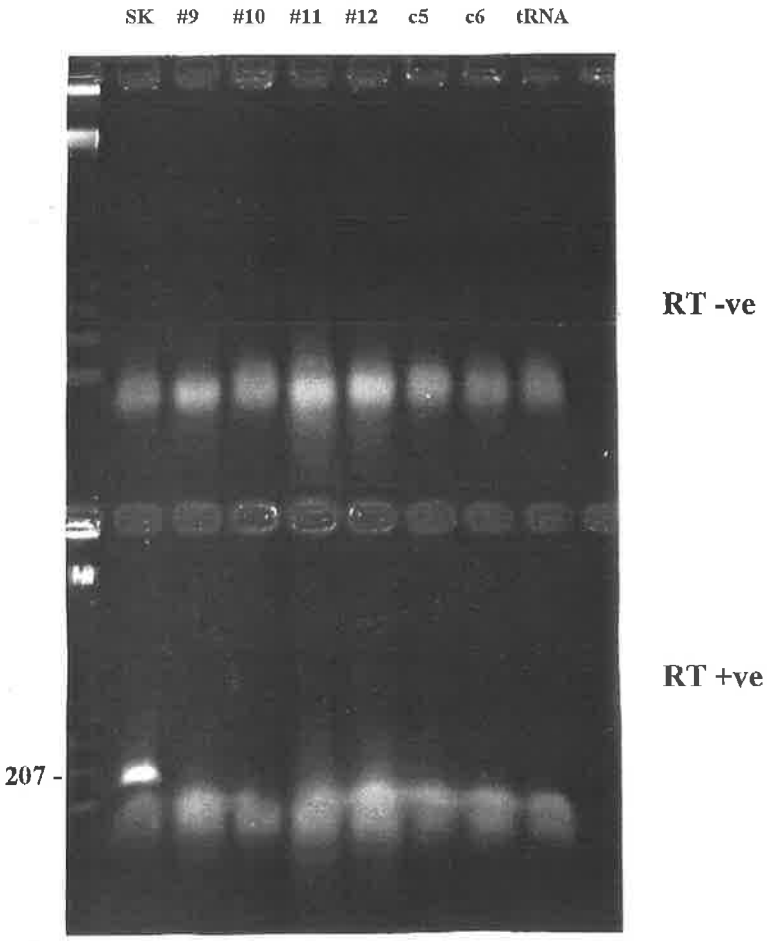



Figure 17. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#9 - #12). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control (SK), whilst 2 ml. samples of whole blood from healthy volunteers (c5, c6) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen only in the positive control sample. No false positive results were seen in any of the RT negative samples.

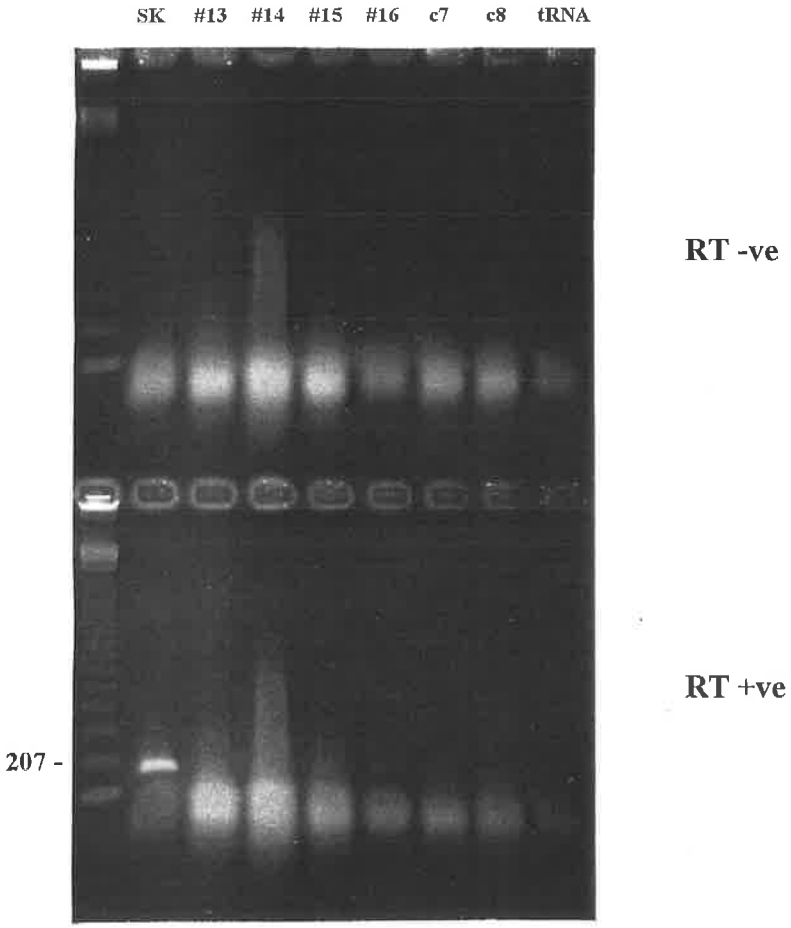



Figure 18. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#13 - #16). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control, whilst 2 ml. samples of whole blood from healthy volunteers (c7, c8) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen only in the positive control sample. No false positive results were seen in any of the RT negative samples.

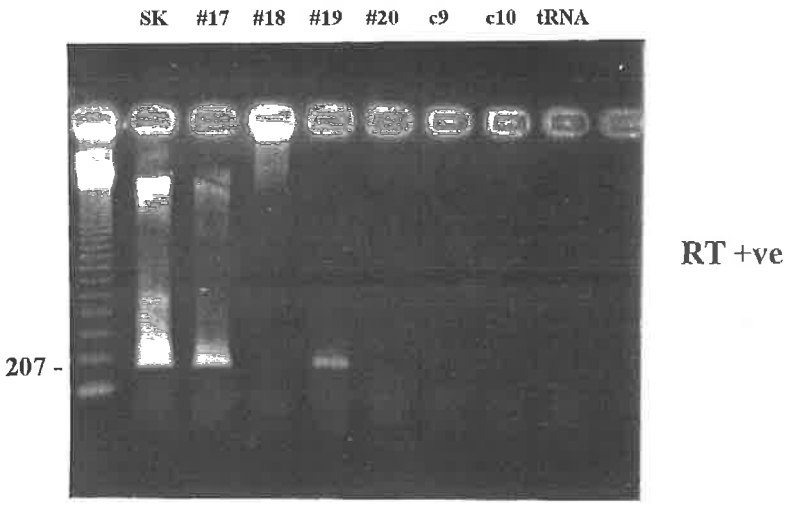



Figure 19. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#17 - #20). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control, whilst 2 ml. samples of whole blood from healthy volunteers (c9, c10) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen in the positive control sample, the sample from patient 17 and the sample from patient 19. No false positive results were seen in any of the RT negative samples (results not shown).

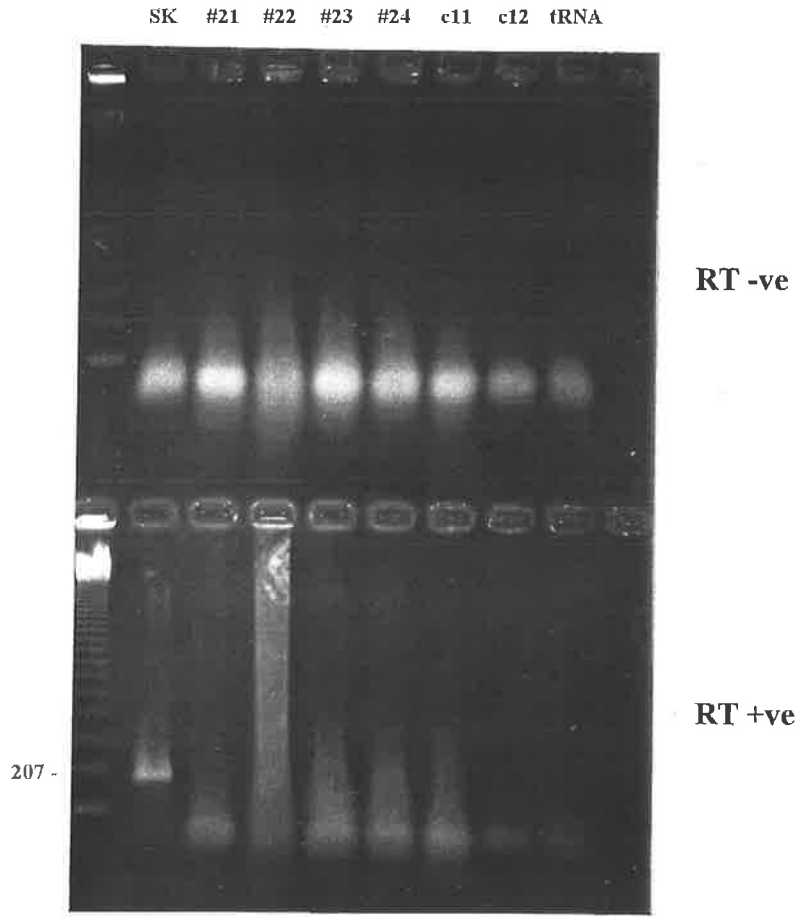



Figure 20. Single round RT/PCR (50 cycles) using inner tyrosinase primers on 2 ug RNA from 2 ml. whole blood samples from patients with known advanced/metastatic melanoma (#21 - #24). One 2 ml. sample of whole blood spiked with 10 SK 19 cells acted as the positive control, whilst 2 ml. samples of whole blood from healthy volunteers (c11, c12) and 1 ug. tRNA acted as negative controls. Detection of the 207 bp PCR product was seen only in the positive control sample. No false positive results were seen in any of the RT negative samples.

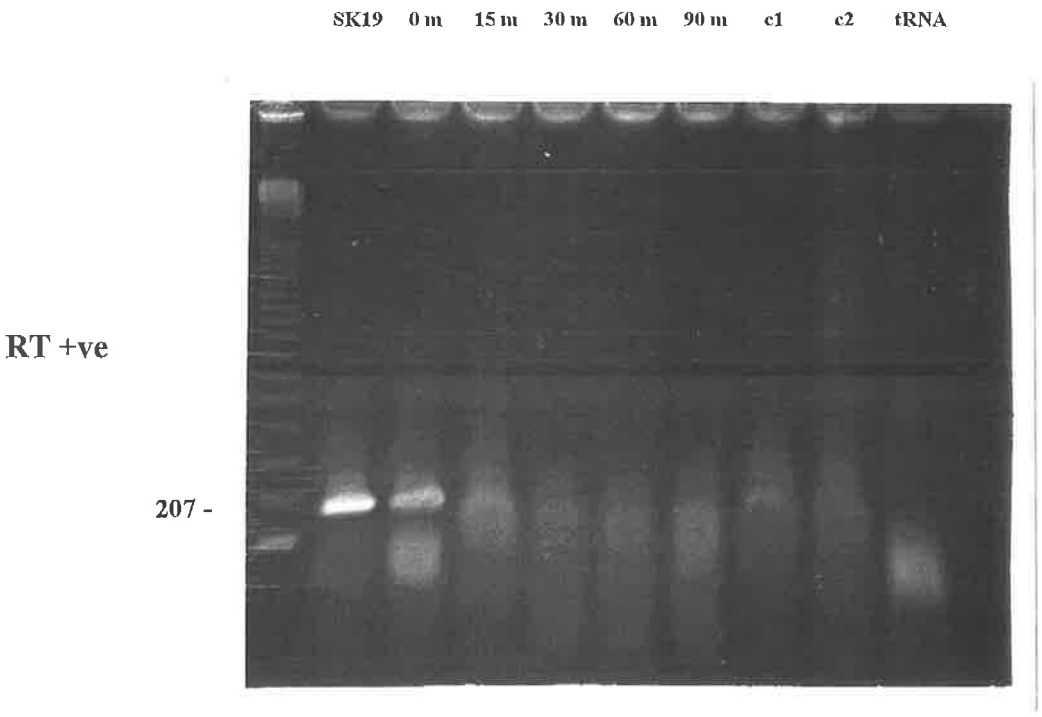



Figure 21. Single round RT/PCR (50 cycles) with inner tyrosinase rimers performed on 10ng SK 19 RNA dissolved in 2 ml. whole blood and incubated at varying intervals (15 minutes - 90 minutes) before whole blood RNA extraction by the Ultraspec (TM) method. 100 ng free SK 19 RNA and 10 ng SK 19 RNA dissolved in whole blood only after the Ultraspec (TM) had been added to the blood sample (0 min) acted as positive controls. 2 ml. whole blood samples from 2 healthy volunteers (c1, c2), and 1 ug tRNA acted as negative controls. Each sample was divided into 2 equal aliquots, and only one aliquot underwent a true everse transcription reaction. The 207 bp PCR product was detected only in the free SK 19 positive control sample and the 0 min. positive control sample. No false positive results were seen in any of the RT negative samples (results not shown).

RNAses in blood rapidly digest free RNA, so that positive results with this method of RT/PCR are due to the presence of intracellular RNA.

## 3.3 DISCUSSION

### 3.3.1 PCR sensitivity and problems of contamination

As PCR is such an exquisitely sensitive method of detecting extremely small amounts of target DNA, false positivity (detection of target DNA which has arisen from exogenous sources rather than the sample itself) becomes an increasingly frequent problem. To adequately control for contamination, the physical transfer of DNA between amplified samples as well as between positive and negative samples must be totally avoided .

Despite the total isolation of PCR preparations and products, the use of autoclaved solutions,the careful aliquotting of reagents into minimal re-use or no re-use containers, the frequent changing of protective gloves, the use of positive displacement pipettes etc. [Kwok and Higuchi 1989], false positive results continued to occur. As the 2 round PCR technique (with nested primers) required obligatory handling at the stage of the preparation of second round of PCR, it seemed that no amount of "contamination free" preparation could completely avoid contamination with PCR product. As this problem did not become manifest until after the pmel 34 gene had been cloned, one assumption is that this gene had contaminated all parts of the laboratory, including the autoclave at the time that it was cloned.

An alternative possibility is that the intermittent false positivity arose from a gradual local environmental increase in target PCR product from the first round of PCR which was subsequently intermittently carried over during manipulation of PCR

products between the two rounds of PCR. Such contamination problems are well recognised [Sarker and Sommer 1990], and various techniques to avoid false positives have been proposed. These are essentially "barrier" techniques most of which are viable in a standard laboratory setting. However if a significant contamination arises within a laboratory, such barrier or isolation techniques can never provide complete safety, due to the robust nature and longevity of the DNA produced. The possibility of intermittent false positivity occurring reinforces the need for stringent negative controls.

### 3.3.2 The necessity of stringent controls for RT/PCR techniques

The "two edged sword" property of PCR means that as PCR is such a sensitive method of detecting rare segments of DNA, it runs a high risk of producing false positive results due to even extremely low levels of contamination. It also reinforces the value of including adequate concurrent controls for every PCR reaction performed.

Each PCR experiment needs meaningful "control" PCRs to proceed concurrently. The "positive" controls should not only include a sample of target RNA in amounts easily detectable by PCR but also target RNA in much smaller amounts approximating the level of sensitivity of the technique. Hence the use of 8 melanoma cell line cells in 2 ml whole blood as a positive control for each clinical batch run of RT/PCR. Only then, can one be certain that the sensitivity of the technique is maintained. Negative controls should include a simple carrier such as water or tRNA. A new "carrier" negative control should be introduced for each procedure which may allow more easy pinpointing of the timing of any contamination. Ideally each clinical sample should be run concurrently with a corresponding RT negative ("sham") control, to ensure that each individual sample has not been contaminated by target cDNA.

Adhering to these strict positive and negative controls has enabled meaningful interpretation of each result, even those which suggested a contamination problem. In this study, during the time in which 2 rounds of PCR (with nested primers) was used, the random nature of the false positive results tended to indicate that contamination was secondary to low level "backgound" contamination, rather than a specific source of contamination.

Unless stringent positive and negative controls are run concurrently with any RT/PCR reaction, a "positive" result may be interpreted as contamination in the absence of stringent concurrent negative controls, and a "negative" result may be interpreted as procedure failure in the absence of meaningful concurrent positive controls.

### 3.3.3 Critical appraisal of this technique of RT/PCR to detect melanoma cells in peripheral blood

The initial technique of dual rounds (with nested primers) of PCR following RT produced very encouraging results in the initial clinical study, with 4 advanced melanoma patient samples out of 7 giving a positive result for detection of tyrosinase [Smith *et al.* 1991]. The problems of contamination and subsequent abandonment of that particular technique has been previously discussed.

The 50 cycle single round RT/PCR technique that was subsequently developed proved to be extremely sensitive (10 - 100 pg RNA or ~ 1 - 2 cells / ml. whole blood), and was not plagued by the problem of false positivity due to contamination. However, the rate of positivity in the clinical setting (3 out of 24 patients with grossly advanced disease) was considerably reduced, suggesting that this technique has no clinical use in the context of it being a sensitive method of detecting metastatic disease [Pittman*et al.*

submitted]. Another group studying patients with uveal melanoma using essentially the same RT/PCR tyrosinase technique also reported an initial high rate of positive detection (3 positive results from 6 patients) [Tobal *et al.* 1993], but a subsequent larger study, performed by the same group failed to detect any positive results in 36 patients with uveal melanoma [Foss *et al.* 1995]. Combining the results of the two studies (in which similar RT/PCR techniques were used to detect metastatic melanoma cells in blood) gives a detection rate of 3 in 60 or 5 %. It seems unlikely that the sensitivity of this test can be significantly improved unless considerably larger quantities of blood are to be examined and the feasibility of examining very large quantities of blood is questionable.

In contrast to this study and the study by Foss *et al.* [1995], Prossart *et al.* [1993] have used a technique of RT/PCR based upon the technique described in 3.2.3.1 in a cohort of 56 patients with melanoma and detected tyrosinase transcripts in 36 of these patients. In addition, there was a significant correlation between between the rate of tyrosinase detection and the clinical stage of the disease. This conclusion has been supported by Vormwald-Dogan *et al.* [1994] who have also used the same technique to show that tyrosinase detection in peripheral blood correlates with clinical stage. In these studies, internal RT negative controls were not routinely performed on all samples examined. There is clearly non-concordance in the predicted rates of circulating tumour cells in melanoma patients as assessed by these studies. Further studies with rigorous controls and possibly independent observation are required to determine the true rate of detection.

The lack of detection of tyrosinase transcripts in samples of blood in which free SK 18 RNA was dissolved at varying times suggests that free RNA does not appear to

circulate in peripheral blood and is probably digested by the numerous RNAses in blood. Therefore the RNA detected by this technique of RT/PCR originates from cells in circulation rather than small quantities of free RNA. The results from this study as well as the results from the melanoma studies by Prossart*et al.* [1993] and Vormwald-Dogan *et al.* [1994] also re-invite the questions as to how frequently tumour cells do actually circulate in the peripheral blood, and what is the clinical significance of the tumour cell detected in circulation.

In the 1950s and 1960s when cytological techniques to screen blood for circulating tumour cells were fashionable, the positive rate of detection was variably reported as 0 - 95 % [Christopherson 1965]. In a critical analysis of these techniques, Goldblatt and Nadel [1965] discovered a high rate of false positivity, and concluded that the true positive detection rate was probably only about 5 %. Using the newer technology of RT/PCR, pilot studies have suggested a circulating melanoma cell detection rate of greater than 50 % of patients with melanoma [Smith *et al.* 1991, Tobal *et al.* 1993, Prossart *et al.* 1993]. Whilst a later study by Vormwald-Dagan *et al.* [1994] supports a high rate of tumour cell detection, other follow up studies [Foss *et al.* 1995, Pittman *et al.* submitted] suggest that the rate may be closer to 5 % which is similar to the figure suggested by several commentators 30 years ago [Goldblatt and Nadel 1965, Christopherson 1965].

It may be postulated that the use of two rounds of PCR with "outer" and "nested" primers provides greater sensitivity and specificity than a single round method of RT/PCR, although the sensitivity achieved in this study using one round of PCR with 50 cycles was certainly of the same order of magnitude as that achieved with two

rounds. Also, the study by Foss *et al.* [1995] in which two rounds of PCR were used failed to detect any expressed tyrosinase RNA in any of the clinical samples examined.

Two other major differences between the RT/PCR technique used in this study compared with others were the DNAse step prior to RT, and the use of RT negative controls for every sample examined. These time and resource consuming steps ensured that contaminating cDNA within an RNA sample was kept to an absolute minimum, as well as ensuring that contaminating cDNA could not produce a false positive result, that could not be simply traced for any given sample. In the other RT/PCR melanoma studies discussed, there was no DNAse step, based upon the premise that minute quantities of contaminating gDNA would not produce PCR bands of the expected size due to the introns contained within. However, contaminating cDNA would produce PCR bands of expected sizes, and the best way to minimise this potential problem is with DNAse treatment of all RNA samples immediately prior to the RT step. The potential for cDNA contamination is even greater when two rounds of PCR are used in light of the increased number of sample manipulations. The studies by Smith *et al.* [1991], Prossart *et al.* [1993], Vormwald-Dagan *et al.* [1994] and Foss *et al.* [1995] used two RT negative control samples per "run" of samples. Although these "RT negative" controls did not produce positive results suggestive of any cDNA contamination, it does not automatically follow that the clinical samples studies were free of cDNA contamination as they were not individually tested. Random contamination with cDNA certainly occurs as discussed in 3.3.2, and in the paper by Foss *et al.*[1995] in which the RT/PCR also required considerable alteration to overcome the problem of random contamination.

Current metastasis theory [Fidler and Hart 1982] implies that tumour cells are shed into the circulation intermittently in a non-continuous, non-random manner, so it may be possible that the positivity rate for this type of examination may be increased if sequential samples are examined [Salisbury 1975]. Even so, the results of this study suggest that it is unlikely that the rate of positivity could be increased sufficiently to propose that the technique will be a routinely reliable sensitive test for micrometastatic disease.

As the rate of positive detection of melanoma cells in peripheral blood in patients with advanced melanoma is so low in this study, no conclusions may be drawn regarding the clinical significance of positive detection in patients with known advanced disease. In all of the patients examined so far there was no suggestion that those patients with positive results had a worse prognosis. This contrasts with the papers by Prossart*et al.* [1993], and Vormwald-Dogen *et al.* [1994] in which there appeared to be a good correlation between likelihood of tyrosinase RT/PCR positivity and clinical stage. Patients with surgically treated early stage melanoma were not examined in this study. Even so, as Engell [1959] and Griffiths *et al.* [1973] discovered many years ago, the simple detection of solid tumour cells in blood of patients at the time of resection of early stage colorectal cancer, did not appear to indicate that metastasis had already occurred, and provided little prognostic information. There is now good evidence that the vast majority of circulating tumour cells in peripheral blood are destroyed fairly rapidly, and that the process of metastasis is therefore a relatively inefficient one [Fidler 1973, Weiss 1992]. A tumour cell in circulation has only reached part of the way to establishing metastasis [Fidler 1990], so detection alone of such a cell gives little insight into the true metastatic potential of that cell.

This clinical evaluation of the technique of RT/PCR for tyrosinase in patients with melanoma suggests that the rate of circulating melanoma cells in patients with melanoma is low, and therefore this technique appears to have little clinical application in the accurate determination of micrometastatic disease. This conclusion is supported by the study of Foss *et al.* [1995] but is in contrast with the more optimistic conclusions reached in the studies by Prossart *et al.* [1993] and Vormald-Dogen *et al.* [1994]. These divergent conclusions suggest that the RT/PCR methods presently in use for detecting tyrosinase expression are not yet sufficiently robust for routine clinical use in the setting of patients with malignant melanoma.

### 3.3.4 Therapeutic implications of detecting circulating melanoma cells

The detection of circulating melanoma cells in the peripheral blood of metastatic melanoma patients provides an excellent model for using RT/PCR to detect tissue specific gene expression as a marker of tumour presence. However from a clinical viewpoint, at present there is relatively little therapeutic gain in such tumour cell detection. This is due to the lack of effective systemic treatments for metastatic disease, either gross or micrometastatic, as demonstrated by studies in both the advanced setting and in the adjuvant setting [Veronesi *et* al 1982, Lakhani *et* al 1990]. Certainly prognostic information may be gained which may be useful to both clinician and patient. However, before a sensitive test of the presence of blood borne metastatic disease can become of major clinical importance, more effective treatments for advanced melanoma are needed. With cytotoxic chemotherapy presently having such a limited effect in

melanoma, there is an urgent need to explore novel biological therapies, as well as novel methods of circumventing the longstanding problem of cytotoxic drug resistance.

Perhaps the most promising line of treatment driven research in malignant melanoma is the increasing understanding of the immunobiology of melanoma which has significantly rekindled enthusiasm for immunotherapies for melanoma [Mukerjhi and Chakraborty 1995]. If these immunotherapies are able to eventually significantly improve the outcome for patients with advanced melanoma, then molecular detection of metastatic circulating melanoma cells in blood may carry greater clinical significance. However, a full discussion of the potential immunomodulatory therapies in melanoma is beyond the scope of this paper.

# 4. CYTOKERATINS / CARCINOMAS

## 4.1 THE SCOPE OF EPITHELIAL TUMOURS

### 4.1.1 Epithelial malignancy in adult oncology practice

Epithelial cancers or carcinomas are the most common malignant tumours in adults and are responsible for by far the most cancer related death [South Aust. Cancer Registry 1994]. The most common epithelial tumours such as lung cancer, breast cancer and colorectal cancer are all tumours that tend to eventually spread via the haematogenous route. In each of these cases early excision of the primary localised tumour offers the best chance of long term survival. However, a significant proportion of patients who undergo "curative" resections for apparently localised cancers eventually relapse with either loco-regional recurrence or distant metastases [Mountain 1977, Copeland *et al.* 1968, Valagussa *et al.* 1978]. It is assumed that this is due to very small volume metastatic disease (micro-metastases) not identified at the time of surgery, despite appropriate staging investigations.

### 4.1.2 The rationale for adjuvant therapies for carcinomas

It is now common for adjuvant therapies (treatment administered following definitive primary therapy to complement the primary therapy) to be employed following definitive treatment of a primary tumour. Adjuvant therapies are now frequently employed in patients who are at greatest risk of developing recurrent disease, despite complete resection of the primary tumour. There is now data available to confirm that adjuvant therapies contribute to improved survival for certain tumour types such as breast cancer and colorectal cancer [EBCTSG 1992, Moertel *et al.* 1992]. In spite of this improvement in survival with adjuvant treatment, there are considerable

numbers of patients who are probably already cured of their malignancy following primary resection of a tumour, who undergo potentially toxic adjuvant chemotherapy because they happen to be in a high risk category. With effective treatments now available, the detection of very small volume disease (micrometastases) may allow adjuvant treatments to be used with best effect in patients who genuinely need them while ensuring that those patients who may already be cured following primary treatment are not subjected to unwarranted therapy. Presently there are no sensitive and specific tests of micrometastatic disease for common epithelial malignancies in adults.

## 4.2 CYTOKERATINS

### 4.2.1 The catalogue of intermediate filaments

The 3 major filament systems within vertebrate cells are (i) actin and associated proteins that provide the contractile forces within cells; (ii) microtubules which aid in the orientation and polarisation of the cell; and (iii) the intermediate filaments (or cytoskeleton).

The majority of vertebrate cells contain a large proportion of structural filaments which are made up of the various members of the family of intermediate filaments [Steinert *et al.* 1985]. Intermediate filaments are morphologically and structurally quite similar to each other, based upon a long α-helical core or rod domain. They also dimerise with similar molecules by interaction along a backbone of hydrophobic residues producing a coiled coil which becomes the building block of polymerised intermediate filament [Crewther *et al.* 1978, Landschulz *et al.* 1988]. However the members of the intermediate filament family are able to be differentiated on immunological, biochemical and possibly functional grounds.

At least 5 main groups have been identified :

(i) vimentin - occurring in cells of mesenchymal origin, astrocytes, Sertoli cells, vascular smooth muscle cells, and many cultured cell lines. The exact cytoskeletal function of vimentin is not clear.

(ii) desmin - typically found in most types of myogenic cells, it is located at the Z-disc of striated muscle or in the dense bodies in smooth muscle apparently anchoring the contractile apparatus.

(iii) neurofilaments - typical of neuronal cells; found in association with glial microtubules, probably providing stability.

(iv) glial filaments - found in astrocytes.

(v) cytokeratins - the characteristic structural filaments of epithelial tissue [Moll *et al.* 1982].

As these intermediate filaments tend to be largely conserved within cell/tissue types [Azumi and Battifora 1987], the presence or absence of specific types of intermediate filaments has been utilised in diagnosing cells of origin in particular tumours.

### 4.2.2 Keratin family of intermediate filaments

Unlike vimentin, desmin and glial types of intermediate filaments which consist usually of one type of sub-unit protein [Steinert *et al.* 1981, Quinlan and Franke 1982], the keratin filaments consist of a family of different proteins [Steinert and Roop 1988]. These keratin subtypes can be present in numerous combinations in different epithelia. It may be possible to characterise different epithelia by the pattern of the various keratin

subtypes. At least 20 different keratins have been identified and each is probably encoded by are separate gene [Nagle 1988, Moll*et al.* 1992].

### 4.2.3 Keratin filament pairing

Keratin filaments are formed by the interaction of a pair of keratin subtypes, one smaller in size (40 - 56 kd) with a relatively acidic pH, and the other larger (53 - 67 kd) and with a relatively basic pH [Steinert and Roop 1988]. For this reason, keratin subtypes tend to be co-expressed in pairs within tissues. For example, keratin 8 (basic) is co-expressed with keratin 18 (acidic). cDNA transfection experiments have shown that the keratin filaments produced by this pairing are not synthesised if either one of the keratin genes (either 8 or 18) is not co-expressed [Domenjoud*et al.* 1988, Kulesh *et al.* 1989]. The exception to this rule is keratin 19, the smallest acidic keratin [Wu and Rheinwald 1981, Stasiak and Lane 1987, Eckert 1988]. A theory about the lack of pairing of keratin 19 is that it exists to address the unbalanced production of basic keratins [Savtchenko *et al.* 1988]. It may non-specifically pair with a number of basic keratin proteins.

### 4.2.4 Keratins as markers of epithelial differentiation

Keratin expression patterns vary with different tissue types. The specific keratin polypeptide pairings may permit further developmental classification of particular epithelial cells [Chan *et al.* 1986, van Muijan *et al.* 1987]. An example of this is the invariable expression of cytokeratins 8 and 18 in embryonic epithelia and simple non-stratified epithelia, but lack of expression in more complex stratified epithelia [Moll*et al.* 1982]. This implies that further characterisation of particular epithelial tissue is

possible. Keratins are generally considered the most reliable of markers of epithelial differentiation and are therefore very useful in the diagnosis and characterisation of epithelial tumours [ Sun *et al.* 1985, Battifora 1988, Trask *et al.* 1990].

### 4.2.5 Keratin expression in non-epithelial tissue as a marker of contamination by epithelial malignancy

As keratins are expressed widely in all epithelial tumour types, it may be possible to seek expression of keratin in non-epithelial tissue such as lymph node tissue [Kainz *et al.* 1993], bone marrow [Schlimok *et al.* 1990] peripheral blood as a means of demonstrating malignant contamination by keratin expressing tumour cells. The presence of keratin containing cells in the peripheral blood may be an indicator of metastatic epithelial tumour cells in circulation. Such information may have major prognostic implications and may therefore have a significant bearing upon the management of malignancy.

### 4.2.6 Monoclonal antibody techniques of keratin detection in bone marrow

The notion of detecting keratin expressing tumour cells in non-epithelial sites as a marker of metastatic disease has been tested clinically using monoclonal antibody techniques on bone marrow in patients with malignancy. A monoclonal antibody technique was able to detect tumour cells in bone marrow of 28 % of patients with advanced epithelial malignancy when triple bone marrow aspirates were performed [Redding *et al.* 1983]. A similar technique in patients with small cell cancer of the lung was able to detect tumour cells in bone marrow of 50 % of patients with limited stage

disease (radiological staging) and 77 % of patients with extensive disease [Berensden*et al.* 1988]. A further study using an immunocytochemical assay for cytokeratin 18 detected tumour cells in bone marrow of 32 % of patients who had undergone curative resection for colorectal cancer [Schlimok *et al.* 1990, Schimlok and Riethmuller 1990]. The detection of tumour cells in bone marrow has also been discussed in 1.5.4.

### 4.2.7 Prognostic implications of bone marrow micrometastases

In each case discussed above (4.2.6) the presence or absence of bone marrow micrometastases had an independent impact on prognosis. If tumour load is important in predicting survival, and low tumour load has a better chance of being completely eradicated, then the finding of micrometastatic disease may suggest the need for intensive eradication techniques if the particular tumour type is sensitive to a particular therapy. Alternatively, the detection of micrometastatic disease may imply that less intensive eradication methods be used if the tumour type is non-responsive to therapies following apparent curative resection. Thus better patient selection for adjuvant therapies may ensue.

Bone marrow takes a considerable proportion of cardiac output (about 600 ml/min) as it is an highly vascularised organ [Weiss 1992]. A significant proportion of aspirated bone marrow is therefore likely to be whole blood. Although bone marrow stroma may significantly influence the attraction of tumour cells into the bone marrow as well as establishment of bone marrow metastases, one may speculate that similar prognostic data could be obtained by screening whole blood rather than bone marrow.

## 4.3 METHODS / RESULTS : IMMUNOFLUORESCENT ANTIBODY DETECTION OF CYTOKERATIN EXPRESSION

### 4.3.1 Preclinical evaluation

#### 4.3.1.1 Antibody selection

The 3 cytokeratin antibodies compared were LP1K (keratin 7), LE41 (keratin 8) and LE61 (keratin 18) [Lane and Alexander 1990]. Primary antibody labelling and secondary antibody labelling was performed as described in 2.8.1, using the human bladder carcinoma cell lines EJ and RT112 as positive controls. Two samples were prepared with the primary antibody labelling omitted from the protocol.

The degree of fluorescence was assessed for each antibody. Genuine fluorescence due to antibody labelling was distinguished from autofluorescence by viewing under FITC (red) light. For the samples in which no primary antibody was used, it was agreed by 3 observers that there was no fluorescence due to antibody labelling. LP1K and LE61 labelling produced the greatest degree of fluorescence (see Figures 22 and 23). LE41 produced considerably less fluorescence (see Figure 24). The LP1K and LE61 antibodies were chosen for further fluorescent antibody labelling experiments.

#### 4.3.1.2 Blood spiking with RT112 cells

8 mls. of whole blood were extracted from a healthy volunteer. This was divided into 1 ml. aliquots (see 2.1.4). 100 cultured RT112 cells were spiked into 4 of the 1 ml. aliquots. Each sample was then prepared for cytospin by the direct red cell lysing technique (2.7.1). Each 1 ml. sample was eventually divided into 5 cytospin preparations and following the cytospin procedure (2.7.3), underwent acetone fixation.

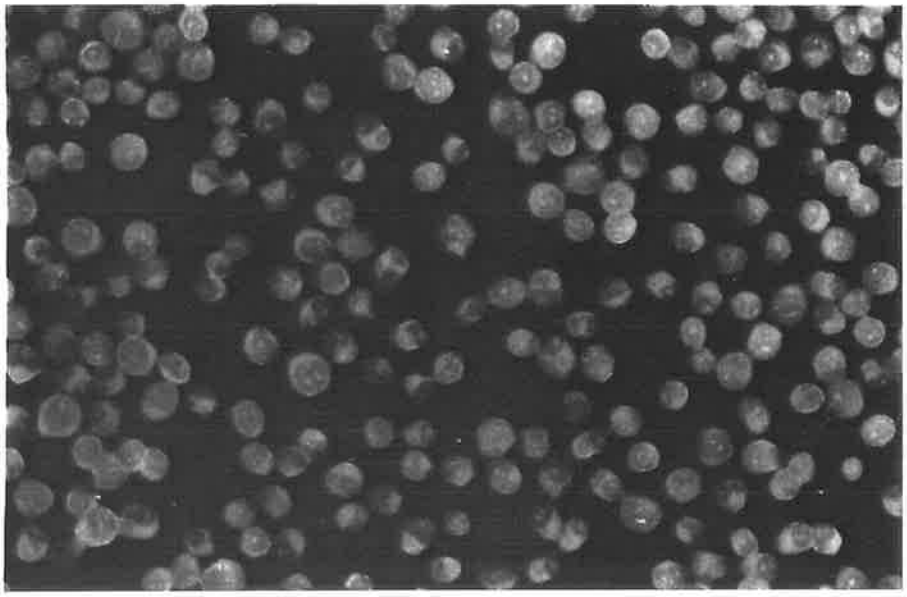


Figure 22. EJ (bladder carcinoma) cells fluoresecent pattern with LP1K as the primary antibody (Axioplan binocular microscope, oil immersion, magnification x 40).

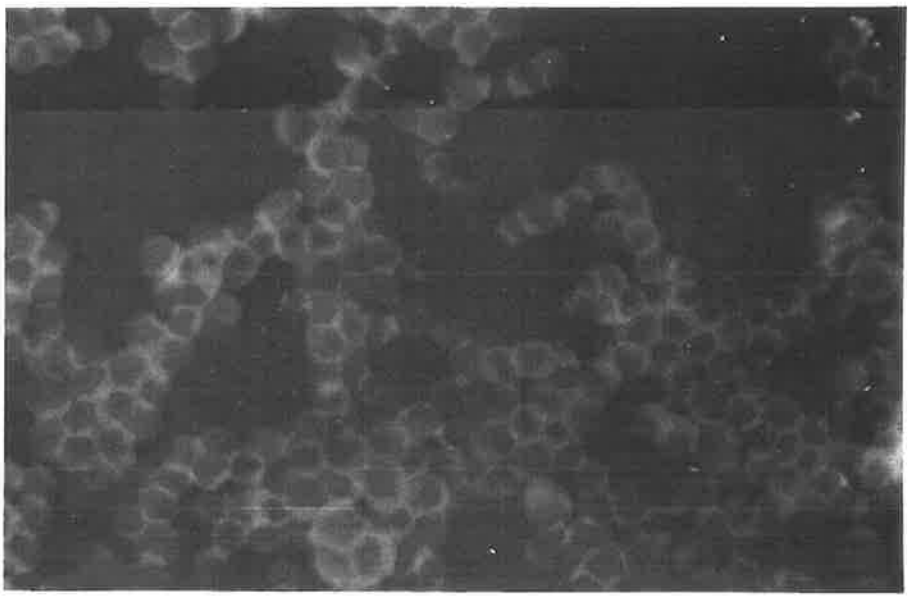


Figure 23. Fluoresecnt pattern of RT112 (bladder carcinoma) cells with LE61 as the primary antibody (Axioplan binocular microscope, oil immersion, magnification x 40).

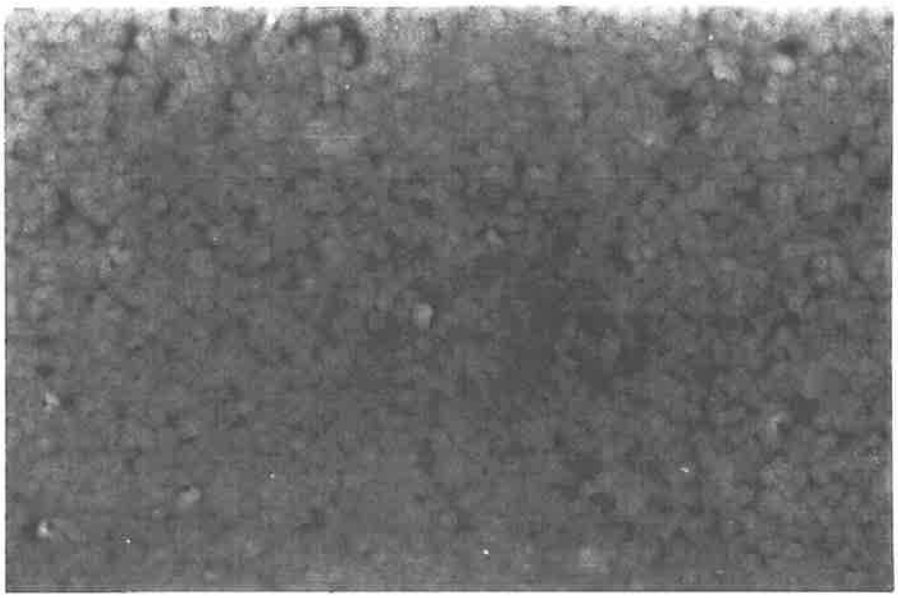


Figure 24. Fluorescent pattern of EJ (bladder carcinoma) cells using LE41 as the primary antibody (Axioplan binocular microscope, oil immersion, magnification x 25).

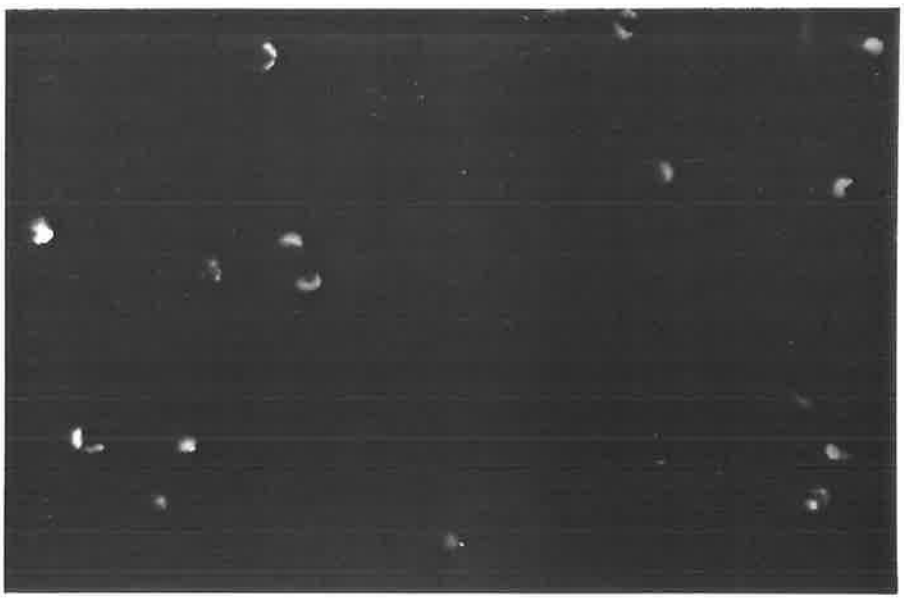


Figure 25. Autofluorescence pattern of polymorphs in whole blood cytospins (Axioplan binocular microscope, oil immersion, magnification x 40).

#### 4.3.1.3 Assessing tumour cell recovery from spiked blood samples

The LP1Kand LE61 primary antibodies were tested on 2 spiked blood samples and 2 normal blood samples each. The number of antibody labelled fluorescent cells per microscope field was tallied. For the normal blood samples which had not been spiked, no antibody labelled fluorescent cells were seen. Polymorph granulocytes were seen to autofluoresce in varying amounts (see Figure 25). Occasionally this was quite prominent but they invariably continued to fluoresce under FITC light unlike genuinely antibody labelled cells (see Figures 26 and 27). For the LE61 labelled preparations, a total of 31 and 27 positively labelled cells were counted in the 2 spiked blood samples analysed. For the LP1K labelled preparations, 34 and 36 positively labelled cells were counted in the 2 preparations. Antibody labelled fluorescing cells were at least a log greater in fluorescent intensity than surrounding autofluorescing polymorphs, and were therefore easily identified (see Figures 26 and 28). The LP1K antibody was used for all subsequent cytospin fluorescent labelling experiments.

#### 4.3.1.4 Comparison of Ficoll extraction vs. direct red cell lysis technique

The technique of ficoll extraction from whole blood was compared with the direct lysis technique. 1 ml of heparinised whole blood was layered onto 3 ml. Ficoll 1027 (TM) and centrifuged at 400 g for 30 minutes. A mononuclear layer of cells was easily visible in the upper transparent layer. These cells were removed and prepared for cytospin analysis as previously described. For 2 samples of whole blood spiked with 100 RT112 (bladder cancer) cells, the total number of positively labelled fluorescent cells identified were 2 and 4, compared with 31 and 34 cells for the direct red cell lysis technique (see Table 3). It was deemed that the 30 - 35 % recovery rate of the direct red

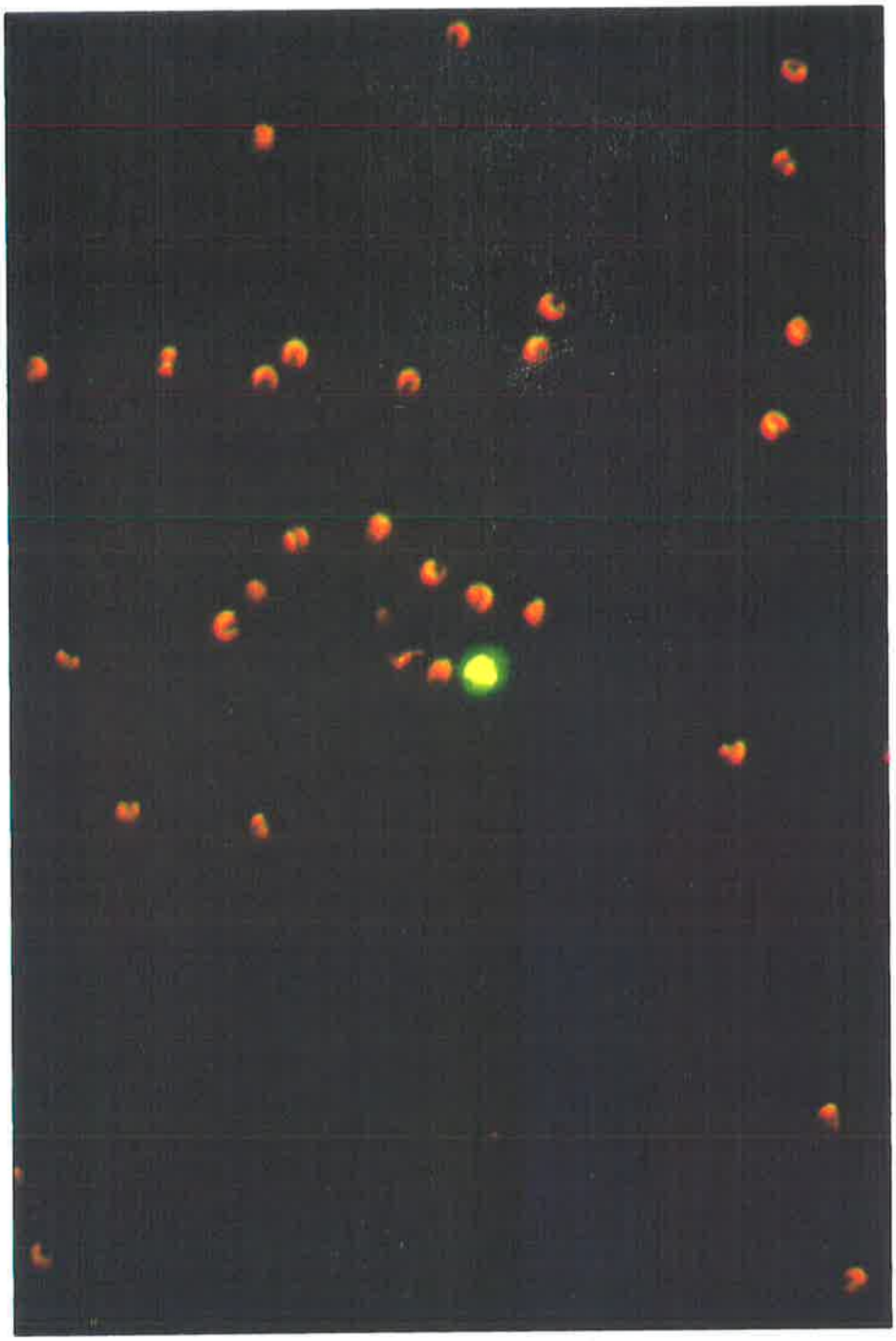


Figure 26 A single RT112 (bladder carcinoma) cell seen (fluorescent green/yellow) amongst autofluorescent polymorphs from a whole blood sample spiked with RT112 cells after cytospin and labelling with the LP1K antibody and secondary fluorescent antibody (Axioplan binocular microscope, oil immersion, magnification x 40). This photograph was double exposed under both fluorescent light and FITC light to demonstrate the differece in the fluorescence pattern between the antibody labelled epithelial tumour cell and the autoflourescent polymorphs.

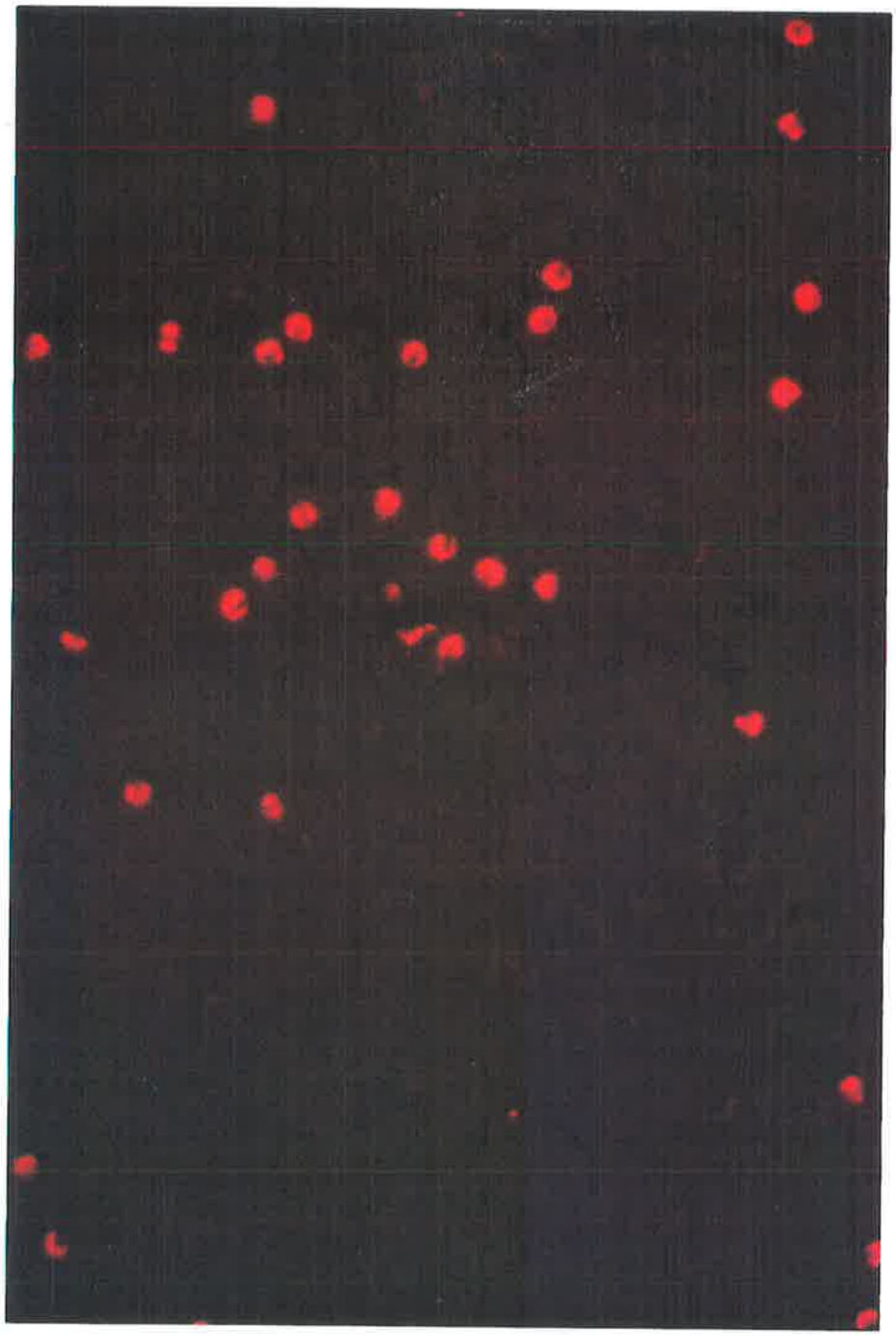


Figure 27. The same field as Figure 26 photographed under FITC light only, demonstrating the autofluorescence of polymorphs and lack of flourescence of the RT112 tumour cell. This technique of viewing samples under both fluorescent light and FITC light allowed discrimination between genuinely positively labelled fluorescent cells and autofluorescent polymorphs or artifacts.

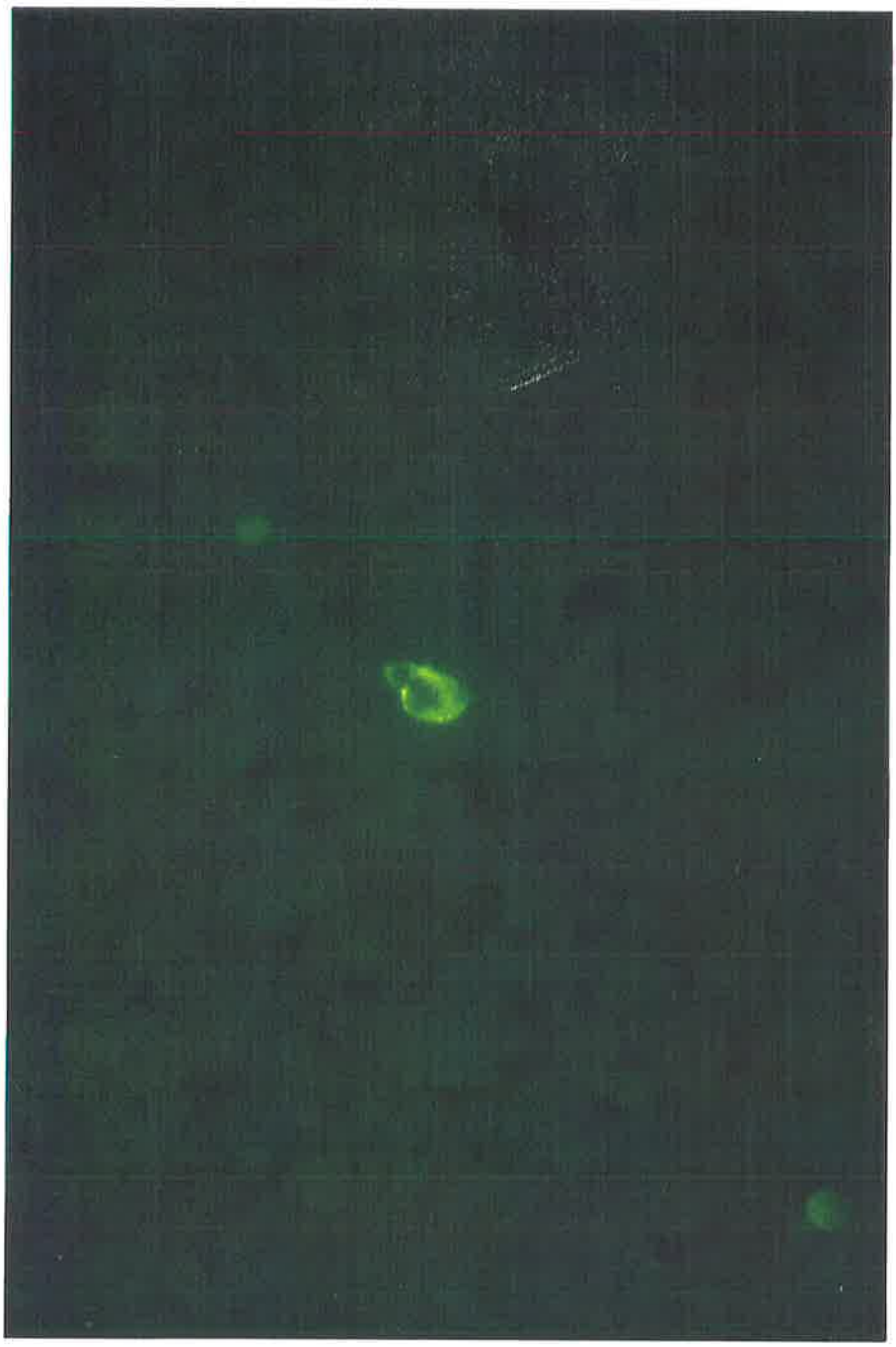


Figure 28. The fluorescent pattern of an EJ (bladder carcinoma) cell easily identified in a whole blood cytospin slide following primary labelling with the LP1K antibody (Axioplan binocular microscope, oil immersion, magnification x 40).

# TABLE 3.

COMPARISON OF BLOOD CELL SEPARATION TECHNIQUES WITH REGARD TO LEVEL OF DETECTION OF RT112 (BLADDER CANCER) CELLS IN BLOOD USING THE CYTOSPIN / ANTIBODY / IMMUNOFLUORESCENCE TECHNIQUE

## DIRECT RED CELL LYSIS

| | #1 | #2 | #3 | #4 | #5 | total (recovery per 100 cells) |
|---|---|---|---|---|---|---|
| Antibody | | | | | | |
| LE61 | 6 | 5 | 7 | 6 | 7 | 31 |
| LP1K | 9 | 6 | 6 | 7 | 6 | 34 |

## FICOLL-HYPAQUE

| | #1 | #2 | #3 | #4 | #5 | total (recovery per 100 cells) |
|---|---|---|---|---|---|---|
| Antibody | | | | | | |
| LE61 | 0 | 1 | 0 | 1 | 0 | 2 |
| LP1K | 1 | 0 | 2 | 0 | 1 | 4 |

The tables depict the significant tumour cell (RT112) loss and therefore decreased sensitivity of the Ficoll-Hypaque technique of cell separation. Each 1 ml. sample of whole blood spiked with 100 RT112 cells was divided into 5 equal aliquots (#1-#5) prior to cytospinning/antibody-fluorescent labelling and microscopy under UV light.

cell lysis technique provided greater chance of detecting circulating tumour cells in peripheral blood than the ficoll technique.

#### 4.3.1.5 Sensitivity assessment by serial spiking

Sensitivity of the technique was established by serial spiking of peripheral blood samples. 1 ml. samples were spiked with 0, 2, 5, 10, 20, 50 and 100 RT112 cells. The samples were then prepared for cytospin in the usual way. The primary antibody used was LP1K. The number of positively labelled fluorescing cells identified from each preparation were 0 cells from the two unspiked aliquots, 0 cells from the 2 cell sample, 2 cells from the 5 cell sample (40 %), 3 cells from the 10 cell sample (30 %), 4 cells from the 20 cell sample (20 %), 14 cells from the 50 cell sample (28 %),, and 30 cells from the 100 cell sample (30 %) (see Table 4).

#### 4.3.1.6 Use of magnetic beads to increase recovery

To ascertain whether the use of magnetic beads would increase the recovery of cells, and decrease the number of cytospin preparations per ml. of blood required to examine, modifications were made to the cytospin and labelling protocol. Whole blood was serially spiked with increasing numbers of RT112 cells. Following lysis of the red cells and washing and resuspending the pellet, the cells were fixed by incubating for 1 hour with methanol/acetone. The cells were centrifuged, washed in PBS with FBS and finally resuspended in 20 ul PBS with FBS. 20 ul LE61 antibody was added and the cells were incubated at room temperature for 60 minutes. The mix was then washed and centrifuged twice in PBSand finally resuspended in 80 ul of PBS with FBS. 20 ul microbeads were added and the mix was gently mixed and incubated at 4c for 30 minutes. 2.5 ul G&M IgG-FITC antibody was added to the mixture and allowed to

# TABLE 4.

## CELL RECOVERY / No. SPIKED EJ CELLS PER Ml. OF BLOOD (USING LP1K ANTIBODY

| | #1 | #2 | #3 | #4 | #5 | TOTAL |
|---|---|---|---|---|---|---|
| **0 CELLS** | 0 | 0 | 0 | 0 | 0 | 0 |
| **2 CELLS** | 0 | 0 | 0 | 0 | 0 | 0 |
| **5 CELLS** | 1 | 1 | 1 | 0 | 1 | 4 |
| **10 CELLS** | 1 | 0 | 1 | 0 | 3 | 5 |
| **20 CELLS** | 1 | 0 | 2 | 0 | 1 | 5 |
| **50 CELLS** | 5 | 3 | 4 | 3 | 5 | 20 |
| **100 CELLS** | 7 | 6 | 8 | 7 | 5 | 33 |

The table depicts the level of detection of spiked EJ (bladder cancer) cells in 1 ml. of whole blood using the cytospin/antibody/immunofluorescence technique of 5 cytospin preparations per ml. of blood. This shows that the level of detection is 5 cells per ml. of blood.

incubate for a further 30 minutes. 5 ml PBS with FBS was added to the tube which was then fixed to a magnet for 30 minutes. While being careful not to disturb the cells which had been magnetised to the side of the tube, the supernatant was removed. The "magnetised" cells were resuspended in 100 ul PBS. The cells in the supernatant were centrifuged, washed and resuspended in 400 ul PBS. A single cytospin preparation was performed on the "magnetised" cells, and 4 cytospin preparations were performed on the cells in the supernatant.

In the "magnetised" preparations, no positively labelled fluorescent cells were identified from any of the samples. In the supernatant cytospins, a total of 3 cells were recovered from the 50 RT112 cell spiked blood sample. The experiment was repeated, and on the second occasion, 1 cell was identified in the "magnetised" cytospin preparation of the 50 RT112 cell spiked blood sample. It was concluded that the use of magnetic beads did not enhance recovery of positively labelled fluorescent cells from blood samples.

#### 4.3.1.7 Tumour cell recovery with less cytospins

An experiment was performed to determine whether decreasing the number of cytospins per sample significantly altered the cell recovery. 1 ml. blood samples were spiked with 0, 2, 5, 10, 20, and 50 RT112 cells, and repeated using EJ (another human bladder carcinoma cell line) cells. Samples were prepared using direct red cell lysis and the LP1K antibody. Samples were prepared using both 2 cytospins per ml. of blood and 1 cytospin per ml. of blood. Using the 2 cytospin technique, the recovery of RT112 cells was 4 from 50 cells (8 %), 2 from 20 cells (10 %), 1 from 10 cells (10 %), 2 from 5 cells (40 %), 0 from both 2 cells and 0 cells. The recovery of EJ cells using the 2 cytospin

technique was 3 from 50 cells (6 %), 2 from 20 cells (10 %), 1 from 10 cells (10 %), and 0 from 5 cells, 2 cells, and 0 cells. For samples prepared using 1 cytospin only, no cells were identified in any preparation with either cell line (see Table 5).

It was concluded that the 1 and 2 cytospins per sample methods of preparation did not improve rates of identification of tumour cells in whole blood samples. Experiments to determine whether a number of cytospins greater than 5 per sample improved the identification rate were not performed. Using the 5 cytospin per 1 ml. whole blood sample, one may expect to identify "positive" blood samples when as few as 5 tumour cells are present in 1 ml. of blood.

### 4.3.2 Clinical evaluation

Samples of blood were obtained from patients with advanced carinoma. These were obtained both from inpatients of the Medical Oncology unit at St. James's University Hospital Leeds, U.K., and patients attending medical oncology outpatient clinics of the same hospital. All patients gave consent for their blood to be examined.

Three broad groups of patients were examined. The 3 groups were breast cancer, colorectal cancer, and "other" carcinomas (including gastric cancer, bladder cancer, ovarian cancer, renal cancer and small cell lung cancer). A "positive" result was determined as the identification of one or more positively labelled fluorescent cells in any one of the 5 cytospin slides per patient sample. The blood preparations were prepared "unblinded", but their analysis under the microscope was "blinded". The results are depicted in Tables 6 - 8.

# TABLE 5.

**Number of cytospins per sample vs. cell recovery #1 (RT112).**

| | 5 spins | 2 spins | 1 spin |
|---|---|---|---|
| **100 cells** | 35 | 8 | 0 |
| **50 cells** | 16 | 3 | 0 |
| **20 cells** | 4 | 1 | 0 |
| **10 cells** | 4 | 2 | 0 |
| **5 cells** | 3 | 2 | 0 |
| **2 cells** | 0 | 0 | 0 |
| **0 cells** | 0 | 0 | 0 |

**Number of cytospins per sample vs. cell recovery #2 (EJ).**

| | 5 spins | 2 spins | 1 spin |
|---|---|---|---|
| **50 cells** | 14 | 3 | 0 |
| **20 cells** | 5 | 2 | 0 |
| **10 cells** | 3 | 1 | 0 |
| **5 cells** | 2 | 0 | 0 |
| **2 cells** | 0 | 0 | 0 |
| **0 cells** | 0 | 0 | 0 |

The tables depict the significant tumour cell loss (and therefore lower detection level) with lower numbers of cytospin preparations per 1 ml. sample of blood spiked with either RT112 (bladder cancer) or EJ (bladder cancer) cells, using the cytospin/antibody/immunofluorescence technique.

#### 4.3.2.1 Breast cancer patients

Of 20 breast cancer patients, there were 2 positive results (see Table 6). One case was that of a 39 year old woman with widespread metastases from breast cancer. Positively staining fluorescent cells were seen in 3 of 5 cytospin slide preparations (see Figure 29). Soon after this specimen was examined she developed bone marrow failure. A bone marrow trephine biopsy showed infiltration with carcinoma. She died shortly afterwards. The second case was that of a 60 year old woman with bone and lung metastases. Several clumps of positively staining tumour cells were identified in 4 of the 5 cytospin slides (see Figure 30).

#### 4.3.2.2 Colorectal cancer patients

Of 19 colorectal cancer patients, there was 1 positive result (see Table 7). This was of a 72 year old man with diffuse pelvic spread of colonic cancer, but with no radiological evidence of either liver or lung involvement. One positively staining fluorescent cell was seen in 1 of 5 cytospin slides (see Figure 31).

#### 4.3.2.3 Other carcinomas

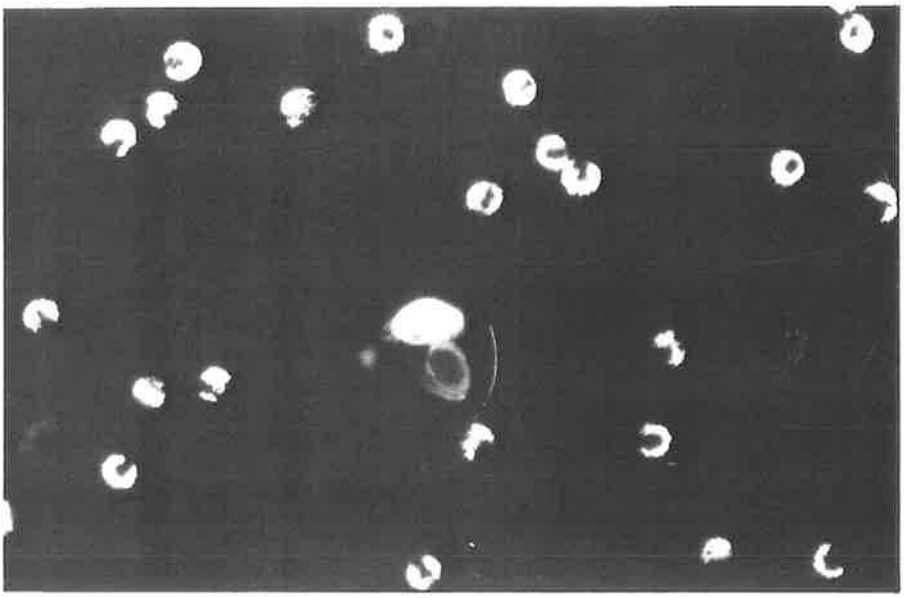
Blood from 14 patients with other epithelial cancers (non breast or colo-rectal), were also examined. These tumours were ovarian cancer,. gastric cancer, renal cell cancer, bladder cancer, small cell lung cancer. There were no positive results (see Table 8).

## TABLE 6.

## CYTOSPIN/ANTIBODY IMMUNOFLUORESCENCE DETECTION OF CIRCULATING CANCER CELLS IN PERIPHERAL BLOOD FOR PATIENTS WITH ADVANCED BREAST CANCER

| AGE | SITES OF DISEASE | CYTOTOXIC CHEMOTHERAPY (in previous 3 mths) | HORMONE THERAPY (in previous 3 mths) | RESULT |
|---|---|---|---|---|
| 35 | BONE | no | yes | NEGATIVE |
| 52 | CHEST WALL | yes | yes | NEGATIVE |
| 38 | BONE; BONE MARROW; LIVER; LUNG | yes | no | **POSITIVE 4 CELLS** |
| 43 | BRAIN | yes | no | NEGATIVE |
| 45 | LIVER; BONE | yes | yes | NEGATIVE |
| 51 | CHEST WALL NECK NODES | yes | no | NEGATIVE |
| 31 | CHEST WALL AXILLARY NODES | yes | no | NEGATIVE |
| 52 | BONE | yes | yes | NEGATIVE |
| 59 | LIVER; LUNG | yes | no | NEGATIVE |
| 60 | PERITONEUM | yes | yes | NEGATIVE |
| 60 | BONE; LIVER | yes | yes | NEGATIVE |
| 49 | NECK NODES | no | yes | NEGATIVE |
| 58 | INFLAM. CA; CHEST WALL; NECK NODES | yes | no | NEGATIVE |
| 81 | BONE | no | yes | NEGATIVE |
| 60 | BRAIN; LUNG; CHEST WALL | yes | yes | NEGATIVE |
| 46 | CHEST WALL; LUNG | yes | yes | NEGATIVE |
| 79 | BONE | yes | yes | **POSITIVE 28 CELLS** |
| 61 | LIVER | yes | no | NEGATIVE |
| 68 | LIVER; BONE | yes | yes | NEGATIVE |
| 78 | BONE | no | yes | NEGATIVE |

29a.



29b.

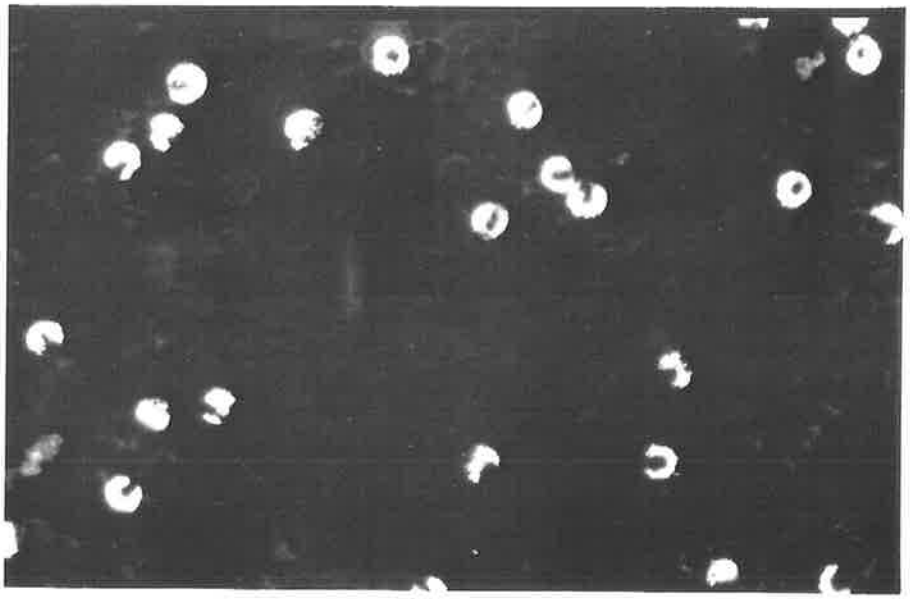
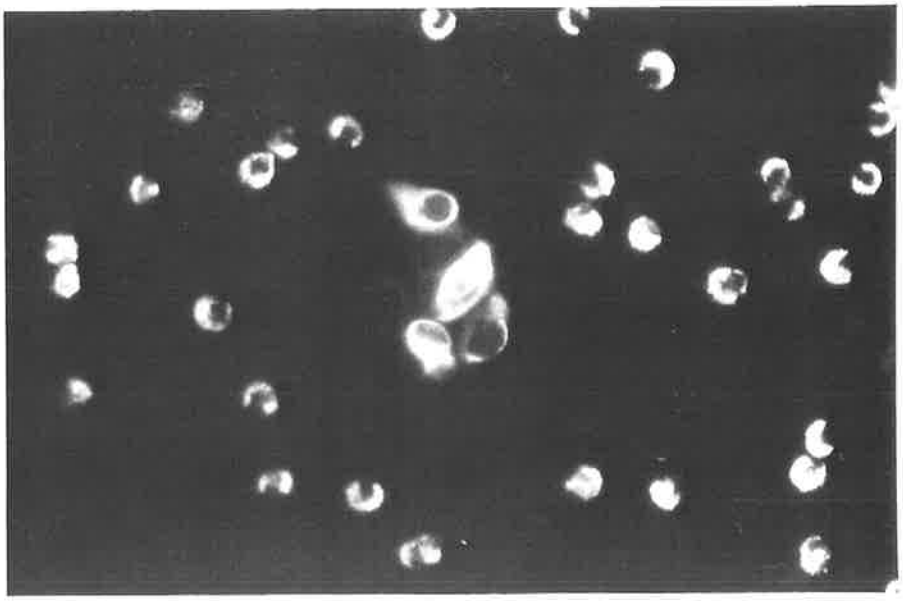


Figure 29. Two positively identified cells observed in a patient with metastatic breast cancer including bone marrow involvement (Axioplan binocular microscope, oil immersion, magnification x 40). The fluorescing cells observed in the centre of Figure 29a. are distinguished from the surrounding autofluorescent polymorphs by virtue of the degreee of fluoresence, the size and morphology, and the absence of autofluoresence properties demonstrated by observing the slides under FITC (red) light as seen in Figure 29b. Positively fluorescing cells were identified in 3 of 5 cytospin preparations from this patient.

**30a.**



**30b.**

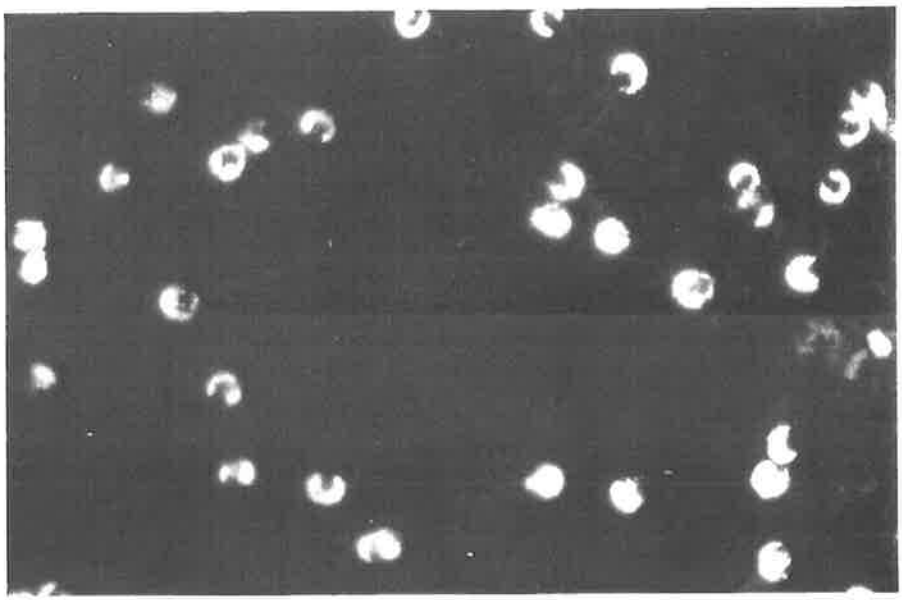


Figure 30. A clump of tumour cells observed in a whole blood cytospin sample from a patient with metastatic breast cancer (bone and lung metastases) (Axioplan binocular microscope, oil immersion, magnification x 40). The fluorescing cells observed in the centre of Figure 30a. are distinguished from the surrounding autofluorescent polymorphs by virtue of the degreee of fluoresence, the size and morphology, and the absence of autofluoresence properties demonstrated by observing the slides under FITC (red) light as seen in Figure 30b. Positively fluorescing cells were identified in 4 of 5 cytospin preparations from this patient

## TABLE 7.

## CYTOSPIN/ANTIBODY/IMMUNOFLUORESCENCE DETECTION OF CIRCULATING CANCER CELLS IN PERIPHERAL BLOOD FROM PATIENTS WITH ADVANCED COLO-RECTAL CANCER

| AGE | SEX | SITES OF DISEASE | 5FU BASED CHEMOTHERAPY (in previous 3 mths) | RESULT |
|---|---|---|---|---|
| 53 | F | LIVER | yes | NEGATIVE |
| 46 | M | PELVIS | yes | NEGATIVE |
| 59 | M | LIVER | yes | NEGATIVE |
| 56 | M | LIVER; PERITONEUM | yes | NEGATIVE |
| 75 | M | PELVIS | yes | **POSITIVE 1 CELL** |
| 52 | M | LIVER | yes | NEGATIVE |
| 70 | M | LIVER; BONE | yes | NEGATIVE |
| 55 | M | LIVER; LUNGS | yes | NEGATIVE |
| 26 | M | LIVER | yes | NEGATIVE |
| 58 | F | BONE | yes | NEGATIVE |
| 46 | M | LIVER | no | NEGATIVE |
| 52 | M | LIVER | no | NEGATIVE |
| 76 | M | PELVIS | no | NEGATIVE |
| 58 | M | LIVER | yes | NEGATIVE |
| 63 | F | LIVER | yes | NEGATIVE |
| 67 | F | PELVIS | no | NEGATIVE |
| 47 | F | LIVER; LUNGS | yes | NEGATIVE |
| 49 | F | BONES | yes | NEGATIVE |
| 50 | M | LIVER | yes | NEGATIVE |

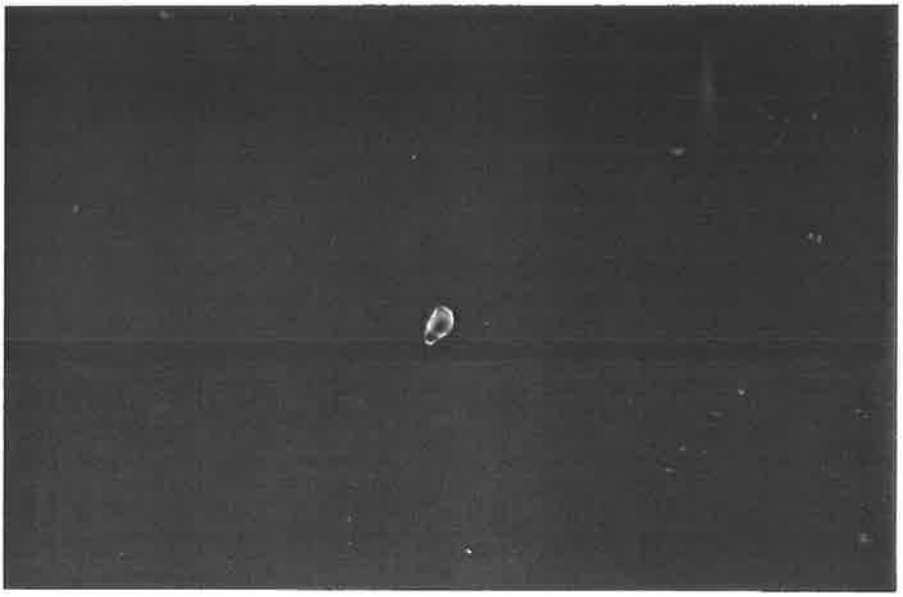


Figure 31. A single positively identified fluorescent cell observed in a whole blood cytospin preparation from a patient with extensive intrapelvic spread of colonic carcinoma (Axioplan binocular microscope, oil immersion, magnification x 40).

# TABLE 8.

## CYTOSPIN / ANTIBODY / IMMUNOFLUORESCENCE DETECTION OF CIRCULATING CANCER CELLS IN PERIPHERAL BLOOD FROM PATIENTS WITH ADVANCED CANCER OF BLADDER, KIDNEY, LUNG, OVARY AND STOMACH.

| AGE | SEX | CANCER TYPE | SITES OF DISEASE | RESULT |
|---|---|---|---|---|
| 59 | M | Bladder cancer | Pelvis; Bones | negative |
| 55 | F | Renal cancer | Lung; Abdo. nodes | negative |
| 54 | F | Renal cancer | L. Kidney; Lungs | negative |
| 48 | M | Gastric cancer | Stomach; Liver | negative |
| 46 | M | Renal cancer | Lungs | negative |
| 72 | M | Renal cancer | Kidneys; Bones | negative |
| 66 | M | Renal cancer | Abdo. nodes; Lungs | negative |
| 64 | F | Ovarian cancer | Peritoneum; Pleura | negative |
| 67 | F | Ovarian cancer | Peritoneum; Abdo. nodes | negative |
| 33 | F | Bladder cancer | Bladder, Abdo nodes, Liver | negative |
| 58 | F | Ovarian cancer | Peritoneum; Abdo. wall | negative |
| 64 | M | Small cell lung Ca. | Lungs; Liver; Bone | negative |
| 70 | M | Small cell lung Ca. | Lungs; Brain | negative |
| 58 | F | Gastric cancer | Stomach; Liver; Abdo. nodes | negative |

## 4.4 METHODS / RESULTS : CYTOKERATIN 8 RT/PCR

### 4.4.1 Primer selection

Oligonucleotide primers were chosen that spanned 2 introns of the human cytokeratin 8 genomic DNA to produce a 244 base pair product [Kraus and Franke 1990]. 2 sense and 2 antisense primers were initially evaluated, with one sense primer traversing the intron between exons 1 and 2, and the other sense primer located on exon 2. The antisense primers were located on exons 4 and 5 (see Figure 32). The primers were tested using routine PCR conditions on cDNA made from RNA from the cultured cell line A431 (human vulval carcinoma) made as per 2.1.2. Different combinations of the sense and antisense primers were evaluated for PCR product band brightness on ethidium bromide stained gels. The primers finally selected for use in further experimentation were:- Cytokeratin 8 sense : AACAACCTTAGGCGGCAGCT, located on exon 2, and Cytokeratin 8 antisense : GCCTGAGGAAGTTGATCTCG, located on exon 4 (see Figure 33).

### 4.4.2 Pseudogene detection

Pseudogenes are DNA segments which are thought to be the result of RNA reverse transcripts into the genome. The cytokeratin family is known to contain several processed pseudogenes (see also 4.5.2.1). The presence of a pseudogene may enable contaminating genomic DNA to produce a false positive result.The first experiment utilised "standard" PCR conditions (see 1.6.6). 1 ug A431 RNA was used per reaction. For one reaction the RT step was omitted. Water and tRNA were used as negative controls. Following the standard protocol a 244 base band was seen for both samples containing the A431 RNA whether or not the RT step was omitted (see Figure 34). No

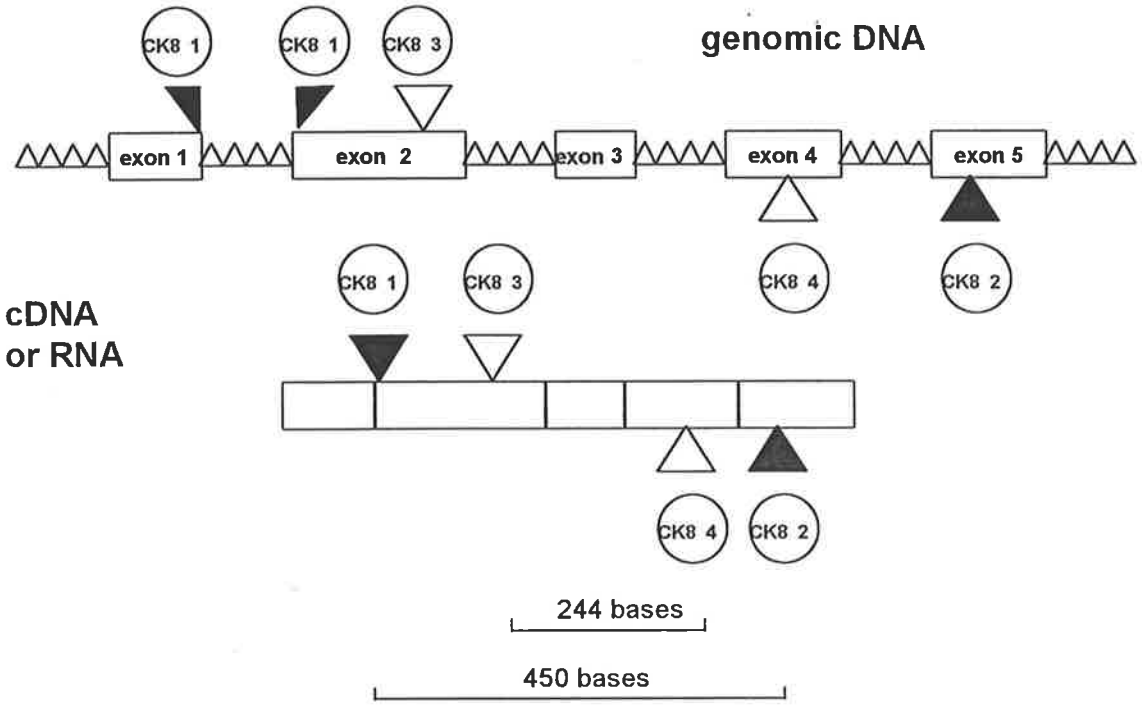



Figure 32. Four human cytokeratin 8 PCR primers were chosen for testing as primers for RT/PCR studies. Each primer was located on separate exons except primer CK8 1 which straddled exons 1 and 2. The pairing of outer primers (CK8 1 and CK8 2) produced a PCR product of 450 bases. The pairing of inner primers (CK8 3 and CK8 4) produced a PCR product of 244 bases.

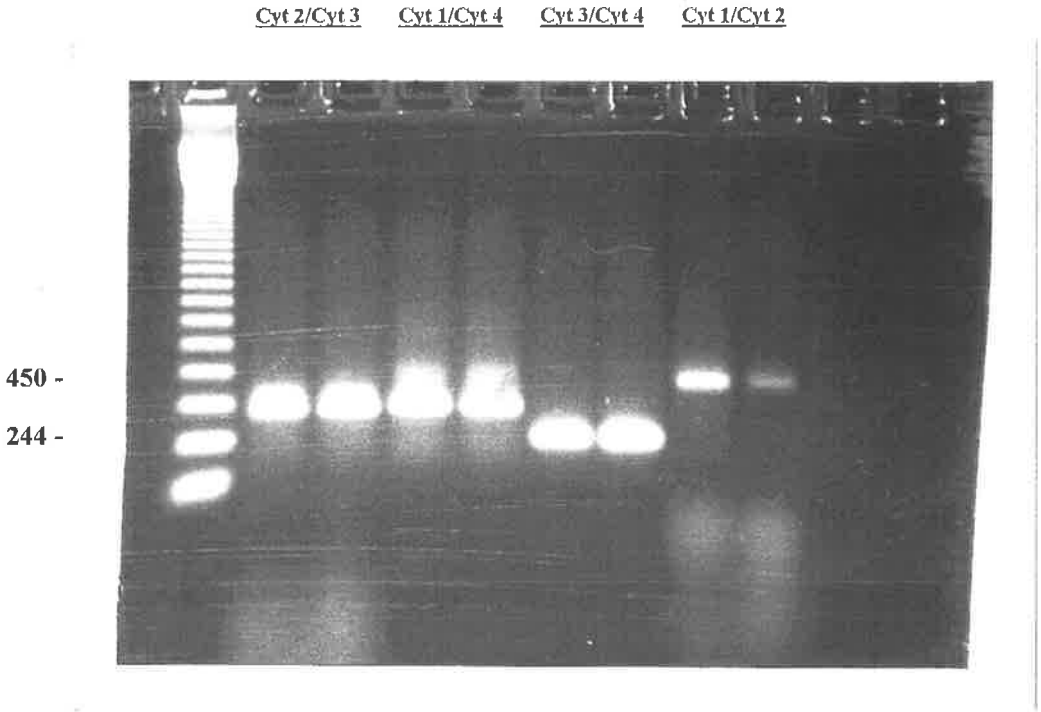



Figure 33. Testing different combinations of cytokeratin primers for RT/PCR using 1 ug RNA extracted from A431 (vulval carcinoma) cells. The combination of cytokeratin 3 and cytokeratin 4 primers produced the brightest and most discrete band.

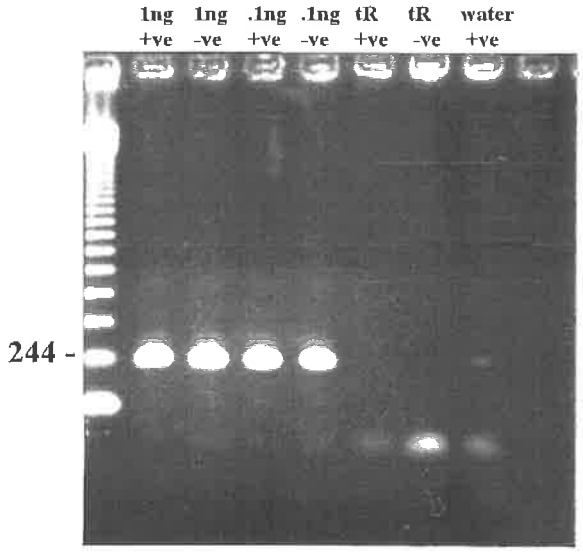



Figure 34. The presence of the cytokeratin 8 pseudogene demonstrated by production of the 244 bp Cyt 8 primer 3 / primer 4 PCR product in samples of A431 (vulval carcinoma) RNA which have not undergone the reverse transcription process. The 244 base pair product must have arisen from contaminating gDNA. The PCR product is the same size as the predicted cDNA sequence, supporting the premise that the cDNA sequence is also contained within gDNA (pseudogene). This demonstrates the need for DNAse treaement of all RNA samples prior to RT/PCR to ensure eradication of all potentially contaminating DNA.

244 base bands were seen in any of the reactions containing only water or tRNA. The conclusion following this experiment was that contaminating gDNA was present in the sample which contained a cytokeratin 8 pseudogene, and therefore all RNA samples need treatment with DNAse prior to the RT step.

### 4.4.3 Optimising DNAse conditions

Optimal DNAse conditions were determined by varying the concentration of DNAse in the mix as well as the time of DNAse treatment. Using a constant treatment time of 45 minutes the amount of DNAse added to the reaction was varied between 0 - 10 units. As little as 0.5 units ofDNAse was seen to completely digest plasmid DNA.

RNA recovery techniques post DNAse treatment were evaluated. The standard technique of phenol/chloroform extraction following DNAse treatment was initially evalauted. RNA recovery was incomplete as measured by both optical densitometry and direct visualisation of RNA electropheresed on a formaldehyde gel (see Figure 35). This step also added between 45 and 60 minutes to the procedure. .

Heating the reaction to 95°c for 5 minutes after the DNAse treatment was complete, as a means of inactivating the DNAse [Dilworth and McCarrey 1992], appeared to afford near complete recovery of RNA (see Figure 36). RNA samples were then treated with 5 units of DNAse for varying lengths of time and RNA integrity was then assessed by optical densitmetry, and by direct visualisation following electropheresis on an agarose gel. No discernible RNA degradation appeared to occur until greater than 45 minutes treatment time (see Figure 37). It was concluded that 5 units of DNAse I incubated with the RNA sample for 30 minutes, followed by heating

**Pre DNAse Post DNAse**

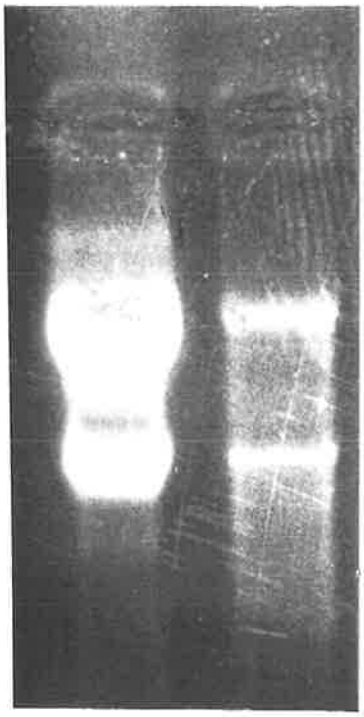


Figure 35. Following DNAse treatment and phenol/chloroform extraction of a 1 ug sample of A431 (vulval carcinoma) RNA, significant loss of RNA signal is observed.

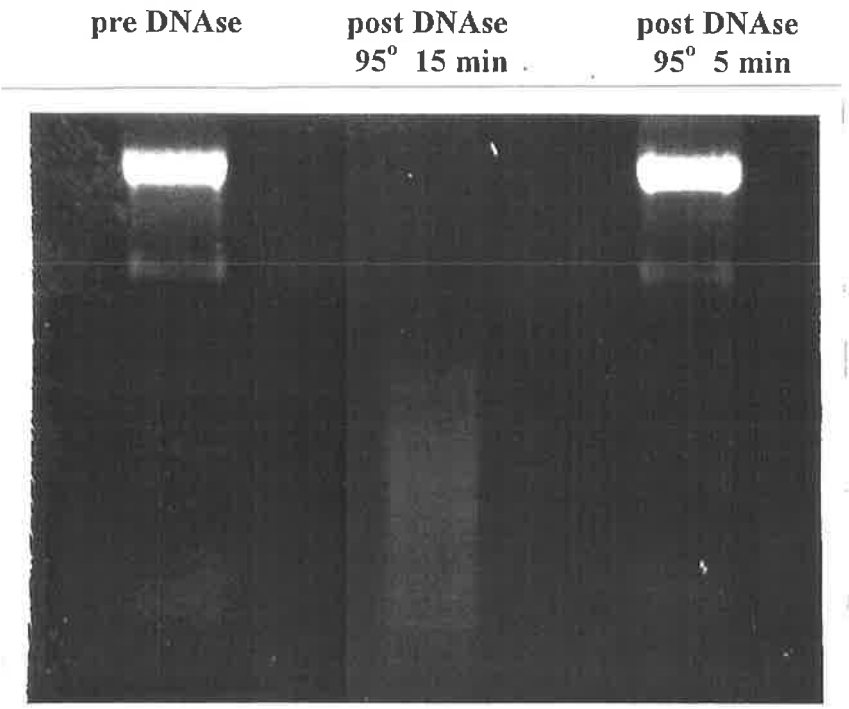



Figure 36. Stopping the DNAse (A431 RNA) reaction by heating at 95° for 15 minutes produces marked degradation of the RNA sample. However stopping the DNAse reaction by heating the sample at 95° for 5 minutes resulted on no discernible degradation of RNA. Checking the optical density of RNA samples pre and post DNAse treatment (in which the reaction was stopped by heating to 95° for 5 minutes) revealed a less than 15 % RNA degradation.

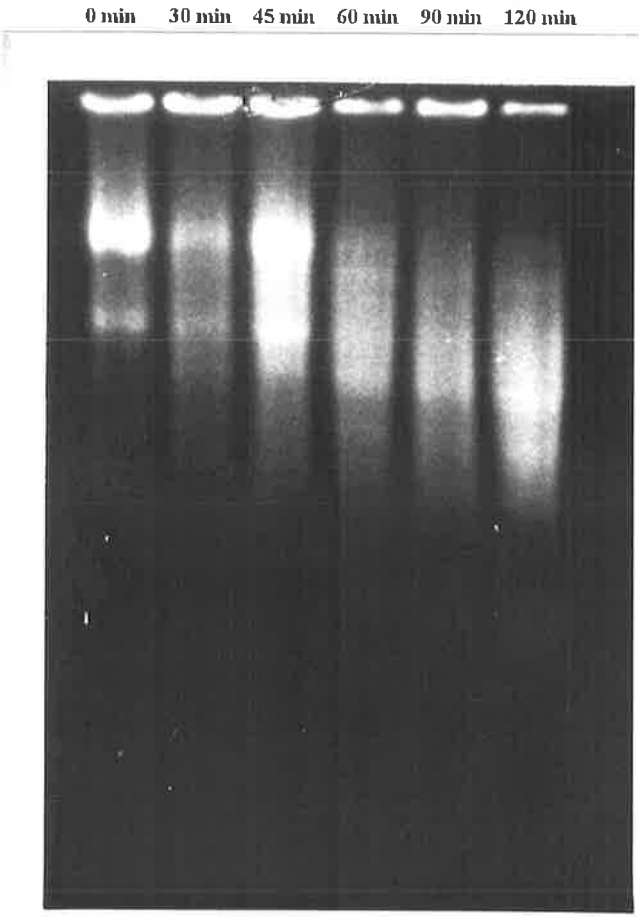



Figure 37. 1 ug samples of A431 (vulval carcinoma) RNA incubated at varying times with 5 units of DNAse (Pharmacia$^{(TM)}$) demonstratating that significant degradation of RNA does not occur until the DNAse reaction continues for at least 45 minutes.

the sample for at 95°c for 5 minutes would become the "standard" DNAse treatment protocol.

### 4.4.4 Assessing reliability of DNAse treatment

4 aliquots of A431 RNA were prepared for RT/PCR using the Cytokeratin 8 primers. 2 aliquots underwent DNAse treatment, and 2 underwent "sham" DNAse treatment with DNAse omitted. Following the DNAse reaction, one aliquot which had been genuinely DNAse treated and one aliquot which had undergone "sham" DNAse treatment underwent the reverse transcriptase reaction. The remaining aliquots underwent a "sham" RT reaction with no RT. All then underwent 30 cycles of PCR. In the "sham" DNAse treatemnt aliquots, PCR product was easily visible on an ethidium bromide treated agarose gel following electropheresis. Only the genuinely DNAse treated aliquot that had not undergone RT failed to produce appropriate 244 base bands on the ethidium bromide stained agarose gel (see Figure 38). It could be concluded that the technique of DNAse treatment followed by RT/PCR ensured that bands produced by this method were amplified cDNA and not contaminating gDNA.

The experiment was repeated using aliqots of A431 RNA contaminated with 1 ug pMel 34 plasmid DNA. Following DNAse/"sham" DNAse and RT/"sham"RT reactions, samples underwent PCR simultaneously Cytokeratin 8 oligonuceotide primers, and tyrosinase oligonucleotide primers. DNAse adequately removed contaminating A431 gDNA, but did not completely digest 1 ug pMel34 plasmid DNA as demonstrated by presence of a 207 bp PCR product in the RT negative sample. Despite apparent complete digestion of plasmid DNA as determined by visualisation on agarose gel, enough plasmid DNA remained intact to be amplified in the PCR reaction

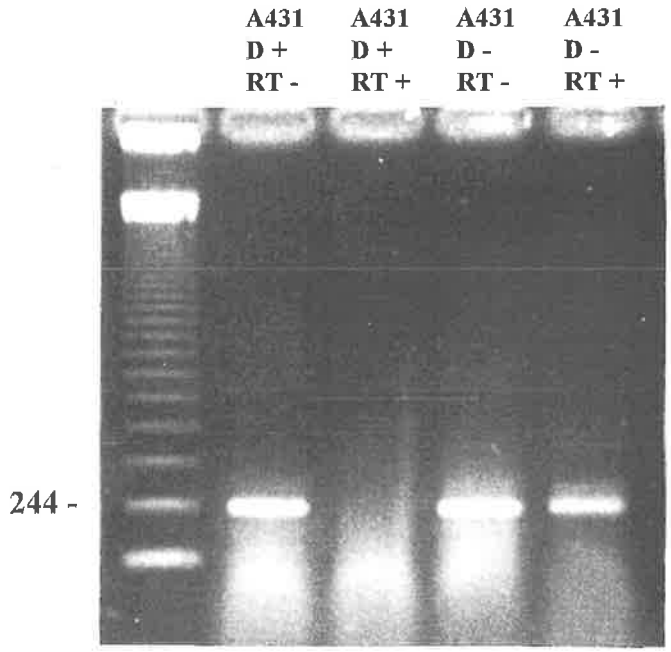



Figure 38. A 1 ug sample of A431 (vulval carcinoma) RNA divided into 2 equal aliquots, only one of which underwent DNAse treatment (D +). These aliquots were again divided into 2 further aliquots. One aliquot from each initial division underwent a "sham" reverse transcription reaction, without the RT (RT -ve). All samples then underwent PCR (single round 30 cycles with cytokeratin primers). Positive 244 bp bands were identified in all samples except the DNAse +ve/RT -ve sample, demonstrating the effectiveness of the DNAse treatment in removing contaminating gDNA (containing the cytokeratin 8 pseudogene) from the RNA sample. The positive result in the DNAse -ve/RT -ve sample confirms the necessity for DNAse treatment of all RNA samples.

(results not shown). It reinforced the necessity of always including RT negative controls with each reaction to ensure that false positive results due to contaminating DNA are detected.

### 4.4.5 Optimising PCR conditions

PCR conditions were varied to determine the most suitable conditions for PCR using the Cytokeratin 8 primers. Final magnesium concentration was varied between 0.3 mmol and 1.5 mmol. Brightest bands were seen when Mg concentration was 0.5 mmol (see Figure 39). dNTP concentration was varied between 50 umol and 400 umol. Brightest bands were seen using 100 umol dNTP (see Figure 40). The amount of oligonucleotide primer added to the PCR reaction was varied between 50 pmol and 450 pmol. The best results were seen using 100 pmol of Cytokeratin 8 oligonucleotide primers. Annealing temperature was varied between 52°c and 58°c. 58°c produced the best results as determined by the brightness and singularity of the band (see Figure 39).

It was determined that the best conditions for the cytokeratin 8 PCR was using 100 umolconcentration of dNTP, 0.5 mmol Mg concentration, a total of 100 pmol Cytokeratin 8 oligonucleotide primer, and an annealing temperature of 58°c.

### 4.4.6 Sensitivity testing with serial RNA dilutions

To test the sensitivity of the technique, serial dilutions of A431 RNA were dissolved in tRNA to make a total amount of 1 ug RNA. Amounts of A431 RNA in ug examined were 0, $10^{-6}$, $10^{-5}$, $10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$, and 1. For each there was both RT positive and RT negative pairs. The sensitivity achieved was between $10^{5}$ and $10^{-6}$ ug

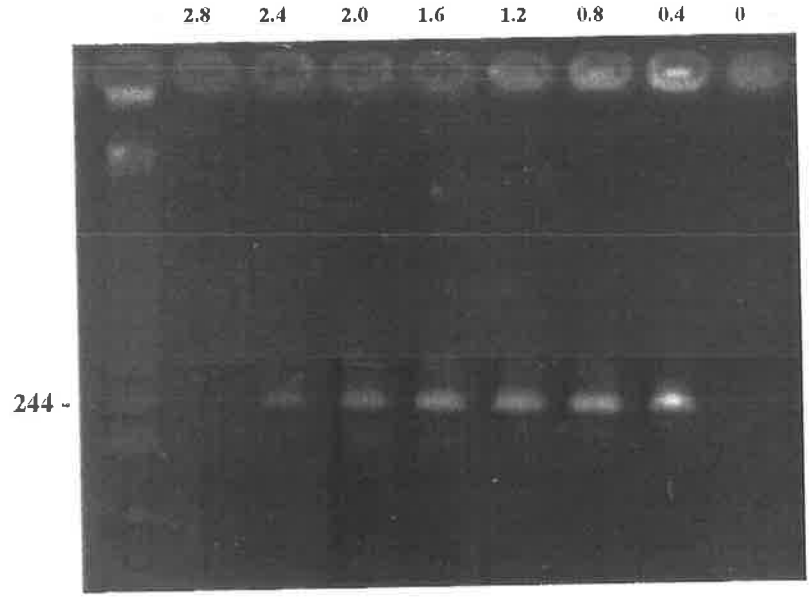



Figure 39. 30 cycles of PCR (using cytokeratin primers) on the same 1ng dilution of A431 (vulval carcinoma) RNA with variable final magnesium concentrations in the PCR buffer (0 - 2.8 mM). The brightest 244 bp band was observed in the 0.4 mM Mg sample, suggesting that this PCR with cytokeratin primers worked optimally with lower magnesium concentrations in the PCR buffer.

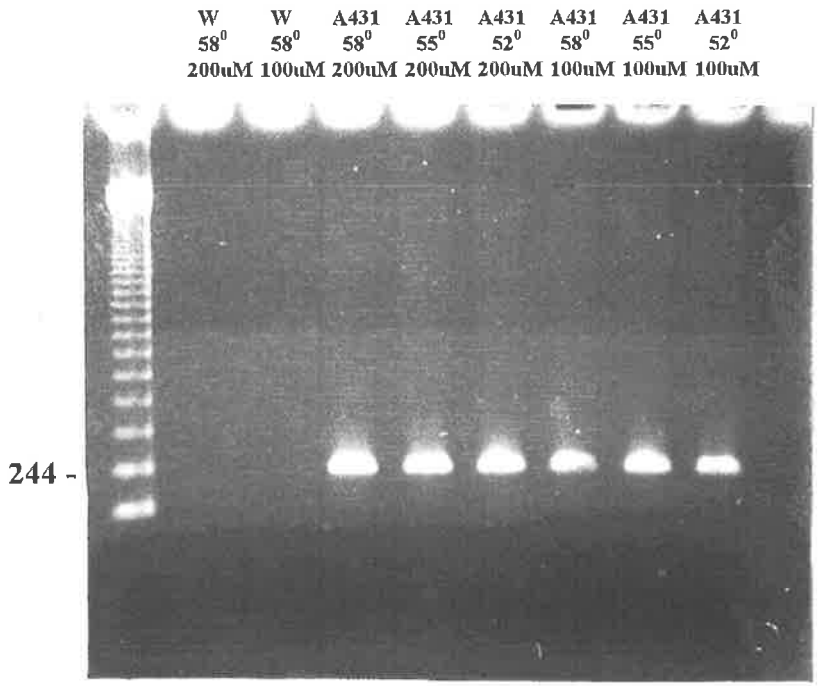



Figure 40. Optimisation of dNTP conentration and annealing temperature simultaneously using 30 cycles of PCR (with cytokeratin primers) on identical 1 ng dilutions of A431 (vulval carcinoma) RNA. dNTP concentration was varied between 100 uM and 200 uM. The annealing temperatures tested were 52°, 55° and 58°. Water (W) was substituted for A431 RNA as the negative controls. The 58° annealing temperature samples gave a marginally qualitatively stronger 244 bp band signal. There appeared to be no difference between the 244 bp band signals for either the 100 uM and the 200 uM dNTP concentrations.

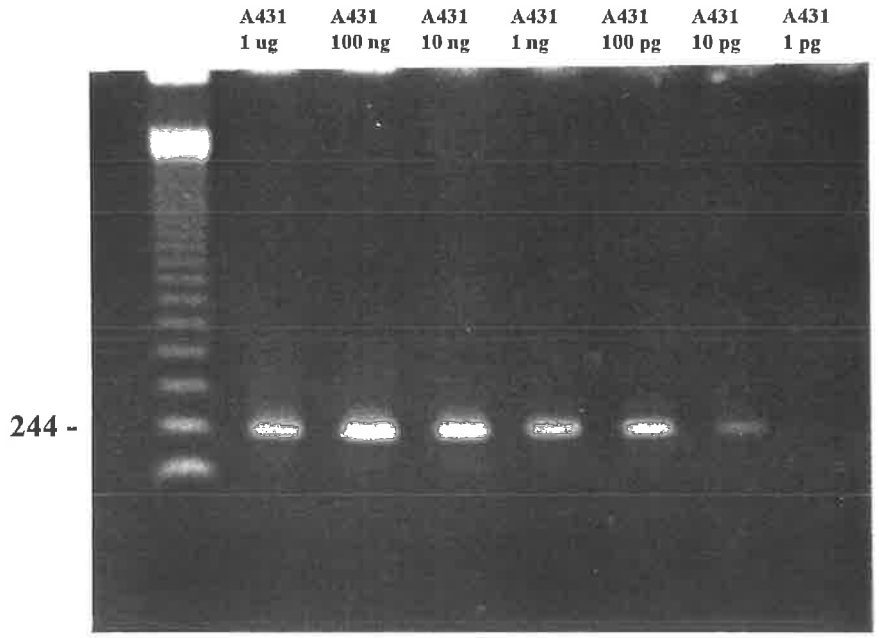



Figure 41a. RT/PCR (30 cycles using cytokeratin primers) performed on serial dilutions of A431 (vulval carcinoma) RNA ranging from 1ug to 1 pg demonstrating that the limit of detection of the 244 bp band is at 10 pg (equivalent to 1 cancer cell).

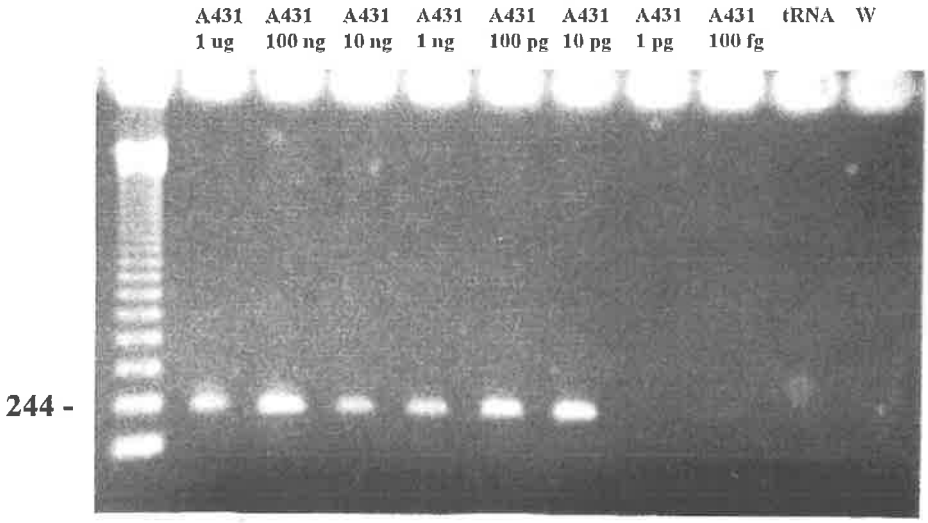



Figure 41b. RT/PCR (30 cycles using cytokeratin primers) performed on serial dilutions of A431 (vulval carcinoma) RNA ranging from 1ug to 100 fg confirming that the limit of detection of the 244 bp band is at 10 pg (equivalent to 1 cancer cell).

(or 10 picograms) of A431 RNA (see Figure 41). This theoretically corresponds to single cell amounts of RNA.

### 4.4.7 Detection of cytokeratin 8 in normal whole blood

To determine whether cytokeratin 8 was expressed in normal peripheral blood cells, 2 ml. samples of peripheral blood from 6 normal healthy volunteers underwent RNA extraction followed by RT/PCR using the Cytokeratin 8 oligonucleotide primers. The RNA viablility was determined by running a 1 ul aliquot of each whole blood RNA sample on a formaldehyde RNA gel and visualising the RNA banding pattern after ethidium bromide staining uder UV light. Any sample that did not produce the appropriate banding pattern was deemed unsuitable for RT/PCR (see Figure 42). RT negative controls were included for each sample. No 244 base bands were seen for any of the RT negative samples. All RT positive blood samples produced 244 base bands. The A431 RNA positive control produced a 244 base band. The tRNA negative controls did not produce a band (see Figure 43). This experiment was repeated using blood samples from 6 different volunteers (see Figure 44). A similar result was obtained. It was concluded that normal peripheral blood cells express cytokeratin 8 mRNA.

An attempt was made to further examine the particular cell types in peripheral blood that express cytokeratin 8 mRNA. Ficoll blood separation into mononuclear cells and polymorphs was performed. Using FACscan analysis, 95 % purity of both mononuclear cell and polymorphs was achieved. In order to determine whether this degree of purification would be able to allow identification of specific cell expression of cytokeratin 8 mRNA, RT/PCR was performed on RNA extracted from each of these

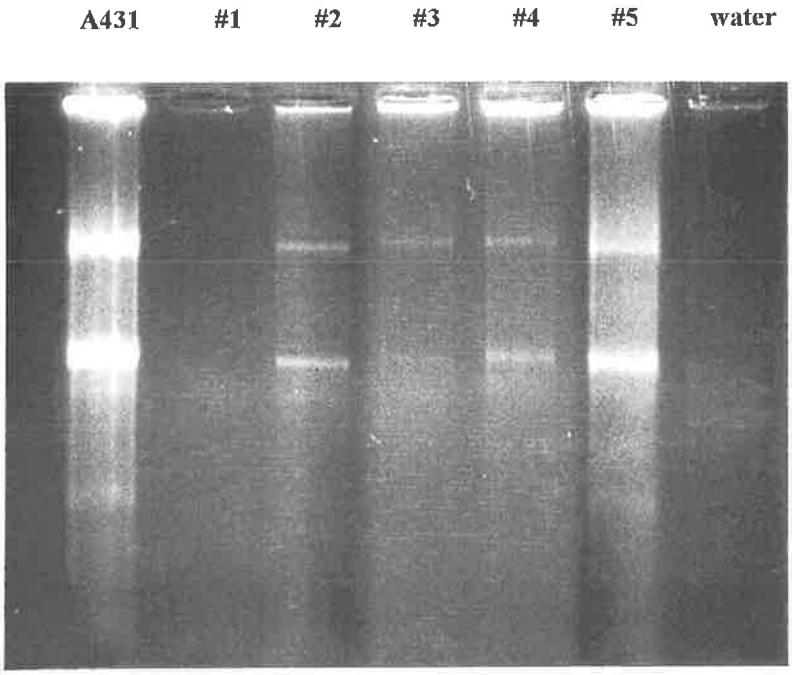



Figure 42. Only whole blood RNA samples which remained intact following DNAse treatment were considered for RT/PCR. In the depicted RNA formaldehyde gels, sample #1 has been degraded and was therefore excluded from RT/PCR analysis.

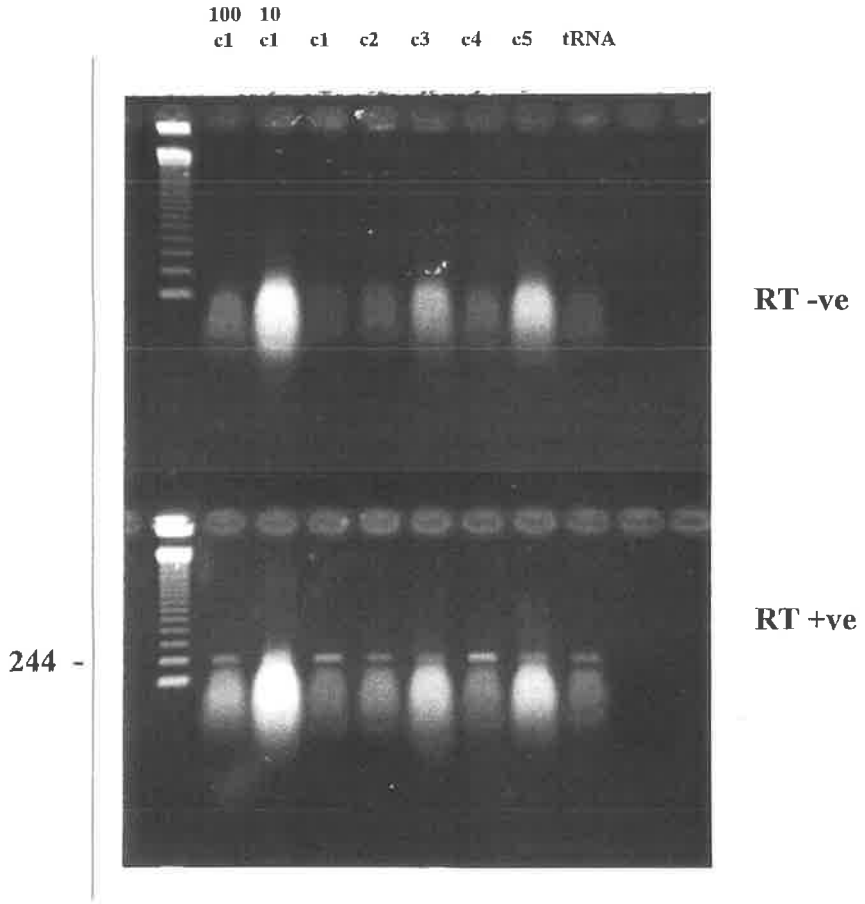



Figure 43. 2 ug RNA samples extracted from whole blood from 6 normal healthy volunteers (c1 - c5) DNAse treated and analysed by RT/PCR (single round of 30 cycles using cytokeratin primers) for evidence of cytokeratin 8 expression. One whole blood RNA sample (c1) had 100 pg and 10 pg A431 (vulval carcinoma) RNA added to 2 ug aliquots of the sample as positive controls. tRNA was used as a negative control. The 244 bp PCR product was identified in all blood samples analysed. There was no detection in the tRNA sample and all RT negative samples.

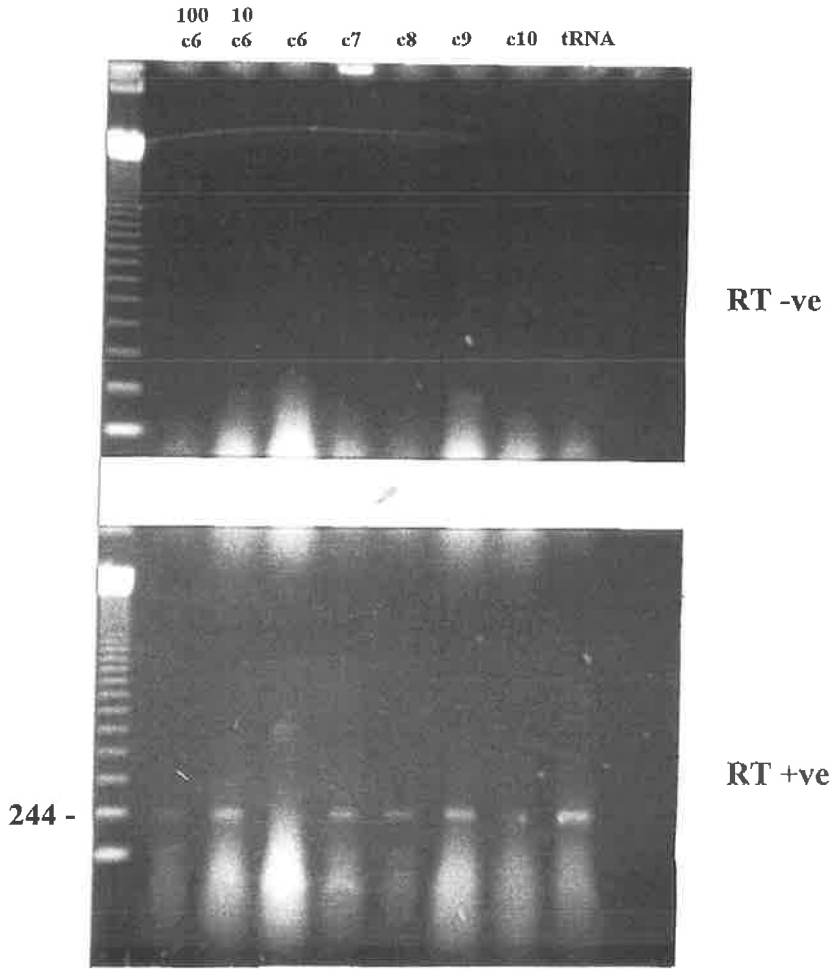



Figure 44. 2 ug RNA samples extracted from whole blood from 6 normal healthy volunteers (c6 - c10) DNAse treated and analysed by RT/PCR (single round of 30 cycles using cytokeratin primers) for evidence of cytokeratin 8 expression. One whole blood RNA sample (c6) had 100 pg and 10 pg A431 (vulval carcinoma) RNA added to 2 ug aliquots of the sample as positive controls. tRNA was used as a negative control. The 244 bp PCR product was identified in all blood samples analysed. There was no positive identification in the tRNA sample ant the RT negative samples.

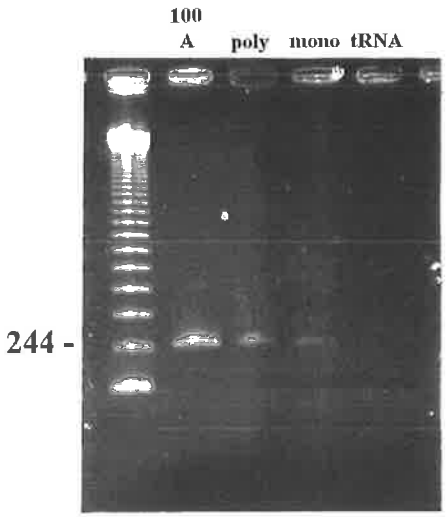



Figure 45. Following red cell lysis of whole blood, the remaining white cell samples underwent Ficoll hypaque cell separation. The cells were separated into polymorph and mononuclear sub-populations of 95 % purity as evaluated by FACscan analysis. RNA was then extracted from these cell populations. The RNA subsequently underwent DNAse treatment and RT/PCR (single round 30 cycles with cytokeratin primers), with 100 pg A431 (vulval carcinoma) RNA as a positive control and tRNA as a negative control. The 244 bp PCR product was observed in both leukocyte populations.

subpopulations of cells. The 244 base band was seen in all subpopulations (see Figure 45).

### 4.4.8 Detection of cytokeratin 8 in non-epithelial cell lines

RNA was extracted from the following cultured cell lines. SKNSH (human neuroblastoma), IMR-32 (human neuroblastoma), SK19 (human melanoma), SK28 (human melanoma), THP1 (human macrophage), Jurkat (human T-lymphocyte) and A431 (human vulval carcinma). RT/PCR was performed on each of the samples., The positive 244 base band was seen in A431, THP1 and SKNSH cell line RNA samples (see Figure 46). In previous experiments (results not shown), RNA from Jurkat cell line and SK19 cell line produced positive results. It was concluded that cytokeratin 8 mRNA is frequently expressed in a number of different non-epithelial human cell types.

### 4.4.9 Sequencing the cytokeratin 8 PCR product

To confirm that the 244 bp PCR product was in fact the target cytokeratin 8 mRNA sequence, RT/PCR was performed on A431 RNA using $^{32}P$ labelled oligonuceotide primers. The PCR products were run on a TAE low melting point agarose gel and the 244 bp band was cut out. The PCR product was purified and chemically sequenced as previously described. Two hundred consecutive bases could be accurately read from the autoradiograph of the sequence:- GGA GAC TCT GGG CCA GGA GAA GCT GAA GCT GGA GGC GGA GCT TGG CAA CAT GCA GGG GCT GGT GGA GGA CTT CAA GAA CAA GTA TGA GGA TGA GAT CAA TAA GCG TAC AGA GAG ATG GAG AAC GAA TTT GTC CTC ATC AAG AAG GAT GGT GGA TGA AGC ATA CAT GAA CAA GGT AGA GCT GGA GTC TCG CCT GGC

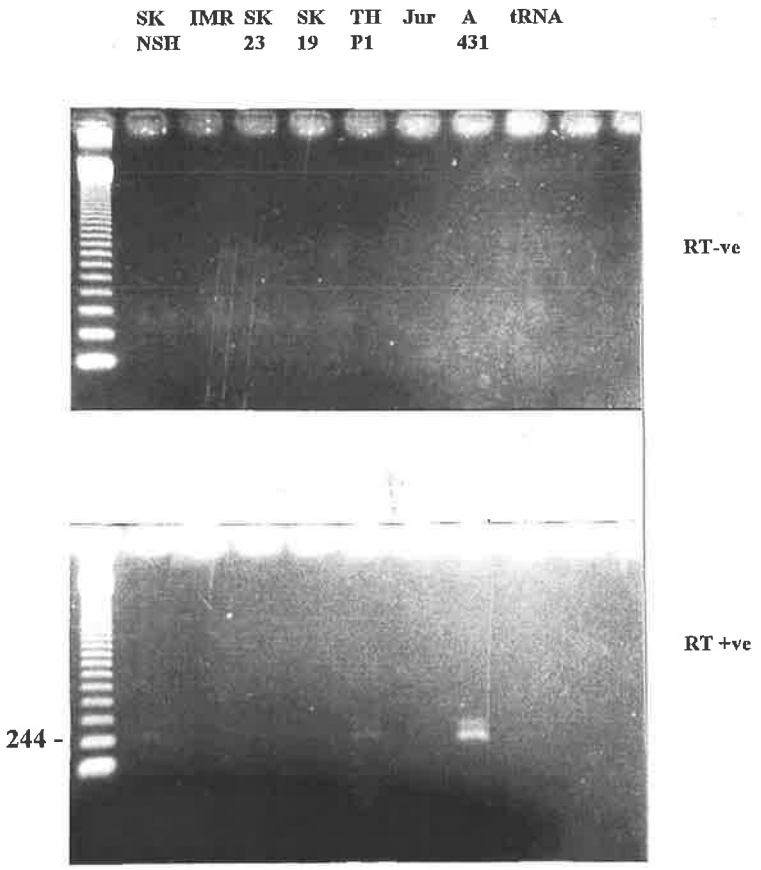



Figure 46. RT/PCR (30 cycles using cytokeratin primers) performed on 10ng samples of human cell line RNA samples : SKNSHand IMR (neuroblastoma), SK23 and SK19 (human melanoma), THP1 (human macrophage), Jurkat (human T-cell), and A431 (human vulval carcinoma)as the positive control. tRNA was the negative control. The 244 bp PCR product was seen in the A431, THP1 and SKNSH samples. there were no positive results in the RT negative controls. In other experiments, both Jurkat and SK19 cell line RNA intermittantly produced positive results.

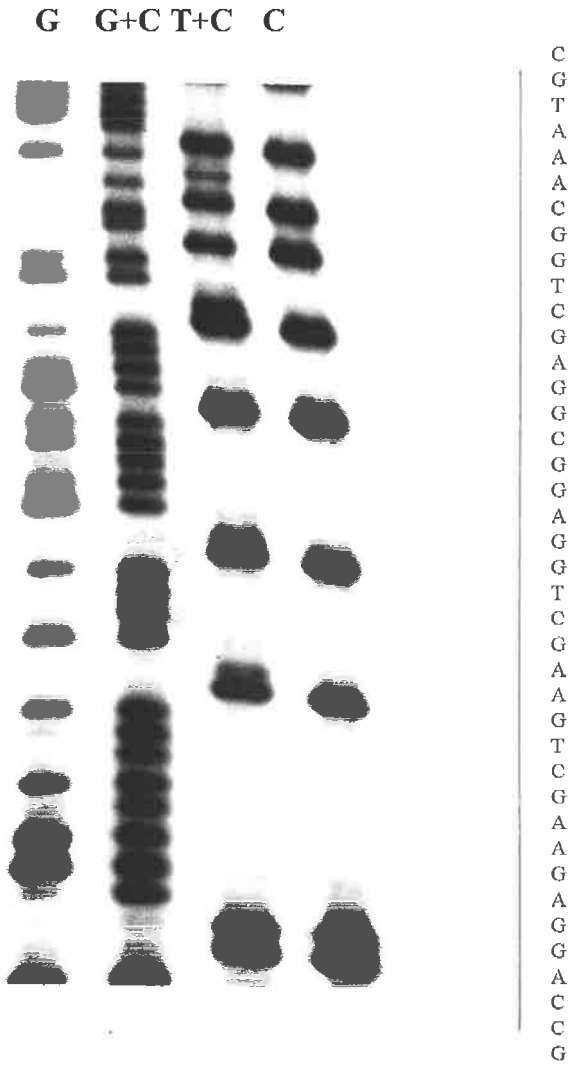



Figure 47. Maxam-Gilbert chemical sequencing of the cytokeratin 8 PCR product depicting 41 bases matching exactly part of the predicted sequence cytokeratin 8 cDNA.

AGG GC. This sequence corresponded exactly to 200 bases of the expected 244 bp sequence of human cytokeratin 8 cDNA (see Figure 47).

## 4.5 DISCUSSION

### 4.5.1 Critical evaluation of the antibody immunofluorescence technique

By ensuring that positive evaluation criteria were strictly adhered to, and by using independent reviewers to scrutinise the results, a sensitivity level of about 5 cells per ml. of whole blood was achieved. Based upon the degree of "cell loss" with this method, this degree of sensitivity is comparable with the numerous cytological techniques that were used in the 1950s and 1960s [Goldblatt and Engell 1965]. At that time, the rate of detection varied widely from < 5 % to > 60 %. The positive detection rate in this series 3 from 53 (5.7 %) and lack of "positive" results in normal blood is more consistant with the more conservatively reported rates from the earlier studies. The positive results were observed to be greater than one log immunofluoresence intensity compared with the autofluoresence of polymorphs and were easily distinguishible from similarly fluorescing artifact creating a relatively "user friendly" microscopic technique. Tumour cell clumping was observed in 2 of the 3 positive samples, and both of these samples had several positive slides. This technique therefore allows a degree of quantification of cell numbers. Although it was not attempted in this experiment, it may be possible to use antibody sorting techniques to isolate these tumour cells (or clumps) from whole blood and further characterise them, especially in comparison with tumour cells from the primary tumour and cells from established secondary sites.

From a prognostic perspective this technique of examining 1 ml. of whole blood appears very limited, with a low level of positivity in patients with grossly advanced disease. Also as only keratin positivity was examined, no information is gained regarding the metastatic potential of the cells detected.

### 4.5.2 Detection of cytokeratin 8 expression in non-epithelial tissue by RT/PCR

#### 4.5.2.1 Pseudogene expression

The detection of a cytokeratin 8 pseudogene was confirmed by the RT negative and DNAse experiments. Processed pseudogenes are DNA segments that are believed to have arisen from the integration of RNA reverse transcripts into the genome. Those pseudogenes that arise from mRNA transcripts lack the intervening sequences (introns) found in the functional genes. Hence thay cannot be differentiated from cDNA sequences based upon size alone [Weiner *et al.* 1986]. It appears that only those genes that are expressed in germ line and pre-germ line cells contain processed pseudogenes. These are often genes for the "housekeeping" proteins such as tubulin, actin, ribosomal proteins etc [Vanin 1985]. Detection of a processed pseudogene usually indicates that the corresponding gene (as functional mRNA) is expressed in early embryo or germ line tissue.

Cytokeratin 8 is expressed in the early embryo with cytokeratin 18 [Jackson*et al.* 1980]. In practical terms this means that although the cytokeratin primer sequences may span one or more intron sequences, producing PCR product bands of different sizes for gDNA and target cDNA thus allowing differentiation between the two, unless all potentially contaminating gDNA is completely eliminated, a processed pseudogene for

cytokeratin 8 may produce the same sized PCR product. Thus strict DNA elimination techniques (and controls to assess DNA elimination) must be employed to avoid false positive results due to detection of the processed pseudogene [Menon*et al.* 1991].

#### 4.5.2.1 Illegitimate transcription

The detection of cytokeratin 8 mRNA expression in non-epithelial tissue (ie. whole blood, non-epithelial tumour cell lines) was confirmed by RT/PCR detection of cytokeratin 8 PCR product in such tissues with careful RT negative controls confirming that contaminating DNA was not present. Regulation of gene expression is one of the basic mechanisms of development in multicellular organisms. Genes may be classified into 2 main groups. There are tissue specific genes which are exclusively expressed at particular developmental stages (these genes encode for proteins involved in functional and phenotypic characteristics of specific cell types), and there are "housekeeping" genes which are generally expressed in all cells (which encode for common structural proteins or ubiquitous enzymes). It has been previously shown, however, that very low levels of expression of certain RNA species may be present in cells for which the protein encoded by the RNA is not expressed [Knapp and Franke 1989]. It seems that these transcripts do not play any specific role in these cells, but their presence indicates a basal level of transcription of tissue-specific genes outside of the tissue where they are normally active. This phenomenon has been termed "illegitimate transcription". A possible mechanism for "illegitimate transcription" is that tissue specific gene transcription is under the control of tissue specific promoters which are in turn, under control of various tissue specific transcriptional factors. However, in the absence of these various factors, gene transcription is extremely low but not totally absent [Chelly

*et al.* 1989]. Using conventional techniques to detect gene product (immunocytochemistry or protein analysis by Western blot) detection of "illegitimately transcribed" gene product is not usually possible. However, with the very sensitive method of RT/PCR to detect RNA, such low level gene transcription has become detectable.

### 4.5.3 Cytokeratins 19 and 20

Cytokeratins 19 and 20 have now also been examined as potential tumour markers in blood using the same technique of RT/PCR used for cytokeratin 8 [Burchill *et al.* 1995]. These analyses were run concurrently with the cytokeratin 8 study. Cytokeratin 19 was targeted as it, like cytokeratin 8, is widely expressed by the simple epithelia of mucosal tissue. Encouraging results using cytokeratin 19 to detect breast cancer cells in lymph node tissue were reported by Datta *et al.* [1994]. Traweek *et al.* [1993] were unable to detect cytokeratin 19 expression in peripheral blood. Using the breast cancer cell line MCF7, a 214 bp band was identified at a sensitivity level of 100 pg total RNA. However when normal whole blood was analysed, using blood of 15 healthy volunteers, the 214 bp CK19 band was detectable in 6 cases (40 %).

The presence of cytokeratin 19 pseudogenes [Bader *et al.* 1986, Savtchenko *et al.* 1988] was shown by positive results in non DNAse treated, RT negative samples. These results are at odds with the data reported by Traweek *et al* [1993] and Datta *et al.* [1994]. The absence of RT negative controls, and RNA quality controls for every sample examined in each of these studies, in conjunction with the relatively small number of patient sample analysed brings into question the validity of the data.. The results form this study [Pittman *et al.* 1993, Burchill *et al.* 1995] are more in keeping

with the study reported by James *et al.* [1993], where cytokeratin 19 expression was inconsistantly demonstrated in normal lymph node tissue and with the results achieved by Schoenfeld *et al.* [1994] who detected cytokeratin 19 expression in normal lymph node tissue by RT/PCR (single round), although this group also found that by lowering the sensitivity of the technique, a distinction could be made between normal "background" expression of cytokeratin 19 and abnormal expression of cytokeratin 19. This final study raises a very interesting prospect in that RT/PCR techniques may be manipulated to become semi-quantitative

Cytokeratin 20, a more recently discovered intermediate filament was chosen as a more discriminatory marker which is expressed particularly in cells of gastrointestinal derivation [Moll *et al.* 1992]. Whilst such a potential tumour marker limits the tumour types for which it may be applicable, it's potential for colorectal cancer or gastric cancer may be considerable. The sensitivity of the technique as demonstrated by detecting a 370 bp band in the colon cancer cell line HT29 was 100 pg of total RNA. Of 15 control whole blood samples from normal healthy volunteers analysed, no positive results (no 370 bp bands) were detected by either ethidium bromide staining or by Southern blot hybridisation. In 6 normal bone marrow samples analysed, again no positive results were obtained [Burchill *et al.* 1995].

In cell spiking experimentaion (when HT29 cells were micro-pipetted into normal whole blood), this RT/PCR method was able to detect down to 100 HT29 cells in 2 ml of whole blood. The 370 bp band generated was shown by Southern blot hybridisation and sequence analysis to be consistant with of the known cytokeratin 20 sequence. There were no positive results identified in any of the corresponding RT negative controls [Burchill *et al.* 1995].

Using the methods discussed cytokeratin 20, not being detectable in normal whole blood makes it the most promising of candidate keratin markers for this method of circulating colonic carcinoma cell detection. Confirmatory studies in patients with colorectal or gastric cancer are required to further evaluate the effectiveness of the method. One potential problem with the technique as it has been presently evaluated is the relative lack of sensitivity. The detection level of 100 cancer cells in 2 ml of blood is one log less than the RT/PCR method used for tyrosinase/melanoma. And the clinical evaluation of this method was only able to detect circulation melanoma cells in 3 of 24 advanced melanoma patients evaluated (see Chapter 3). This level of detection is also only as sensitive as the immunofluorescence cytospin method of detecting tumour cells in peripheral blood (see 4.3) which only gave 1 positive result from 19 samples of whole blood from patients with advanced colorectal cancer examined. Nevertheless, that cytokeratin 20 does not appear to be expressed normally in whole blood or bone marrow must encourage further close evaluation not only of clinical samples, but also of methods which may improve the sensitivity of the technique which may yield higher levels of detection.

### 4.5.4 Correlation with other studies

The detection of keratin gene expression in non-epithelial tissue by RT/PCR has now also been demonstrated by Traweek *et al.* [1993]. In this particular study, cytokertain 8 and 18 were detected in total RNA extracted from sarcoma cells, lymphoma tissue, endothelial cells, leukaemia cells, fibroblasts, normal lymph node tissue, normal bone marrow, and normal peripheral blood cells. It also appears that under certain conditions, such as neoplasia, control of gene expression becomes less

stringent or "leaky", which may account for the occasional finding of keratin protein detection in lymphomas [Sewell *et al.* 1986, Gustmann *et al.*1991], melanomas [Trejdosiewicz et al 1986, Miettinen and Franssila 1988, Hendrix *et al.* 1992], and sarcomas [Meittinen and Rapola 1989]. In several of these examples, the expression of keratin was associated with decreased differentiation and increased malignant potential. Another study by James et al [1993] confirmed the intermittant expression of cytokeratin 19 RNA in normal lymph node tissue by RT/PCR, a finding confirmed by Schoenfeld *et al.* [1994]. However, a study by Datta *et al.* [1994] has suggested that cytokeratin 19 was not expressed in normal lymph node tissue (by RTR/PCR) and that it was discovered in patients with various epithelial malignancies. In this study, primer sequences were selected to incorporate differences between cytokeratin 19 mRNA and one processed cytokeratin 19 pseudogene at the 3' end. This however does not exclude amplification of other cytokeratin 19 pseudogenes that do not differ at the 3' end, and as such probably limits the value of this technique. This study may also be criticised in that RT negative controls appear not to have been performed on each sample tested. Therefore the legitimacy of each positive result may be questioned.

### 4.5.5 Clinical relevance of results

These results, when taken in the context of the clinical aims of the study, suggest that cytokeratin 8 and 19 RNA expression, as measured by RT/PCR cannot be used to differentiate epithelial tumour cells from haemopoietic cells when such a sensitive method is rigorously applied. The study began with the assumption that cytokeratin 8 or 19 would not be expressed in cells of haemopoietic origin. This study and several others performed at approximately the same time suggest that there is, at least, intermittent

expression of cytokeratin RNA in non-epithelial tissue. As such, the results achieved by Datta *et al.* [1994] should be viewed with caution. If cytokeratin is intermittently expressed in non-epithelial cells, then the occasional finding of cytokeratin expression in clinical samples becomes clinically meaningless. If however, manipulation of the sensitivity of PCR results in a "window" of abnormal expression which reflects tumour cell presence, then potential for clinical use still exists.

The potential for cytokeratin 20 as a marker for gastrointestinal malignancy still holds promise. A possible RT/PCR cytokeratin 20 marker, although less generally applicable than the more widespread cytokeratins (such as 8, 18, and 19) may be used to further delineate colorectal cancer or gastric cancer patients at greatest risk of developing metastatic disease who would require adjuvant therapies.

### 4.5.6 Search for more clinically relevant markers of metastasis in solid tumours

Other potentially clinically useful tissue specific tumour markers which may be considered include theexpression of the gene coding for prostate specific antigen (PSA) in patients with prostate cancer, as has been already demonstrated by Moreno *et al.* [1992] in peripheral blood, and by Deguchi *et al.* [1993] in lymph node tissue. Such sensitive techniques of detecting PSA are tempered by the ongoing controversy of optimal management of early stage prostate cancer or patients with asymptomatic raised PSA levels, diveen the long natural history of asymptomatic prostate cancer. Carcinoembryonic antigen (CEA) expression as detected by RT/PCR has been investigated by Gerhard *et al.* [1994] with encouraging results, showing a high level of sensitivity with a low false positive rate.

Two different targets for RT/PCR have been analysed in neuroblastoma. PGP-9.5, a protein related to neurone specific enolase (NSE) has been reported as a potentially useful marker of disease activity [Mattano *et al.* 1992]. However a more recent study of the same marker by Norris *et al.* [1994] found levels of PGP-9.5 expression in normal haemopoietic tissue raising doubts as to its usefulness as a tumour marker. Three separate studies have used tyrosine hydroxylase, the first enzyme in the catecholamine synthesis pathway, to detect neuroblastoma cells [Naito *et al.* 1991, Norris *et al.* 1994 and Burchill *et al.* 1994]. The sensitivity is high at 1 cell in $10^7$ blood cells [Burchill *et al.* 1994] and as yet, no transcription of tyrosine hydroxylase has been demonstrated in normal haemopoietic tissue. There is also some correlation of detection with clinial outcome, and larger cohort studies and longitudinal studies are awaited with interest.

Other potential candidate genes include milk fat globulin for breast cancer, or albumin expression for hepatocellular carcinoma. The advantage of such markers is that they are more highly specific proteins which are less likely to be expressed by other cells, so that the problems associated with "illegitimate transcription" may be circumvented. However, such proposals may equally be criticised in that the proteins may be so specialised that a significant proportion of tumours may cease to express the RNA as they become less well differentiated. For example, it may be that only very well differentiated breast cancers may express milk fat globulin RNA, when the majority of less well differentiated tumours do no express it. Thus the utility of the test is devalued.

Such tissue specific gene expression such as tyrosinase, tyrosine hydroxylase, and cytokeratin expression simply uses a non-malignant aspect of the tissue as a marker. As has been previously documented, the detection of tumour cells in blood per se gives

no actual information about the metastatic potential of such a cell. There is good evidence that the metastatic process is relatively inefficient, and as such the majority of tumour cells that enter the general circulation never develop into secondary tumour deposits. Ideally, a marker should not only reliably detect ciculating tumour cells, but should provide information about the metastatic potential of the cell and carry significant prognostic information. The possibilities for such a marker are discussed in the next chapter.

# 5. CD 44

## 5.1 CD 44 EXPRESSION AND RELATIONSHIP TO TUMOUR METASTASIS

### 5.1.1 Introduction / Metastatic process

The clinical implications of metastasis and the cascade of events believed to involved in the metastatic process has been previously discussed in 1.3. An important component of successful metastasis is the cell surface antigen - stromal tissue interaction. It appears that within a heterogenous tumour cell population, a greater antigenic load favours survival of metastasising colonies. Part of the "survival equipment" that a successful metastasising tumour cell colony must carry is an ability to evade the host immune defence mechanisms [Fidler 1990]. One theory as to how this evasion takes place is that evolved tumour cells express certain proteins that "disguise" the tumour cells in such a way as to allow them to evade the normally efficient immune system. A number of cell adhesion molecules have been proposed as possible candidates for such a function. One of these is the*CD44* molecule.

### 5.1.2 Identification of *CD44*

The *CD44* molecule originally described by Dalchau *et al.* (1980), was shown to be present on T cells, granulocytes, and cortical thymocytes. This molecule was subsequently mapped to a gene located on the short arm of human chromosome 11 [Goodfellow *et al.* 1982]. Several investigators identified the same molecule independently. The antigen was variously named PGp-1, In(Lu) related p80, F10-44-2, ECM-III, HUTCH-1, and Hermes 1-3. [Goodfellow *et al.* 1982, Leterte *et al.* 1985, Jalkanen *et al.* 1986a, Telen *et al.* 1986, Carter and Wayner 1988, Holmes and Morse

1988, Golstein *et al.* 1989]. When it was confirmed that these antigens all reacted with the same antibody, the molecule was renamed *CD44* [Haynes *et al.* 1989]. Antibody reactivity to *CD44* has subsequently been extensively detected not only on lymphocytes, but also on fibroblasts and epithelial cells [Berg*et al.* 1989].

### 5.1.3 CD44 characterisation

The *CD44* molecule is synthesised as a 37 kDa molecule [Stamenkovic *et al.* 1989]. It is processed by glycosylation to an 80kDa form or by addition of chondroitin sulphate to a 180-200 kDa form [Jalkanen *et al.* 1988]. It is found in a wide variety of cells. The functions of CD44 are yet to be clearly defined, but it appears to be involved in the binding to high endothelium, binding to collagen, fibronectin and hyaluronate to confer cell-matrix contacts [Jalkanen *et al.* 1986b, Idzerda *et al. 1989,* Aruffo *et al.* 1990, Miyake *et al.* 1990], and signal transfer in lymphocytes and macrophages [Haynes *et al.* 1989, Denning *et al.* 1990, Harn *et al.* 1991, Conrad *et al.* 1992]. Thus *CD44* has earned the reputation for being involved in the process of lymphocyte "homing". This means that the protein aids in the trafficking of lymphocytes from tissues including blood, into lymphatic channels and into lymph node tissue.

### 5.1.4 "Splice variant" CD44

Two isoforms of the *CD44* molecule have been shown to correspond to *CD44* expressed on haemopoietic cells and epithelial cells respectively [Brown *et al.* 1991, Stamenkovic *et al.* 1991, Cooper *et al.* 1992]. The different isoforms arise by differential splicing of exons into the *CD44* molecule [Jackson *et al.* 1992]. Even larger *CD44* variants have been shown to be expressed by several metastatic tumour cell lines

[Hofmann *et al.* 1991]. The presence of certain *CD44* splice variants in certain tumour cell lines appears to confer predisposition to metastatic behaviour. Gunthert *et al.*[1991] showed that the so-called "metastatic" variant *CD44* transfected into non-metastasising rat carcinoma lines produced carcinoma cells which readily metastasise. The "metastatic" variant *CD44* has been found to be also transiently expressed by B and T lymphocytes, and macrophages after antigenic stimulation and in the post-natal period [Arch *et al.* 1992]. One theory as to why the"metastatic" variant *CD44* appears to have a normal physiologic function during lymphocyte activation is that metastasising tumour cells may be evading normal host immunological defences by mimicking normal lymphocyte "homing" behaviour.

### 5.1.5 Clinical relevance of differential CD44 expression

A report by Matsumura and Tarin [1992] suggested that the identification of abnormal splice variants of *CD44* may help differentiate between malignant and non-malignant tissue. In a small pilot clinical study, such detection permitted differentiation between metastatic tumours and non-metastatic tumours. In this study, whereas there were no non-neoplastic tissue samples of 15 examined that displayed increased spliced variant *CD44*, all 34 clinical examples of neoplastic tissue showed increased spliced variant *CD44*. There also appeared to be a distinct pattern between neoplastic tissue which was metastatic as opposed to non-metastatic samples. The possibilities of such a test are enormous. Patients whose primary tumour tissue is found to be positive for a particular alternative splice variant of *CD44* may be at significantly greater risk of metastatic relapse than patients with "negative" tumours and may therefore be eligible

for more radical adjuvant therapy. Like other tests of "over-expression", this test was reliant upon observer interpretation of "over-expression".

The detection of either "epithelial" variant *CD44* or "metastatic" variant *CD44* in peripheral blood of patients with malignancy (specifically epithelial malignancy) may have major prognostic implications, and therefore therapeutic impications. Whilst the "epithelial" variant *CD44* may be simply another example of tissue specific gene expression to screen blood for tumour cells, the detection of "metastatic" variant *CD44* may provide significantly greater information about the biology of the tumour cell identified. The strategy was dependant upon the premise that the "epithelial" and "metastatic" variant *CD44* is not expressed in normal peripheral blood.

## 5.2 METHODS / RESULTS : CD44 RT/PCR

In an attempt to ascertain whether such an approach may be used to detect such variant *CD44* expressing tumour cells in peripheral blood, RT/PCR was performed on RNA extracted from normal peripheral blood using primers spanning the variable region of the *CD44* gene and compared with the RT/PCR product obtained from RNA extracted from several tumour cell lines.

### 5.2.1 Optimising CD44 RT/PCR conditions

Oligonucleotide primers were chosen to span the variable domains of the published *CD44* gene sequence [Matsumura and Tarin 1992]. The primers selected were as follows :- CD44 sense = ATCACCGACAGCACAGACAGA. CD44 antisense = CCAAGATGATCAGCCTTTCT. A PCR product of 187 bases would be produced if the expressed CD44 amplified contained no splice variants. With the addition of one or

more of the variable domains, PCR products of increasing size would be obtained (see Figure 48).

To optimise PCR conditions for the *CD44* primers, variable magnesium concentrations in the PCR reaction were tested on A *CD44* clone cDNA sequence with an expected PCR product size of about 1220 bases. Once a threshold of magnesium concentration of 1 mM was reached, the signal of the PCR product obtained did not increase with increased magnesium concentration (see Figure 49). For the purposes of the remaining *CD44* experiments a magnesium concentration of 1.6 mM was used. Optimal primer concentrations (0.25 um) and dNTP concentrations (200 um) were also established by variation of each parameter individually. Upon completion of the RT/PCR processes, gels containing the PCR product were visualised under UV light as previously described.

The PCR products were transferred to nitrocellulose by the Southern Blot technique [Southern 1975], and the blots were then probed using digoxigenin labelled probes corresponding to different variable domains. The method for digoxigenin labelled probing is described in 2.5.3.

### 5.2.2 CD44 expression in normal peripheral blood

On RNA extracted, using the Ultraspec™ method (see 2.2), from peripheral blood of 6 normal healthy volunteers, following RT/PCR using the *CD44* primers a bright band was seen in all samples at the expected 187 base level (see Figure 50). Also seen for all samples were considerably fainter bands at the 250 (approx.) base level, the 420 (approx.) base level and the 490 (approx.) base level. The RNA was not treated with RNAse, and in the RT negative control experiments, a considerable number of

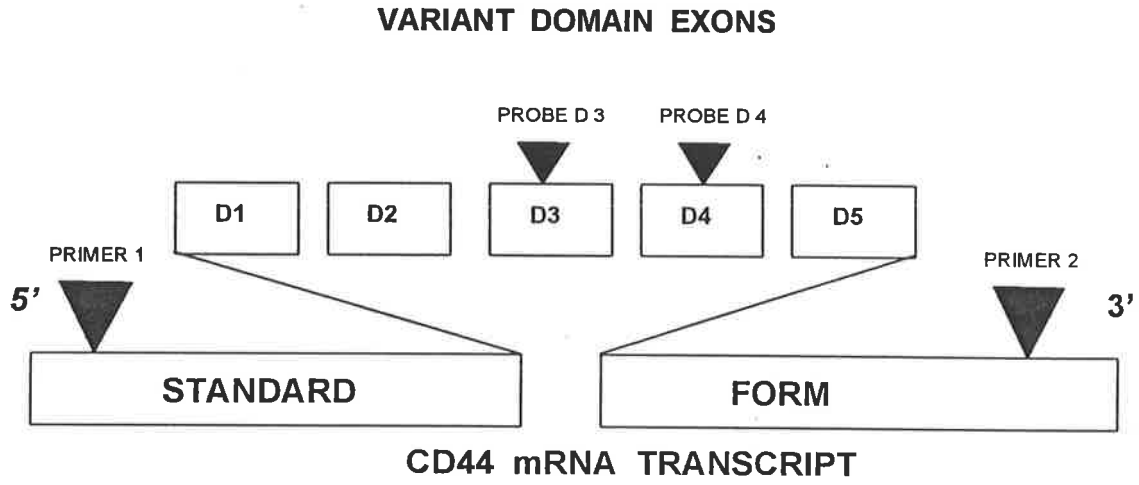



Figure 48. Illustration of the "standard form" CD44 mRNA transcript with variant exon domains, depicting CD44 PCR primers (primer 1 and primer 2) located outside the known variable region, and digoxigenin labelled oligonucleotide probes within variable domains 3 and 4 (D3 and D4).

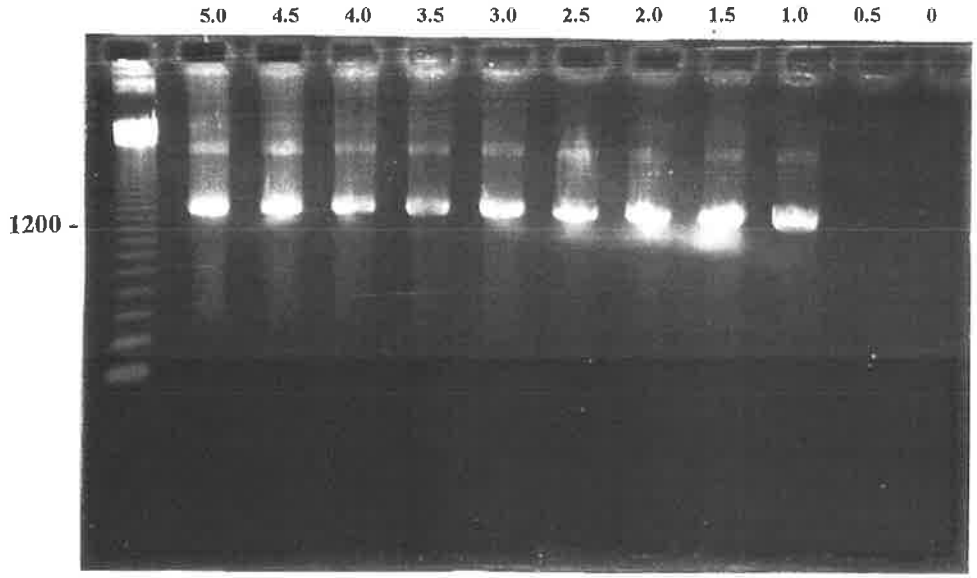



Figure 49. Optimal magnesium concentration for CD44 oligonucleotide primers spanning 5 variable domains of the CD44 cDNA was first tested by varying magnesium concentrations within the PCR buffer. 1ng of CD44R1 (clone) DNA was used as the target. A relatively uniformly bright band of 1200 bp PCR prduct was produced in all lanes when the magnesium conentration was 1.0 mM or greater. It appeared that once the threshold of 1.0 mM Mg was reached, further increases in Mg concentration made no difference to the PCR.

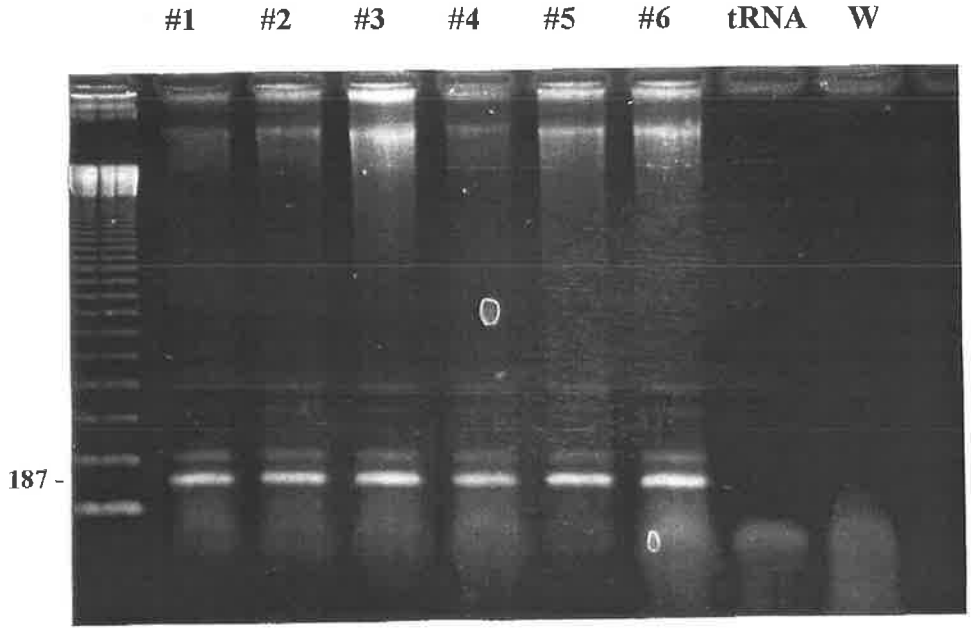



Figure 50. RNA extracted from the whole blood of 6 normal healthy volunteers underwent RT/PCR using primers from outside the the region containing 5 variable domains. tRNA and water were used as the negative controls. Uniform banding patterns were seen in all blood RNA samples with the predominant band being the expected 187 bp size. Fainter bands were seen at approximately 250 bp, 420 bp and 490 bp.

consistently spaced very faint bands were seen in each sample with a more obvious band visualised at the 1200 base (approx.) level. This would be quite consistent with expected 1200 base PCR product of the *CD44* gDNA. With the exception of the 420 base product which may correspond with the *CD44* cDNA with domain 2 inserted the band sizes did not correspond with any of the 5 known *CD44* variations.

### 5.2.3 CD44 expression in epithelial tumour cell lines

The same experiment was repeated with an EJ (bladder carcinoma) cell line RNA and HT29 (colon carcinoma) cell line RNA to determine whether tumour line RNA would preferentially express alternatively spliced *CD44* RNA. Examination of the ethidium bromide stained gel under UV light revealed that the EJ cell line RNA produced faint bands corresponding to those seen with the normal peripheral blood samples. The HT29 cell RNA however produced an additional bright band corresponding to approximately 590 bases (see Figure 51). This corresponds to the expected 586 base *CD44* PCR product which include the domain 4 and domain 5 inserts.

Both normal blood PCR product gels and tumour cell PCR product gels were blotted and probed with digoxigenin labelled probes (see 2.5.3). When the experiment was performed using a probe from within a non-variable region of the *CD44* sequence, a similar grouping of bands to those seen on the ethidium bromide stained gel were observed, with the EJ cell line RNA producing a similar banding pattern to normal peripheral blood RNA and HT29 producing a bright band corresponding to the 586 base *CD44* PCR product containing both variable domains 3 and 4 (see Figure 52). When the same gel was probed with the domain 4 (epithelial associated) probe, a similar group of

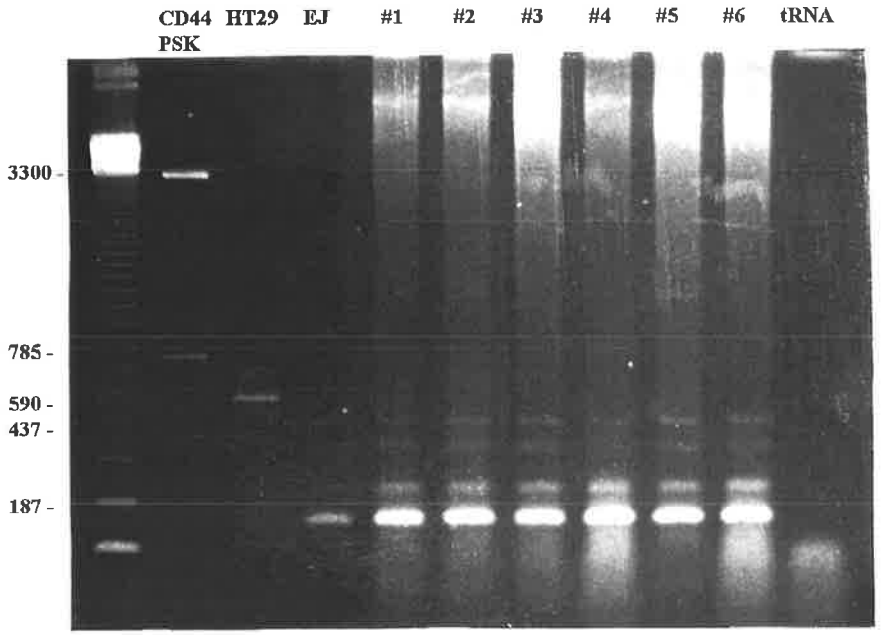



Figure 51. RTR/PCR (30 cycles using CD44 primers spanning the 5 variable domains) performed upon 1 ng samples of RNA from whole blood of 6 healthy volunteers (#1 - #6), and RNA from the human epithelial tumour cell lines HT29 (colon carcinoma) and EJ (bladder carcinoma). 30 cycles PCR was performed upon 1 ng of CD44 PST/PSK plasmid DNA digest. All 6 blood samples produced similar banding patterns with the predominant band at 187 bp (no additional domains). A similar banding pattern was seen with the EJ sample. However, the HT29 sample did not produce a 187 bp band, but produced a single band at approximately 590 bases, corresponding to a CD44 PCR product containing variable domains 4 and 5 (the epithelial variant CD44). The digested plasmid CD44 produced expected bands at 437 bases, 785 bases and 3300 bases.

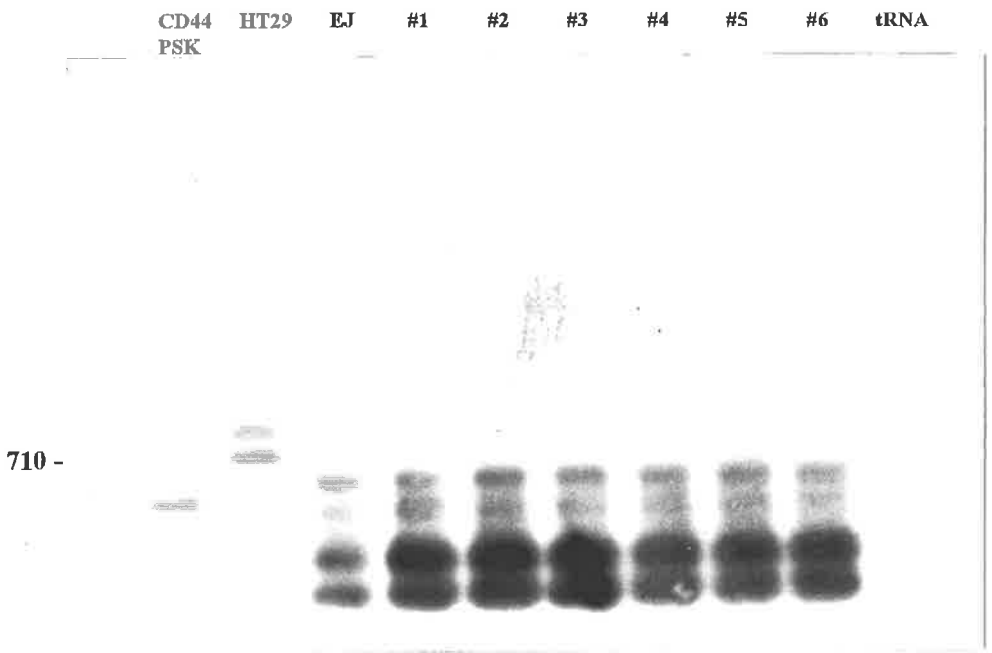



Figure 52. The PCR gel from figure 40 blotted and then hybridised with the digoxigenin labelled CD44 probe from the non-variable region produced he same main banding pattern seen on the ethidium bromide gel for all blood samples and the EJ sample. An extra band was seen in the HT29 sample at approximately 710 bases. This could corresepond with a CD44 sequence containing the variable 1, 4 and 5.

bands were visualised for both normal whole blood RNA and epithelial tumour cell line RNA (see Figure 53). When the gel was probed with the domain 3 (metastatic variant) probe the HT29 differentially expressed a band at about 550 bases. However the EJ cell line RNA did not produce any apparent alternately spiced variant bands (see Figure 54).

## 5.3 DISCUSSION

### 5.3.1 Evaluation of this technique of determining CD44 expression in blood

The unravelling of *CD44* and its relationship to metastatic disease continues at a rapid pace. *CD44* has been shown to play an important role in cell adhesion in the normal system. Naturally occurring splice variants of the *CD44* molecule appear to render different specific adhesive properties on to individual cells. It is likely that over expression of these naturally occurring splice variants confer onto tumour cells the ability to metastasise, possibly by altering cell surface receptor recognition, thus allowing cells to enter the lymphatic system without being eliminated by the normal immunological surveillance system.

From a diagnostic viewpoint, the detection of over-expression of the variant *CD44* mRNA is a significantly different situation from the detection of tyrosinase mRNA or tyrosine hydroxylase mRNA in peripheral blood in the settings of melanoma and neuroblastoma respectively. In the tyrosinase model for metastatic melanoma, the blood samples were considered to be either positive or negative, and therefore either containing melanoma cells or not. For the*CD44* model, splice variant mRNA occurs naturally given an appropriate stimulus. Hence there would be a continuum of

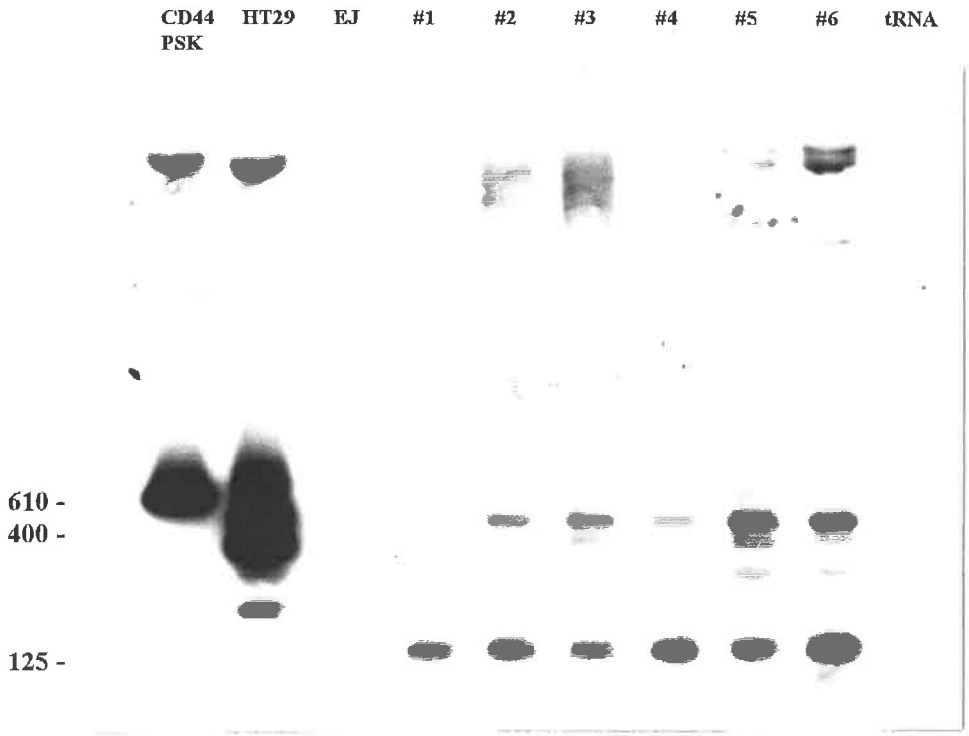



Figure 53. The PCR gel from figure 40 blotted and hybridised with the digoxigenin labelled CD44 probe from within variable domain 4. All blood samples produced similar banding patterns, with the brightest band at approximately 125 bases and a lesser band at about 530 bases (these bands may correspond to the size of domain 1 alone, and CD44 containing variable domains 1 and 4 respectively). The HT29 produced a different banding pattern with a very prominant band at about 400 bases (corresponding to the CD44 sequence containing the variable domain 4) and a second prominant band at about 530 bases. The CD44 plasmid digest produced one prominant band at about 610 bases.

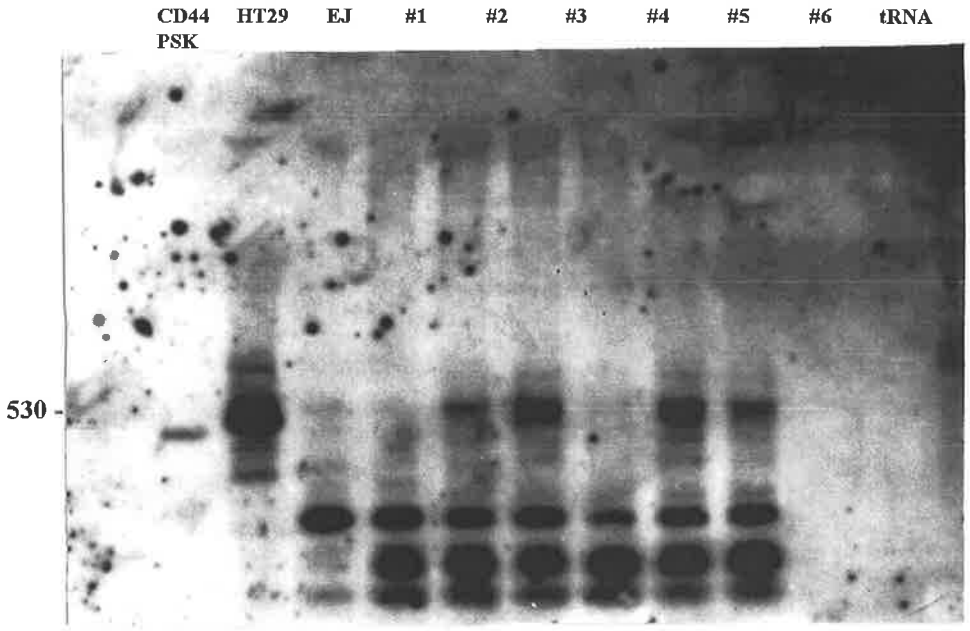



Figure 54. The PCR gel from figure 40 blotted and hybridised with the digoxigenin labelled CD44 probe from within variable domain 3. All blood samples and the EJ sample produced similar banding patterns. The HT29 sample produced a prominant band at about 530 bases.

expression from normal unstimulated, through specifically stimulated, to abnormally expressed as in the metastatic state. There is therefore the need for interpretation of the degree of expression as being either indicative of metastasis or not. Thus, the results of Matsumura and Tarin [1993] in which whole blood expression of "metastatic" splice variant *CD44* mRNA was found in a proportion of patients with malignancy and not in normal healthy volunteers are entirely dependant upon observer interpretation of the degree of expression.

In this small study, multiple variants of the *CD44* mRNA appear to be expressed in normal whole blood. At the time this pilot study was undertaken, 5 variant exons were described. The number of described CD 44 variant isoforms has now increased to at least 20 [Fox *et al.* 1994]. The implication of this is that the presence or absence of a band on a PCR gel of appropriate size is not going to predict for presence or absence of metastatic cells in peripheral blood. Quantitative estimations of intensity of specifically probed bands may be indicative of metastatic tumour cells, but even these results would need to take into account certain inflammatory states which may produce similar degrees of over-expression of "metastatic" variant mRNA.

### 5.3.2 Future implications for CD44 evaluation with regard to diagnosis, prognostication and treatment

That some primary tumours, including some non-Hodgkins lymphomas and colon carcinomas expressing variant *CD44* are associated with a higher rate of metastasis and worse prognosis [Wielenga *et al.* 1993], in conjunction with observations that metastatic deposits from the same host often express variant *CD44* to an even greater degree suggests that the acquisition of variant *CD44* expression probably occurs

early in the malignant transformation process, and greater degrees of abnormal expression may correlate with progressive malignant phenotype .

Whilst *CD44* variant expression appears to be common amongst numerous tumour types [Birch *et al.* 1994], it is not expressed by all malignant tumours and may be readily expressed by lymphoid cells in response to immunogenic stimulation [Herrlich *et al.* 1993, Koopman *et al.* 1993]. Whilst splice variant *CD44* expression is expressed on metastasising tumour cells, it is also indicative of differential stages of development of leucocytes [Dougherty *et al.* 1991] . It is hypothesised that whilst lymphocytes acquire immunogenic properties to defend the host against foreign antigen in response to that antigen, then perhaps tumour cells acquire similar immunogenic properties to facilitate the metastatic process [Kahn 1992]. The successful metastasising tumour cell colony may therefore not already be imbued with the necessary immunogenic properties, but acquire them sequentially during the metastatic process.

This relatively recent information about the metastatic process does not necessarily stray from the Paget "seed and soil" hypothesis [1889] and may be included as part of the cascade, tumour cell / matrix interaction hypothesis of Fidler and Hart [1982] where environmental influences pressure immunological changes in the tumour cell colony which aid in the physical events of metastasis (such as lymphatic trafficking) as well as the immunological events that allow evasion of the host immune system.

Despite the obvious promise of the findings of the study by Matsumura and Tarin [1993], presently it appears that there are distinct limitations for the use of*CD44* in the diagnosis of metastatic tumour cells in peripheral blood. This is largely because there is such a wide variation in the possible splice variant combinations of*CD44* in

normal tissue including lymphocytes related to specific antigenic states [Arch *et al.* 1992]. As *CD44* variants are expressed normally (at low levels) in normal haemopoietic tissue, and circulating tumour cells in peripheral blood are quite rare, it seems unlikely that a quantitative test of variant *CD44* expression could be discriminating enough for general clinical use.

It appears more likely that evaluation of specific variant *CD44* expression in primary tumour tissue will provide more information about the likelihood of metastases being already present, or the likely metastatic potential of the primary tumour. Wielenga *et al* [1993] and Mulder *et al* [1994] have demonstrated that specific variant *CD44* expression in primary colorectal cancer appears to be associated with a greater propensity to metastatic spread and worse patient survival. This prognostic variable appeared to be independent of Dukes' stage, although variant *CD44* expression may be associated with mutant *p53* expression which also carries prognostic information [Mulder et al 1995]. It remains to be seen whether the prognostic information observed gained by evaluation of variant *CD44* expression in primary tumours will be any more discriminating than other molecular prognostic markers such as *p53* under expression or variant expression, or *c-erbB-2* over-expression or *ras* over-expression. Rather than there being one molecular single marker of tumour prognosis, a panel of markers (including growth factor gene up-regulation), may be combined to more closely correlate with disease progression [Tarin and Matsumura 1993].

Monoclonal antibody therapy against variant *CD44* is a potential therapeutic manoeuvre as has been shown by Seiter *et al.* [1993]. In this study a monoclonal antibody raised against the metastasis specific domain of variant *CD44* was able to retard growth of metastatic tumour in the animal model. Potential monoclonal antibody

therapies or gene transfer therapies may one day utilise this interesting variation between malignant and non-malignant tissue.

# 6. DISCUSSION AND CONCLUSIONS

## 6.1 CRITICAL EVALUATION OF RT/PCR TUMOUR CELL DETECTION TECHNIQUES

### 6.1.1 Evaluating the efficacy of solid tumour markers

In this context, the term tumour marker refers to a blood test that measures presence of a specific solid tumour and its level of activity. The relative merit of any tumour marker is a function of : - (i) its sensitivity, (ii) specificity, (iii) reproducibility and ease of administration of the test, and (iv) to what extent its result influences clinical decisions.

### 6.1.2 Conventional solid tumour markers

RT/PCR tumour cell detection techniques need evaluation with respect to the more conventionally accepted "standard" tumour markers presently used in clinical oncological practice. These include α-feto protein (α-FP) in the setting of non-seminomatous germ cell testis tumours and hepatocellular carcinoma [Purves *et al.* 1970, Lange *et al.* 1976], β-human chorionic gonadotrophin (β-HCG) in testicular/trophoblastic tumours [Bagshawe 1965], Ca125 in ovarian cancer [Bast *et al.* 1983], prostate specific antigen (PSA) in prostate cancer [Oesterling 1991], and carcino-embryonic antigen (CEA) in colorectal, other GI cancers and breast cancer [Oh and McLean 1977, Mayer *et al.* 1978]. Serum markers are also occasionally used in the setting of pancreatic cancer, gastric cancer, breast cancer, brain tumours and small cell lung cancer, although their relative lack of both sensitivity and specificity makes their general utility questionable [Steinberg *et al.* 1986, Safi *et al.* 1991, Johnson *et al.* 1993, Pinhorn 1993].

Of all of the commonly used tumour markers, α-FP and β-HCG in the setting of testicular tumours, have the greatest utility. α-FP is an oncofoetal protein produced in foetal development by the liver, yolk sac and gastrointestinal epithelium; it is synthesised as a secretory protein by tumour cells [Gitlin 1975, Kurman*et al.* 1977]. β-HCG is a glycoprotein hormone produced by trophoblastic tissue [Braunstein *et al.* 1973]; the highest levels of secretion are seen in the setting of pregnancy, testicular tumours and trophoblastic tumours [Braunstein *et al.* 1973, Braunstein *et al.* 1976]. Their sensitivity (either one or both being elevated) is of the order of magnitude of 90 % of patients with active non-seminomatous testis tumour, and increasing tumour markers in the setting of testicular tumours is almost invariably associated with disease activity, and very high levels at the time of diagnosis has prognostic value [Moore*et al.* 1978]. α-FP elevations are not specific however, with elevation occasionally being related to liver parenchymal disease [Bloomer *et al.* 1975]. The immunoassay for the markers are reliable and reproducible. Normal levels of these markers do not necessarily indicate absence of disease activity however, as populations of malignant cells may not secrete either protein. The main reason why these markers have such great utility is that testicular germ cell tumours are particularly responsive to treatment. So that elevation of the markers following primary treatment enables early potentially curative treatment to be undertaken.

Ca125, a glycoprotein associated in the embryo with amnion and coelomic epithelium and in the adult, with epithelium of endometrium, endocervix and fallopian tubes [Bast *et al.* 1981] is elevated in about 80% of women with ovarian epithelial cancer. It is by no means specific however, with elevations associated with other epithelial malignancy including pancreatic cancer, lung cancer, and gastrointestinal

cancer [Bast *et al.* 1983]. It may also be elevated in non-malignant conditions involving the peritoneum, such as in the immediate post-operative period or inflammatory diseases such as pancreatitis. For the majority of ovarian cancer patients who present with elevations of Ca125, the marker may act as a useful measure of disease status in primarily treated, asymptomatic patients, who have no radiological evidence of disease recurrence. The assay is now well established, and in general use. At present, it may be considered a less useful test in the setting of ovarian cancer than isα-FP and β-HCG in the setting of testicular tumours, because treatment for relapsed ovarian cancer is considerably less successful than treatment of relapsed testicular tumour

Prostate specific antigen (SPA) is a sensitive but non-specific test of invasive prostate cancer. The sensitivity and specificity of the assay may be varied by altering the "normal" cut-off level for the protein. At a sensitivity of about 65 % (for all stages of disease), the specificity of the assay is about 77 %. At a specificity of greater than 95 %, the sensitivity falls to 23 % [Oesterling 1991]. Following primary therapy of prostate cancer, it may be used to monitor disease activity. The assay is widely used. However, its usefulness is tempered by the continuing controversy as to optimal management of prostate cancer. The natural history of most prostate cancers is frequently quite indolent, so that immediate treatment based upon elevation of PSA alone may not be warranted. As a screening test PSA is again limited because of the knowledge that a significant proportion of elderly men have asymptomatic indolent prostate cancer which may never become a clinical problem, hence positively diagnosing prostate cancer in asymptomatic elderly men following PSA evaluation may not lead to immediate therapy.

Carcinoembryonic antigen (CEA) is a glycoprotein synthesised by columnar epithelial cells [Go *et al.* 1975]. It is a very commonly used tumour marker which is

commonly used in the setting of colorectal cancer. Patients with liver metastases from colonic cancer frequently have very high levels of CEA, which may be in part related to the hepatic clearance of the protein [Lurie *et al.* 1975]. It is also elevated in pancreatic cancer, gastric cancer, lung cancer, and breast cancer. Moderate elevations may be seen in a variety of non-malignant conditions including hepatitis/cirrhosis, colitis, pancreatitis and gastritis/peptic ulceration [Hansen *et al.* 1974]. By the criteria used to evaluate tumour markers already discussed, the low specificity (limiting its use as a screening test) and the lack of useful treatment available in the setting of relapsed disease markedly limits the clinical utility of this marker, except for prognostication, especially in the peri-operative period [Gion *et al.* 1993].

### 6.1.3 RT/PCR "marker" tests

Approximately 8 hours or one full working day is required for RT/PCR analysis of a blood sample, from the time of the blood sample being extracted to the time in which the gel is analysed under UV light. This is calculated as: RNA extraction from whole blood, ~ 1 hour; DNAse/RT set up and reaction, ~1.5 hours; PCR set up and reaction, ~ 3 hours; gel electropheresis/ethidium bromide staining, ~ 2.5 hours. A turn-around time of approximately one working day compares favourably with similar turn-around times for many "standard" tumour marker tests which are often immuno-assay based, many of which are already semi-automated. If Southern blotting and radioactive probing with autoradiography is added, the total time is increased by 36 - 48 hours. From a clinical perspective, these are rapid turn-around times for tests of prognostic significance. Perhaps the more important issue is whether or not the RT/PCR "tests" provide worthwhile information.

Using the same evaluation criteria discussed in 6.1.1, RT/PCR sdid tumour markers published at this date are definitely in their infancy. The numbers of samples examined, both pre-clinical and clinical, provide no indication of sensitivity in the clinical setting. Although the in-vitro sensitivity of the tests (variably quoted as between 2 - 100 cells per ml of blood) [Smith*et al.* 1991, Matsumura and Tarin 1992, Datta *et al* 1994, Burchill *et al.* 1995] are encouraging, it is not yet known what proportion of patients with malignancy will produce positive results. It is also not yet known what proportion of patients with asymptomatic/micrometastatic disease will produce positive results, and what the clinical implications of such a positive result will be in this setting.

All reports up to now studies from single centres, with very small numbers evaluated, so the reproducibility is certainly not yet known. An example of the difficulties with reproducibility is the conflicting results and conclusions reached by various authors regarding the frequency and clinical value of detecting circulating malignant melanoma cells in blood by RT/PCR using tyrosinase as the mRNA target [Prossart *et al.* 1993, Vormwald-Dogan *et al.* 1994, Foss *et al.* 1995 and Pittman *et al.* submitted] These conflicting results demonstrate that the present methods are not yet sufficiently robust for meaningful results to be obtained and hence the methods are certainly not yet ready for routine use. Rather like the circulating cancer cell cytological studies of the 1950s and 1960s [Goldblatt and Nadel 1965], the initial high rates of detection have been more recently tempered by subsequent inability to reliably reproduce results at the single centres that reported the initial findings [Foss*et al.* 1994, James *et al.* 1993, Pittman *et al.* submitted]. None of these have been systematically investigated for reproducibility in multi-centre study. This has led to protocols being

frequently changed and updated in an effort to improve reliability and reproducibility of each method.

Ultimately if such "tests" are to become routinely used, then the results of these tests must eventually have a bearing on clinical management rather than simply being of academic interest. This aspect of evaluation is probably more related to adequacy of present treatments of solid tumours rather than the prognostic value of the test. Hence, at present the detection of circulating melanoma cells in peripheral blood will not result in alteration in management. But if useful treatments are developed for recurrent melanoma, or even effective adjuvant treatments are developed, then such a test may become extremely useful in clinical management. Certainly in the paediatric setting of neuroblastoma, while gross metastatic disease is usually associated with a poor prognosis, micro-metastatic disease may be amenable to chemotherapy, so that there appears to be significant clinical potential for tyrosine hydroxylase as a marker of micrometastatic disease [Naito *et al.* 1991, Burchill *et al.* 1994]. As adjuvant treatments have already proven to be effective for early stage breast cancer and locally advanced colorectal cancer [EBCTSG 1992, Moertel *et al.* 1993], the detection of circulating micrometastatic breast cancer or colorectal cancer cells may allow more expeditious use of adjuvant therapies in these settings.

Molecular biological oncological evaluation is becoming more specific, allowing greater molecular classification, as is seen in the increasingly complex classification of non-Hodgkin's lymphoma [Harris *et al.* 1994]. There is speculation that molecular classification of lymphomas may eventually lead to each individual lymphoma patient having a unique classification so that individual markers of micrometastatic disease may eventually be used for each individual lymphoma patient.

Advances in molecular biological techniques are providing increasing sensitivity and specificity of such tests [Chou *et al.* 1992]. Certainly, direct amplification of common genetic alterations in both blood and bone marrow samples is becoming increasingly frequent in the diagnosis and management of lymphomas and leukaemias [Chang*et al.* 1993]. The cautionary side to this is that in many instances, previously generally accepted notions of genetic control are brought into question. In essence, this means that if one looks hard enough for a genetic characteristic, it is likely to turn up in unexpected places because of extraordinary genetic capacity of each cell.

RT/PCR techniques may be considered only one step "deeper" than immunohistochemical techniques in that they are identifying the message that controls protein production. The cascade of events that lead to a particular protein being produced means that each step back to identifying genetic controlling mechanisms requires molecular techniques of greater sensitivity. Such exquisitely sensitive techniques run a greater risk of detecting "background' effects, and as such results need to be viewed with considerable caution.

## 6.2 PRESENT AND FUTURE EVALUATION OF TUMOUR CELLS IN PERIPHERAL BLOOD

### 6.2.1 Cytological method

The field of evaluating circulating solid tumour cells in blood has been revisited after a significant break in time. The re-emerging interest is not associated with a greater interest in the significance of the circulating tumour cell so much as the improvement in technology that may enable such evaluation.

In the cytospin/immunofluorescence method of blood analysis (see 4.3), more traditional cytological techniques were used, but with an updated adaptation of the immunofluorescence techniques originally described by Herbeuval *et al.* [1965], which allows greater differentiation between fluorescing and non-fluorescing cells. With the development of flow cytometric technology, larger volumes of blood may be able to be analysed more quickly, and possibly with greater sensitivity [Beck and Raamaekers 1990]. One problem with this concept is that the qualitative component of direct visualisation (to discriminate between definite tumour cells and artefact) may be lost.

A combination of both techniques may be possible with initial flow cytometric analysis allowing great sensitivity in the detection of a particular labelled antibody and only those samples positively identified would be subject to discriminating "qualitative" fluorescent microscopy. In this format, such a method appears time consuming and clumsy. But even with present technology, the initial screening process of flow cytometry would add relatively little time to the process, and would be likely to reduce overall time by screening out those samples which do not require cytospin microscopy analysis. Although the phenotypic differences between malignant solid tumour cells and normal blood cells are reasonably discernible, such methods relying upon final differences in protein production are inherently qualitative. However at present, they probably provide the greatest degree of discrimination.

### 6.2.2 RT/PCR methods

There are inherent problems with the use of molecular genetic techniques that attempt to identify small numbers of tumour cells in peripheral blood. These relate to the great heterogeneity of solid tumour biology [Dear and Kefford 1990, Bishop 1991], so

that relatively few unique and consistent tumour biological characteristics exist compared with the biological similarities that exist between normal cells and malignant cells. Even using strict controls, false positive results due to external contamination is a genuine problem and requires consideration in any study using PCR techniques [Karovsky 1990, Shuldiner *et al.* 1991]

Each characteristic which is found to be a feature of tumour cells may be found to be a normal characteristic of some non-malignant cells if given appropriate stimulus (eg: proliferation, migration). The difference being that in the non-malignant setting, the process is under fine homeostatic genetic control, so that when the stimulus ceases, the cascade of events that produce the final effect ceases and the stimulated behaviour ceases. In the malignant setting, proper control of these processes is lost, so that gene product is continuously up-regulated and over-amplified and the abnormal activity of the cell continues.

This means that whilst phenotypic differences between cells are relatively easy to distinguish, the genetic alterations that produce phenotypic changes are less definitely distinguishable, and are more related to differences in order of magnitude.

Presently, RT/PCR technology means that detection of target RNA (cDNA) is a qualitative "all or nothing" measurement rather than being a quantitative measurement. This poses significant problems. RNA expression is not definitely switched on and off, but is quantitatively regulated; hence it is likely that if the sensitivity of the test is great, then there is increased likelihood that RNA transcripts will be detected, even though the protein product of that transcript is not expressed by the cell/tissue examined. This may be overcome if quantitative RT/PCR techniques become better developed. Competitive RT/PCR techniques have been developed which may provide some level of quantitation

[Dallman and Porter 1991, Siebert and Larrick 1992]. Such techniques rely upon comparison of target PCR product formation with control PCR product formation. However, the robustness and reliability of such techniques yet to be proven.

## 6.3 THERAPEUTIC IMPLICATIONS OF CIRCULATING SOLID TUMOUR CELLS

At the height of the interest in cytological examination of blood to detect circulating tumour cells in the early 1960s, although there were numerous reports relating to the frequency of detection of tumour cells and methods to improve detection rates, relatively few reports discussed the long term prognosis of patients discovered to have circulating tumour cells at the time of primary treatment of cancer. Of those that did follow-up positively identified patients, relatively little prognostic information was discovered [Engell 1959, Griffiths *et al.* 1973]. In Engell's study [1959], over half of the patients found to have circulating tumour cells at the time of surgery for colorectal cancer were alive and apparently disease free 5 - 9 years later. In the Griffiths study [1973], patients who had circulating tumour cells found at operation had a non-significant trend towards a better 5 years survival compare with patients in whom no circulating tumour cells were found.

Even taking into account the positive identification problems present at the time, it is clear that the presence of tumour cells in blood per se does not always equate with poor prognosis and short survival. This is in stark contrast to the follow-up studies of patients in whom tumour cells have been identified in bone marrow [Schlimok *et al.* 1990, Dearneley *et al.* 1991, Mansi *et al.* 1991]. The straight forward interpretation of this clinical data is that the cells discovered in blood at the time of treatment of the

primary tumour are essentially in transit, and may or may not have the capacity to form secondary colonies, whereas the cells discovered in bone marrow at that time are likely to have already begun to form secondary colonies. Alternatively, interactions between endothelial surface antigens of larger blood vessels and those of tumour cells may be qualitatively less important in the metastatic process than the interactions between the surface antigens of bone marrow stroma and those of tumour cells.

It is known from animal experiments that the rate of successful metastasis from intravenously or subcutaneously implanted tumour cells is very low [Fidler 1970, Liotta *et al.* 1974, Weiss 1986]. Using radiolabelled B16 melanoma cells, Fidler [1970] calculated that by 24 hours following entry into the circulation, < 1 % of cells were viable. Further analysis demonstrated that < 0.1 % of intravenously implanted cells survived to produce metastases. Rather than successful secondary colony producing tumour cells acquiring the genetic changes enabling them to successfully colonise, many commentators now believe that primary tumours contain a heterogeneous population of tumour cells with regard to metastasising ability, and that only the very small proportion of metastasis capable cells eventually form secondary colonies [Poste and Fidler 1979, Nicolson 1988, Fidler and Hart 1982]. Once secondary colonies have established, characteristics that enable the primary tumour to metastasise may eventually be lost by some "stable" metastatic colonies. Hence there may be great heterogeneity of metastasis capability of tumour cells isolated from a secondary site. Other biological factors are also likely to be variable in metastatic colonies explaining the variable growth rates of different metastatic colonies in the clinical setting as well as the variable response of various metastatic colonies to systemic therapy.

What can be inferred by these data with reference to treatment implications is that significant therapeutic gains may be made if treatment is targeted at that particular population of cells which has the greatest metastatic potential. Present cytotoxic treatment strategies for malignancy utilise the differences in growth rate to achieve responses [De Vita 1993]. The majority of adult solid tumour cells are not in cell cycle, so that this strategy may only affect a small proportion of the tumour cells. One of the main effects of presently commonly used biological cancer treatments is to attempt to enhance host immunity mainly through up-regulation of cytokines to stimulate cytotoxic T-cell activity. This process is likely to be dependent upon tumour cells bearing MHC class I antigens being presented to cytotoxic T-cells. As most tumour cells lack MHC class I antigens [Eisenbach *et al.* 1986], again only a small proportion of tumour cells are likely to be affected.

By targeting only that proportion of the tumour cells that produces most of the deleterious effects of malignancy greater gains may be achieved. Target phenotypes for solid tumours may include aberrant *CD44* expression, down regulation of *nm23*, and down regulation or abnormal expression of *p53*. Potential mechanisms of therapy include monoclonal antibody therapy directed against target protein, "gene therapy" whereby a specific DNA sequence is transfected into target tumour cells, or with the use of drugs that mimic the effect of the normal cellular controlling mechanisms.

With regard to tumour surveillance, an alternative strategy may be based upon the individual patient's tumour biology. As tumours are phenotypically heterogenous, analysis of each tumour may enable the individual genetic differences between the tumour and host to be identified, and these differences more accurately exploited using RT/PCR technology

## 6.4 CONCLUSIONS AND FUTURE PERSPECTIVES

The techniques developed in this study apply a combination of known molecular technologies to the diagnostic problem of detection of micrometastatic disease. The notion of detection of tumour cells in blood is not a new concept. The ability to accurately deliver on the promise, like previous studies is not yet known. Whilst exciting progress is made in molecular biological techniques such as the development of PCR technology, new problems arise, which are mainly related to the exquisite sensitivity of the techniques.

The techniques applied in this study show that it is possible to accurately discriminate very small numbers of tyrosinase expressing melanoma cells from normal blood cells using RT/PCR technology. The finding that cytokeratins 8 and 19 are normally expressed in blood cells prevent their use as markers, and call into question recent papers describing their use in this setting. The non-detection of cytokeratin 20 in blood holds considerable promise with regard to monitoring patients with colorectal cancer. The detection of tyrosine hydroxylase expressing neuroblastoma cells in peripheral blood by RT/PCR may yet prove to be a useful diagnostic and prognostic tool in paediatric oncology.

The methodology followed in this study provides a method for subsequent RT/PCR diagnostic techniques. Critical analysis of subsequent publications in which RT/PCR techniques may be based upon the principles followed in this study. For RT/PCR "assays" to be truly valid, certain criteria must be met with regard to methodology. These criteria include the inclusion of adequate concurrent RNA viability controls, concurrent positive sensitivity controls, DNAse positive and negative controls,

and concurrent RT negative controls to ensure that positive results are truly positive and negative results are truly negative.

Positive identification of tumour cells in peripheral blood per se may not provide any prognostic information when used in the setting of known primary disease, or soon after it's primary treatment (rather like the lack of useful information gained by checking serum tumour markers in the immediate post-operative period). The detection of circulating tumour cells during follow up however, may be of great clinical importance. The application of RT/PCR technology to this problem may significantly advance diagnostic capacity. Before these questions can be asked in the form of large well controlled prospective clinical trials, the techniques of RT/PCR (when applied to tests for malignant contamination of tissues such as blood, bone marrow and lymph node tissue) need to be carefully refined to ensure that they stand up to rigorous critical analysis.

Presently, the ability to detect smaller and smaller numbers of malignant cells may not necessarily translate into improved outcome for those patients in whom positive identification of micrometastatic disease is made. For example, whilst the detection of micrometastatic disease in germ cell tumours as demonstrated by positive tumour markers has resulted in more successful treatment with chemotherapy and radiotherapy, the same cannot be said for lung cancer despite the availability and use of tumour markers in this setting. Unfortunately the majority of solid tumours fall into the second category, in which chemotherapy treatments have little impact on survival. However, as new therapies develop, such molecular information may become more useful.

Such identification of micrometastatic disease may allow more accurate identification of patients at risk of relapse in the setting of those tumours for which

adjuvant chemotherapies have already shown survival benefit, so that those patients at lowest risk of recurrence may be spared the potential toxicities of adjuvant treatments, whilst those patients at especially high risk may be offered more intensive therapy or novel therapies thereby allowing a more rational and cost effective allocation of resources.

The molecular identification of micrometastatic disease may also prove useful as a surrogate endpoint in data collection about response to treatment and relapse risk. Many tumours have a long natural history, meaning that the determination of the impact of new therapies on both disease free survival and overall survival is considerably delayed while awaiting maturation of survival data. A molecular marker that can reliably predict relapse could significantly hasten the evaluation of new therapies. This may be especially applicable to the rapidly evolving field of high dose chemotherapy (with bone marrow support). Whilst clinical complete responses have readily identified in this setting, the clinical value in terms of improving survival and possibly increasing cure rates is certainly not yet proven. The use of exquisitely sensitive methods to detect viable disease after high dose chemotherapy, and also to detect viable tumour cells in haematopoietic progenitor cells used for bone marrow rescue may allow more rapid evaluation of high dose chemotherapy approaches so that inappropriate applications of this technique are quickly halted.

In conclusion, techniques of molecular identification of very small numbers of tumour cells are evolving at a rapid rate, possibly redefining what constitutes a complete remission. The techniques of detecting small numbers of tumour cells are evolving at a faster rate than the evolution of more effective treatments for the majority of cancers. The next steps required in the field of molecular detection of tumour cells in peripheral

blood, is a critical evaluation of the robustness of each new technique developed, as well as an in depth appraisal of the predictive power of the techniques to allow them to be applied to the emergent therapies.

## REFERENCES


Albino, A.P. 1988. The status of oncogenes in malignant melanoma. In: Bagnara, J.T.(ed.) *Advances in pigment cell research.* New York. Alan R. Liss, Inc. 361-390.

Albino, A.P., Le Strange, R., Oliff, A.I., Furth, M.E. and Old, L.J. 1984. Transforming ras genes from human melanoma. A manifestation of tumour heterogenicity. *Nature* 308: 69 - 72.

Albino, A.P., Nanus, D.M., Mantle, I.R., Cordon-Cardo, C., McNutt, N.S., Bressler, J. and Andreff, M. 1989. Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with different phenotype. *Oncogene 4*: 1363 - 1374.

Amer, M.N., Al-Sarraf, M. and Vaitkevicius, V.K. 1979. Clinical presentation, natural history and prognostic factors in advanced melanoma. *Surg. Gynaecol. Obstet.* 149: 168 - 192.

Ando, S., Ando, O., Suemoto, Y. and Mishima, Y. 1993. Tyrosinase gene transcription and it's control by melanogenic inhibitors. *J. Invest. Dermatol.* 100 (suppl):150 - 155.

Angerer, L.M. and Angerer, R.C. 1991. Localisation of mRNA by in situ hybridisation. *Methods Cell. Biol.* 35: 37 - 71.

Annotation 1964. *Lancet* ii: 630.

Antman, K.H. 1994. Dose intensive adjuvant therapy in breast cancer. *A.S.C.O. Educational Booklet* 74 - 79.

Arch, R., Wirth, K., Hofmann, M., Ponta, H., Matzku, S., Herrlich, P. and Zoller, M. 1992. Participation in normal immune responses of a metastasis inducing splice variant of CD44. *Science* 257: 682 - 685.

Arnold, A., Cossman, J., Bakhashi, A. Jaffe, E.S., Walddmann, T.A. and Korsmeyer, S.J. 1983. Immunoglobulin gene rearrangements as unique clonal markers in human lymphoid neoplasms. *N.Engl. J. Med.* 309: 1593 - 1598.

Arruffo, A., Stamenkovic, I., Melnick, M., Underhill, C.B. and Seed, B. 1990. CD44 is the principle cell surface receptor for hydronate. *Cell* 61: 1303 - 1313.

Azumi, N. and Battifora, H. 1987. The distribution of vimentin and keratin in epithelial and nonepithelial neoplasms. *Am. J. Clin. Pathol.* 88: 286 - 296.

Bader, B.I., Magin, T.M., Hatzfeld, M. and Franke, W.W. 1986. Amino acid expression and gene organisation of cytokeratin no. 19, an exceptional tail-less intermediate filament protein. *EMBO J.* 5:1865 - 1875.

Bagshawe, K.D. 1965. Clinical applications of hCG. *Adv. Exp. Med. Biol.* 76: 313 - 324.

Bakker, A.B.H., Schreurs, M.W.J., de Boer, A.J., Kawakami, S.A., Rosenberg, S.A., Adema, G.J. and Figdor, C.G. 1994. Melanocyte lineage-specific antigen gp100 is recognised by melanoma derived tumour infiltrating lymphocytes. *J. Exp. Med.* 179: 1005 - 1009.

Balch, C.M., Murad, T.M., Soong, S.J., Ingalls, A.l., Richards, P.C. and Maddox, W.A. 1979. Tumour thickness as a guide to surgical management of clinical stage 1 melanoma patients. *Cancer* 43: 883 - 888.

Bale, S.J., Chakravarty, A. and Green, M.H. 1986. Cutaneous malignant melanoma and familial dysplastic nevi: evidence for autosomal dominance and pleiotropy. *Amer. J. Hum. Genet.* 38: 188 - 196.

Barsky, S.H., Rao, C.N., Williams, J.E. and Liotta, L.A. 1984. Laminin molecular domains which alter metastasis in a murine model.*J. Clin. Invest.* 74: 843 - 848.

Barton, D.E., Kwon, B.S. and Franke, U. 1988. Human tyrosinase gene mapped to chromosome 11 (q14-q21), defines second region of homology with mouse chromosome 7. *Genomics* 3: 17 - 24.

Bast, R.C., Feney, M., Lazarus, H., Nadler, L.M., Colvin, R.B. and Knapp, R.C. 1981. Reactivity of a monoclonal antibody with human ovarian carcinoma.*J. Clin. Invest.* 68: 1331 - 1337.

Bast, R.C. Jr., Klug, T.L. St. John, E., Jenson, E., Nilhoff, J.M., Lazarus, H., Berkowitz, R.S., Leavitt, T., Griffiths, C.T., Parker, L., Zurawski, V.R. and Knapp, R.C. 1983. A radioimmunoassay using an antibody to monitor the course of epithelioid ovarian cancer. *N. Engl. J. Med.*309: 883 - 887.

Battifora, H. 1988. Clinical applications of the filamentous proteins. *Amer. J. Surg. Pathol.* 12 (suppl. 1): 4 - 42.

Beardmore, G.L. 1972. The epidemiology of malignant melanoma in Australia. In: McCarthy, W.H. (Ed.)*Melanoma and skin cancer.* Sydney. V.C.N. Blight. 40 - 64.

Beck, D., Maritoz, O., Philip, I., Fontaniure, B., Gentilhomme, D., Bailly, C., Zucker, J.M., Gentet, J.C., Kemshead, J. and Philip, T. 1986. Histological and immunological analyses are complementary for the detection of neuroblastoma cells in bone marrow. *Br. J. Cancer* 54: 637 - 642.

Beck, S., O'Keefe, T.O., Coull, J.M. and Koster, H. 1989. Chemiluminescent detection of DNA. Application for DNA sequencing and hybridisation:*Nucl. Acids Res.* 17: 5115 - 5123.

Beck, J.L.M. and Ramaekers, F.C.S. 1990. Application of antibodies in the flow cytometric analysis of benign and malignant tumours.*Semin. Cancer Biol.* 1:181-188.

Bernards, R., Schrier, P.I. Houweling, A., Bos, J.L. and van der Eb, A.J. 1983. Tumourigenicity of cells transformed by adenovirus type 12 by evasion of T-cell immunity. *Nature* 305: 776 - 779.

Berensden, H.H., De Leij, L., Postmus, P.E., Ter Haar, J.G., Poppoma, S. and The, T. 1988. Detection of small cell lung cancer metastases in bone marrow aspirates using monoclonal antibody directed against neuroendocrine differentiation antigen. *J. Clin. Pathol.* 41: 273 - 276.

Berg, E.L., Goldstein, L.A., Jutila, M.A., Nakache, M., Picker, P.R., Wu, N.W., Zhou, D. and Butcher, E.C. 1989. Homing receptors and vascular adhesion: cell adhesion molecules that direct lymphocyte traffic. *Immunol. Rev.* 108: 5 - 18.

Bicknell, R. and Harris, A.L. 1991. Novel growth regulatory factors and tumour angiogenesis. *Eur. J. Cancer* 27: 785 - 789.

Birch, M., Mitchell, S. and Hart, I.J. 1991. Isolation and characterisation of human melanoma cell variants expressing high and low levels of CD44. *Cancer Res.* 51: 6660 - 6667.

Bishop, J.M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* 52: 301 - 354.

Bishop, J.M. 1991. Molecular themes in oncogenesis. *Cell* 64: 235 - 248.

Bloomer, J.R., Waldmann, T.A., McIntire, K.R. and Klatskin, G. 1975. Alpha-fetoprotein in non-neoplastic hepatitic disorders. *J. Amer. Med. Assoc.* 233: 38 - 41.

Boehringer Mannheim Biochemica 1992. *DIG luminescent detection kit manual.*

Braunstein, G.D., Vaitukaitis, J.L. Carbone, P.P. and Ross, G.T. 1973. Ectopic production of human chorionic gonadotropin by neoplasms. *Ann. Int. Med.* 78: 39 - 45.

Braunstein, G.D., Rasor, J., Adler, D., Danzer, H. and Wade, M.E. 1976. Serum human chorionic gonadotropin levels throughout normal pregnancy. *Amer. J. Obstet. Gynoecol.* 126: 678 - 681.

Brodeur, G.M. 1990. Neuroblastoma - clinical applications of molecular parameters. *Brain Pathol.* 1: 47 - 54.

Brodeur, G.M., Seager, R.C., Schwab M. et al 1984. Amplification of N-MYC in untreated human neuroblastoma correlates with advanced disease stage. *Science* 224: 1121 - 1124.

Brown, C.E. and Warren, S. 1938. Visceral metastasis from rectal carcinoma. *Surg. Gynaecol. Obstet.* 66: 611 - 621.

Brown, T.A., Bouchard, T., St.John, T., Wayner, E. and Carter, W.G. 1991. Human keratinocytes express a new CD44 core protein (CD44E) as a heparan-sulfate intrinsic membrane proteoglycan with additional exons. *J. Cell. Biol.* 113: 207 - 221.

Burchill, S.A., Bradbury, M.F., Smith, B., Lewis, I.J. and Selby, P. 1994. Neuroblastoma cell detection by reverse-transcriptase polymerase chain-reaction (RT-PCR) for tyrosine hydroxylase messenger-RNA. *Int. J. Cancer.* 57: 671 - 675.

Burchill, S.A., Bradbury, M.F., Pittman, K., Southgate, J., Smith, B. and Selby, P. 1995. Detection of epithelial cancer cells in peripheral blood by reverse-transcriptase-polymerase chain reaction. *Br. J. Cancer* 71: 287 - 291.

Cairns, J. 1981. The origin of human cancers. *Nature* 289: 353 - 357.

Candar, Z., Ritchie, A.C., Hopkirk, J.F. and Long, R.C. 1962. The prognostic value of circulating tumour cells in patients with breast cancer. *Surg. Gynaecol. Obstet.* 115: 291 - 294.

Cannon Albright, L., Goldgar, D., Meyer, L., Lewis, C., Anderson, D., Fountain, J., Hegi, M., Wiseman, R., Petty, E. and Bale, A. 1992. Assignment of a locus for familial melanoma MLM to chromosome 9p13 - p32. *Science* 258: 1148 - 1152.

Carter, W.G. and Wayner, E.A. 1988. Characterisation of the class II collagen receptor, a phosphorylated transmembrane glycoprotein expressed in nucleated human cells. *J. Biol. Chem.* 263: 4193 - 4201.

Chan, R., Rossito, P.V., Edwards, B.F. and Cardiff, R.D. 1986. Presence of proteolytically processed keratins in the culture medium of MCF-7 cells. *Cancer Res.* 46: 6353 - 6359.

Chang, K.-S., Fan, Y.-H., Stass, S.A., Estey, E.H., Wang, G., Trujillo, J.M., Erickson, P. and Drabkin, H. 1993. Expression of *AML1-ETO* fusion transcripts and detection of minimal residual disease in t(8;21)-positive acute myeloid leukemia. *Oncogene* 8: 983 - 988.

Chelly, J., Concordet, J-P., Kaplan, J-C. and Kahn, A. 1989. Illegitimate transcription: transcription of any gene in any cell type. *Proc. Natl. Acad. Sci. USA* 86: 2617 - 2621.

Cherry, J.M. 1991. DNA sequencing. In: Asubal, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K.(eds.) *Current protocols in molecular biology.* Vol.1. New York. John Wiley and Sons Inc. 7.0.1- 7.7.31.

Chou, Q., Russell, M., Birch, D.E., Raymond, J. and Bloch, W. 1992. Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications. *Nucl. Acids Res.* 20:1717 - 1723.

Christopherson, W.M. 1965. Cancer cells in the peripheral blood; a second look. *Acta Cytol.* 9:169 - 174.

Chomczynski, P. 1989. The RNAzol B method. *Cinna/Biotecz Bulletin* 3.

Chomczynski, P. and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156 - 159.

Clark Jr., W.H. 1991. Tumour progression and the nature of cancer. *Br. J. Cancer* 54: 631 - 644.

Clark Jr., W.H., Elder, D.E., Guerry, D., Epstein, M.N., Greene, M.H. and Van Horn, M. 1984. A study of tumour progression: the precursor lesion of superficial spreading and nodular melanoma. *Hum. Pathol.* 15: 1147 - 1165.

Cleary, M.L. and Sklar, J. 1985. Nucleotide sequence of a t(14:18) chromosomal bp in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci. USA* 82: 7439 - 7443.

Clifton, E.E. and Agostino, D. 1962. Factors affecting the development of metastatic cancer. Effect of alterations in clotting mechanism. *Cancer* 15: 276 - 283.

Coen, D.M. 1991. The polymerase chain reaction: introduction. In: Asubal, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) *Current protocols in molecular biology.* Vol. 2. New York. Cold Spring Harbor. 15.0.3 - 15.1.7.

Cohen, S.M., Onhuma, T., Ambinder, E.P. and Hollander, J.F. 1986. Lomustine, bleomycin and cisplatinum in patients with metastatic malignant melanoma. *Cancer Treat. Rep.* 70: 688 - 692.

Commis, R.L. 1976. DTIC in the management of melanoma; a perspective. *Cancer Treat. Rep.* 60: 165 - 176.

Conrad, P., Rothman, B.L., Kelley, K.A. and Blue, ML. 1992. Mechanism of peripheral T cell activation by coengagement of CD44 and CD2. *J. Immunol.* 149:1833 - 1839.

Cooper, D.L., Dougherty,G., Harn, HJ., Jackson, S., Baptist, E.W., Byers, J., Datta, A., Phillips, G. and Isola, N.R. 1992. The complex CD44 transcriptional unit: alternative splicing of three internal exons generates the epithelial form of CD44. *Biochem. Biophys. Res. Commun* 182: 569 - 578.

Cooper, G.M. 1984. Activation of cellular transforming genes in neoplasms. *Prog. Cancer Res. Ther.* 31: 1 - 6.

Copeland, E.M. 1968. Prognostic factors in carcinoma of the colon and rectum. *Amer. J. Surg.* 116: 875 - 880.

Cordon-Cardo, C., Fuks, Z., Drobnjak, M., Moreno, C., Eisenbach, L. and Feldman, M. 1991. Expression of HLA-A,B,C antigens on primary and metastatic tumour cell populations of human carcinomas. *Cancer Res.* 51: 6372 - 6380.

Cote, R.J., Rosen, P.P., Hakes, T.B., Sedira, M., Bazinet, M., Kinne, D.W., Old, L.J. and Osborne, M.P. 1988. Monoclonal antibodies detect occult breast carcinoma metastases in the bone marrow of patients with early stage disease. *Amer. J. Surg. Pathol.* 12: 333 - 340.

Coulie, P.G., Brichard, V., Van Pel, A., Wolfel, T., Schneider, J., Traversari, C., Mattei, S., De Plaen, E., Lurquin, J.P., Szikora, J.P., Renauld, J.C. and Boon, T. 1994. A new gene coding for a differentiation antigen recognised by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180: 35 - 42.

Cross, N.C.P., Feng, L., Chase, A., Bungey, J., Hughes, T.P. and Goldman, J.M. 1993. Competitive polymerase chain-reaction to estimate the number of bcr-abl transcripts in chronic myeloid-leukemia patients after bone marrow transplantation. *Blood* 82: 1929 - 1936.

Crewther, W.G., Inglis, A.S. and McKern, N.M. 1978. Amino acid sequence of α-helical segments from s-carboxymethylkerateine A. *Biochem. J.* 173: 365 - 171.

Dalchau, R., Kirkley, J. and Fabre, J.W. 1980. Monoclonal antibody to a human brain-granulocyte-T lymphocyte antigen probably homologous to W3/13 antigen of the rat. *Eur. J. Immunol.* 10: 745 - 749.

Dalla-Favera, R. Franschini, G. Martinotti, S. Wong-Stahl, F., Gallo, R. and Croce, C.M. 1982. Chromosomal assignment of the human homologues of feline sarcoma virus and avian myeloblastosis virus onc genes. *Proc. Natl. Acad. Sci. USA* 79: 4714 - 4717.

Dallman, M.J. and Porter, A.C.G. 1991. Semiquatitative PCR for the analysis of gene expression. In; McPherson, M.J., Quirk, P. and Taylor G.R. (Eds.)*PCR: a practical approach.* Oxford. Oxford University Press. 215 - 224.

Danforth, D.N. 1992. The role of axillary lymph node dissection in the management of breast cancer. *Principles and Practice of Oncology; PPO Updates* 6 (2): 1 - 16.

D'Arcy, C., Holman, J., Armstrong, B.K. and Heenan, P.J. 1983. A theory of the etiology and pathogenesis of human cutaneous malignant melanoma. *J. Natl. Cancer Inst.* 71: 651 - 656.

Datta, Y.H. Adams, P.T., Drobyski, W.R., Ethier, S.P., Ferry, V.H. and Roth, M.S. 1994. Sensitive detection of occult breast cancer by the reverse transcriptase polymerase chain reaction. *J. Clin. Oncol.* 12: 475 - 482.

Davies, B., Brown, P.D., Crimmin, M., East, N. and Balkwill, F.R. 1993. A synthetic metalloprotease inhibitor decreases tumour burden and prolongs survival of mice with ovarian cancer xenografts. *Cancer Res.* 53: 2087 - 2091.

Day, C.L., Mihm, M.C., Lew, R.A. Kopf, A.W., Sober, A.J. and Fitzpatrick, T.B. 1982. Cutaneous melanoma: prognostic guidelines for physicians and patients. *CA* 32: 113 - 122.

De Franchis, R., Cross, N.C.P., Foulkes, N.S. and Cox, T.M. 1988. A potent inhibitor of Taq polymerase copurifies with human genomic DNA. *Nucl. Acids Res.* 16:10355.

Deguchi, T., Doi, T., Ehara, H., Ito, S., Takahashi, Y., Nishino, Y., Fugihiro, S., Kawamura, T., Komeda, H. and Horie, M. 1993. Detection of micrometastatic prostate-cancer cells in lymph nodes by reverse-transcription-polymerase-chain-reaction. *Cancer Res.* 53: 5350 - 5354.

Delage, R., Soiffier, D.J., Dera, K., and Ritz. J. 1991. Clinical significance of bcr-abl gene rearrangement detected by polymerase chain reaction after allogeneic bone marrow transplantation in chronic myelogenous leukaemia. *Blood* 78: 2759 - 2767.

De Vita, V.T. Jr. 1993. Principles of Chemotherapy. In: De Vita, V.T., Hellman, S. and Rosenberg, S.A. (eds.) *Cancer: Principles and Practice of Oncology.* (4th edition) Philadelphia. J.B. Lippencott 276 - 293.

Dear, T.N. and Kefford, R.F. 1990. Molecular oncogenetics of metastasis *Mol. Aspects Med.* 11: 243 - 24.

Dearneley, D.P., Ormerod, M.G. and Sloane, J.P. 1991. Micrometastases in breast cancer: long term follow-up of the first patient cohort. *Eur. J. Cancer* 27: 236 - 239.

Denning, S.M., Le, P.T., Singer, K.H. and Haynes, B.F. 1990. Antibodies against the CD44 p80 lymphocyte homing receptor molecule augment human peripheral blood T cell activation. *J. Immunol.* 144: 7 - 15.

Dicker, A.P., Volkenandt, M. and Albino, A.P. 1990. Mutational analysis of human NRAS genes in malignant melanoma: rapid methods for oligonucleotide hybridisation and manual and automated direct sequencing of products generated by the polymerase chain reaction. *Genes Chromosomes Cancer* 1:257 - 269.

Dilworth, D.D. and McCarrey, J.R. 1992. Singlestep elimination of contaminating DNA prior to reverse transcriptase PCR. *PCR Methods Applic.* 1:279 - 282.

Dionne, L. 1965. The pattern of blood-borne metastasis for carcinoma of rectum. *Cancer* 18: 775 - 781.

Dippold, W.G., Lloyd, K.O., Li, L.T.C., Ikeda, H., Oettgen, H.F. and Old, L.J. 1980. Cell surface antigens of human melanoma; definition of six antigenic systems with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* 77: 6114 - 6118.

Domenjoud, L., Jorcano, J.L., Breuer, B. and Alonso, A. 1988. Synthesis and fate of keratins 8 and 18 in nonepithelial cells transfected with cDNA. *Exp. Cell Res.* 179: 352 - 361.

Dougherty, G.J., Lansdorp, P.M., Cooper, D.L. and Humphries, R.K. 1991. Molecular cloning of CD44R1 and CD44R2, two novel isoforms of the human CD44 lymphocyte "homing" receptor expressed by hemopoietic cells. *J. Exp. Med.* 174:1 - 5.

Dracopoli, N.C., Houghton, A.N. and Old, L.J. 1985. Loss of pleomorphic fragments in human melanoma: implications for tumour heterogeneity. *Proc. Natl. Acad. Sci. USA* 82: 1470 - 1474.

Duffy, M.J. 1992. The role of proteolytic enzymes in cancer invasion and metastasis. *Clin. Exp. Metastasis* 10:145 - 155.

Dutcher, J.P., Gaynor, E.R., Boldt, D.H., Dorashaw, J.H., Mier, J., Sparamo, J., Fisher, R.I., Weiss, G., Margolin, K., Aronson, F.R., Hawkins, M. and Atkins, M. 1991. A phase II study of high dose continuous infusion interleukin-2 with lymphokine activated killer cells in patients with metastatic melanoma. *J. Clin. Oncol.* 9: 641 - 648.

Early Breast Cancer Triallists Collaborative Group. 1992. Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. *Lancet* 339: 1 - 15, 71 - 85.

Eckert, R.L. 1988. Sequence of the human 40kDa keratin reveals an unusual structure with very high sequence identity to the corresponding bovine keratin. *Proc. Natl. Acad. Sci. USA* 85:1114 - 1118.

Eisenbach, L., Kushtai, G., Plksin, D. and Feldman, M. 1986. MHC genes and oncogenes controlling the metastatic phenotype of tumour cells. *Cancer Rev.* 5: 1 - 18.

Elwood, J.M. and Lee, J.A.H. 1975. Recent data on the epidemiology of malignant melanoma. *Semin. Oncol.* 2: 149 - 154.

Engell, H.C. 1959. Cancer cells in the blood. A five to nine year follow up study *Ann. Surg.* 149: 457 - 461.

Ewing, J. 1928. *Neoplastic Diseases* 3rd ed. Philadelphia. W.B. Saunders.

Fahy, E., Kwoh, D.Y. and Gingeras, T.R. 1991. Self-sustained sequence replication (3SR): an isothermal transcription-based amplification system alternative to PCR. *PCR Methods Applic.* 1:25 - 33.

Fentiman, I.S. 1990. *Detection and treatment of early breast cancer.* London. Dunitz.

Fidler, I.J. 1970. Metastasis: quantitative analysis of distribution and fate of tumour cell emboli labelled with $^{125}$I-5-iododeoxyuridine. *J. Natl. Cancer Inst.* 45: 773 - 782.

Fidler, I.J. 1973. The relationship of embolic homogenicity, number, size and viability to the incidence of experimental metastasis. *Eur. J. Cancer* 9: 223 - 227.

Fidler, I.J. 1990. Critical factors in the biology of human cancer metastasis: Twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res.* 50: 6130 - 6138.

Fidler, I.J. and Balch C.M. 1987. The biology of cancer metastasis and implications for therapy. *Curr. Prob. Surg.* 24: 137 - 209.

Fidler, I.J. Gersten, D.M. and Hart, I.R. 1978. The biology of cancer invasion and metastasis. *Adv. Cancer Res.* 28: 149 - 250.

Fidler, I.J. and Kripke, M.L. 1977. Metastatic results from pre-existing variant cells within a malignant tumour. *Science* 197: 893 - 895.

Fidler, I.J. and Hart, I.R. 1982. Biological diversity in metastatic neoplasms: origins and implications. *Science* 217: 998 - 1003.

Fidler, I.J. and Radinsky, R. 1990. Genetic control of cancer metastasis. *J. Natl. Cancer Inst.* 82:166 - 168.

Field, J.K. and Spandidos, D.A. 1990. The role of RAS and MYC oncogenes in human solid tumours and their relevance to prognosis. *Anticancer Res.* 10: 1 - 22.

Finney, M. 1991. The polymerase chain reaction. In: Asubal, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith J.A. and Struhl, K.(eds) *Current protocols in molecular biology*. Vol. 2. New York. John Wiley and Sons Inc., 15.0.1- 15.7.6.

Fisher, E.R. and Fisher, B.. 1965. Experimental study of factors influencing development of hepatic metastases from circulating tumour cells. *Acta Cytol.* 9:146 - 159.

Folkman, J. 1986. How is blood vessel growth regulated in normal and neoplastic tissue? C.H.A Clowes memorial lecture. *Cancer Res.* 46: 467 - 473.

Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A., Goldgar, D.E., Breast Cancer Linkage Consortium. 1994. Risks of cancer in *BRCA1*-muation carriers. *Lancet* 343: 692 - 695.

Foss, A.J.E., Guille, M.J., Occleston, N.L., Hykin, P.G., Hungerford, J.L. and Lightman, S. 1995. The detection of melanoma cells in peripheral blood by reverse transcription-polymerase chain reaction. *Br. J. Cancer* 72: 155 - 159.

Fountain, J.W., Karayiorgou, M., Ernstoff, M.S., Kirkwood, J.M., Vlock, D.R., Titus-Ernstoff, L., Bouchard, B., Vijaysaradhi, S., Houghton, A.N., Lahti, J., Kidd, V.J., Housman, D.E. and Dracopoli, N.C. 1992.Homologous deletion of human chromosome 9p21 in melanoma. *Proc Natl. Acad. Sci. USA* 89: 10557 - 10561.

Fox, S.B., Fawcett, J., Jackson, D.G., Collins, I., Gatter, K.C., Harris, A.L., Gearing, A. and Simmons, D.L. 1994. Normal human tissues in addition to some tumours express multiple different CD44 isoforms. *Cancer Res.* 54: 4539 - 4546.

Friend, S.H., Bernards, R., Rogelj S., Weinberg, R.A., Rapaport, J.M., Albert, D.M. and Dryja, T.P. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323: 643 - 646.

Frost, J. K. 1965. Cancer cells in circulating blood: a comparison with cytologic criteria for other sites. *Acta Cytol.* 9: 83 - 93.

Frost, P. and Levin B. 1992. Clinical implications of metastatic process. *Lancet* 339:1458 - 1461.

Fuks, Z. 1980. Patterns of spread of ovarian carcinoma: relation to therapeutic strategies. *Adv. Biosci.* 26: 39 - 51.

Ganesan, T.S., Rassool, F., Guo, A.P., Th'ng, K.H., Dowding, C., Hibbin, J.A., Young, B.D., White, H., Kumara, T.O., Galton, D.A. and Goldman, J.M. 1986. Rearrangement of the bcr gene in Philadelphia chromosome-negative chronic myelogenous leukaemia. *Blood* 68: 957 - 960.

Gatter, K.C., Alcock, C., Heryet, A. and Mason, D.Y. 1985. Clinical importance of analysing tumour of uncertain origin with immunohistological techniques. *Lancet* i: 1302 - 1305.

Gatter, K.C., Ralfkiaer, E., Skinner, J., Brown, D., Heryet, A., Pulford, K.A.F., Hou-Jensen, K. and Mason, D.Y. 1985. An immunocytochemical study of malignant melanoma and its differential diagnosis from other malignant tumours. *J. Clin. Pathol.* 38: 1353 - 1357.

Gelfand, D.H. 1989. Taq DNA polymerase. In: Erlich H.A. (ed.)*PCR technology: principles and applications for DNA amplification.* New York. Stockton Press. 17 - 22.

Gerhard, M., Juhl, H., Kalthoff, H., Schreiber, H.W., Wagener, C. and Naumaier, M. 1994. Specific detection of carcinoembryonic-antigen-expressing tumour cells in bone marrow aspirates by polymerase chain reaction. *J. Clin. Oncol.* 12: 725 - 729.

Gion, M., Ruggeri, G., Mione, R., Marconato, R., Casella, C., Nosadini, A., Simoncini, E., Belloli, S., Dal Zennaro, E. and Bruscagnin, G. 1993. A new approach to tumour marker assessment by perioperative determination in breast and colorectal cancer. *Int. J. Biol. Markers* 8: 8 - 13.

Gitlin, D. 1976. Normal biology of alpha fetoprotein. *Ann. NY Acad. Sci.* 259: 7 - 16

Go, V.L.W., Ammon, H.W., Holtermuller, K.H., Krag, E. and Phillips, S.F. 1975. Quantitation of carcinoembryonic antigen-like activities in normal human gastrointestinal secretions. *Cancer* 36 (suppl.): 2346 - 2350.

Goldblatt, S.A. and Nadel, E.M. 1965. Cancer cells in the circulating blood: a critical review. *Acta Cytol.* 9: 6 - 20.

Goldstein, L.A., Zhou, D.F.H., Picker, L.J., Minty, C.N., Bargatze, R.F., Ding, J.F. and Butcher, E.C. 1989. A human lymphocyte homing receptor, the Hermes antigen, is related to cartilage proteoglycan core and link proteins. *Cell* 56 1063 - 1072.

Goodfellow, P.N., Banting, G., Wiles, M.V. Tunncliffe, A. Parker, M., Solomon, E., Dachau, R. and Fabre, J.W. 1982. The gene MIC4 which controls expression of the antigen defined by monoclonal antibody F 10.44.2 is on human chromosome 11. *Eur. J. Immunol.* 12: 659 - 663.

Greenberg, M.E. and Bender, T.P. 1991. Preparation and analysis of RNA. In. Asubal, F.M., Brent, R., Kingston, R.E., Moore, D.D.,. Seidman, J.G, Smith, J.A. and Struhl K., (eds.) *Current protocols in molecular biology.* Vol1. John Wiley and Sons Inc., New York. 4.0.1 - 4.10.9.

Gribben, J.G., Freedman, A.S., Neuberg, D., Roy, D., Blake, K.W., Woo, S.D., Grossbard, M.L., Rabinowe, S.N., Coral, F., Freeman, G.J., Ritz, J. and Nadler, L.M. 1991. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. *N. Engl. J. Med.* 325:1525 - 1533.

Griffiths, J.D., McKinna, J.A., Rowbotham, H.D., Tsoladakis, P. and Salisbury, A.J. 1973. Carcinoma of the colon and rectum: circulating malignant cells and five year survival. *Cancer* 31: 226 - 236.

Griffiths, J.D. and Salisbury, A.J. 1963. The fate of the circulating*Walker 256* tumour cell injected intravenously in rats. *Br. J. Cancer* 17: 546 - 557.

Griffiths, J.D. and Salisbury, A.J. 1965. *Circulating cancer cells.*Springfield. Charles C. Thomas.

Grillo, M. and Margolis, F.L. 1990. Use of reverse transcriptase polymerase chain reaction to monitor expression of intronless genes. *Biotechniques* 9: 262 - 268.

Gullick, W.J. Love, S.B. Wright, C. Barnes, D.M., Gusterson, B., Harris, A.L. and Altman D.G. 1991. c-erbB-2 protein over-expression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br. J. Cancer* 63: 434 - 438.

Gunthert, U., Hofmann, M., Reber, S., Zoller, M., HauBmann, I., Matzku, S., Wenzel, A., Ponta, H. and Herrlich, P. 1991. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 65:13 - 24.

Gustmann, C., Altmannsberger, M., Osborn, M., Griesser, H. and Feller, A.C. 1991. Cytokeratin expression and vimentin content in large cell anaplastic lymphomas and other non-Hodgkin's lymphomas. *Amer. J. Pathol.* 138:1413 - 1422.

Hansen, H.J., Snyder, L.J., Miller, E., Vandevoorde, J.P., Miller, O.P., Hines, L.R. and Burns, J.J. 1974. Carcinoembryonic antigen (CEA) assay. A laboratory adjuvant in the diagnosis and management of cancer. *J. Human Pathol* 5: 139 - 147.

Harbour, J.W., Lai, S-L., Whang-Peng, J., Gadzur, A.C., Minna, J.D. and Kaye, F.J. 1988. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* 241: 353 - 357.

Harn, H-J., Isola, N. and Cooper, D.L. 1991. The multispecific cell adhesion molecule CD44 is represented in reticulocyte cDNA. *Biochem. Biophys. Res. Commun* 178:1127 - 1134.

Harris, C.C. and Hollstein, M. 1992. p53 tumour suppressor gene. *Principles and Practice of Oncology Updates* 6 (10): 1 - 12.

Harris, J.R., Morrow, M. and Bonnadonna, G. 1993. Cancer of the breast. In: De Vita, V.T., Hellman, S. and Rosenberg, S.A. (eds.) *Cancer: Principles and Practice of Oncology* (4th edition) Philadelphia. J.B. Lippencott 1264 - 1332.

Harris, N.L., Jaffe, E.S., Stein, H., Banks, P.M., Chan, J.K.C., Cleary, M.L., Delsol, G., De Wolf-Peeters, C., Falini, B., Gatter, K., Grogan, T.M., Isaacson, P.G., Knowles, D.M., Mason, D.Y., Muller-Hermelink, H-K., Pileri, S.A., Piris, M.A., Ralfkiaer,E. and Warnke, R.A. 1994. A revised European-American classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood* 84: 1361 - 1392

Hart, I.R. 1989. Immune profile in metastasis. *Curr. Opin. Immunol.* 1: 900 - 903.

Hart, I. R. and Easty, D. 1991. Identification of genes controlling metastatic behaviour. *Br. J. Cancer* 63: 9 - 12.

Haynes, B.F., Telen, M.J., Hale, L.P. and Denning S.M. 1989. CD44- A molecule involved in leukocyte adherence and T-cell activation. *Immunol. Today* 10: 423 - 428.

Henderson, I.C. 1994. Clinical decision making in the use of adjuvant systemic therapy. *ASCO Educational Booklet* 6 - 10.

Hendrix, M.J.C., Seftor, E.A., Chu, Y-W., Seftor, R.E.B., Nagle, R.B., McDaniel, K.M., Leong, S.P.L., Yohem, K.H., Leibovitz, A.M., Meyskens Jr., F.L., Conaway, D.H., Welch, D.R., Liotta, L.A. and Stetler-Stevenson, W. 1992. Coexpression of vimentin and keratins by human melanoma tumour cells: correlation with invasive and metastatic potential. *J. Natl. Cancer Inst.* 84:165 - 174.

Hennessy, C., Henry, J..A., May, F.E.B., Westly, B., Angus, B. and Lennard, T.W. 1991. Expression of the anti-metastatic gene nm23 in human breast cancer; an association with good prognosis. *J. Natl. Cancer Inst.* 83: 281 - 285.

Hensen, D.E., Ries, L., Freedman, L.S. and Carrigia, M. 1991. Relationship among outcome, stage of disease and histological grade for 22,616 cases of breast cancer; the basis for a prognostic index. *Cancer* 68: 2142 - 2149.

Herbeuval, R., Duheille, J. and Goedert-Herbeuval C. 1965. Diagnosis of unusual blood cells by immunofluorescence. *Acta Cytol.* 9:73 - 82.

Herlyn, D., Iliopoulos, D., Jensen, P.J., Parmiter, A., Baird, J., Hotta, H., Adachi, K., Ross, A.H., Jambrosic, J., Koprowski, H. and Herlyn, M. 1990. In vitro properties of human melanoma cell metastasis in nude mice. *Cancer Res.* 50: 2296 - 2302.

Herlyn, M. 1990. Human melanoma: development and progression. *Cancer Metastasis Rev.* 9: 101 - 112.

Herlyn, M., Kath, R., Williams, N., Valyi-Naji, I. and Rodeck, U. 1990. Growth-regulatory factors for normal, premalignant and malignant human cells in vitro. *Adv. Cancer Res.* 54: 213 - 234.

Herrlich, P., Zoller, M., Pals, S.T. and Ponta, H. 1993. CD44 splice variants: metastases meet lymphocytes. *Immunol. Today* 14: 395 - 399.

Heydermann, E. and McCartney, J.C. 1985. Epithelial membrane antigen and lymphoid cells. *Lancet* i: 109.

Hirayama, R., Sawai, S., Tagaki, Y., Mishima, Y., Kimura, N., Shimada, N., Esaki, Y., Kurashima, C., Utsuyama, M. and Hirokawa, K. 1991. Positive relationship between expression of anti-metastatic factor (nm23 gene product or nucleoside diphosphate kinase) and good prognosis in human breast cancer. *J. Natl. Cancer Inst.* 83: 249 - 1250.

Hockenberry, D., Nuuez, G., Milliman, C, Schreiber, R.D. and Korsmeyer, S.J. 1990. Bcl-2 is an inner mitochondrial membrane protein that blocks cell death. *Nature* 348: 334 - 336.

Hofmann, M., Rudy, W., Zoller, M., Tolg, C., Ponta, H., Herrlich, P. and Gunthert, U. 1991. CD44 splice variants confer metastatic behaviour in rats: homologous sequences are expressed in human tumour cell lines. *Cancer Res.* 51: 5292 - 5297.

Holmes, K.L. and Morse, H.C. 1988. Murine hematopoietic cell surface antigen expression. *Immunol. Today* 9: 344 - 350.

Hood, D.L. Petros, R.E. , Edinger, M., Fazio, U. and Tubbs, R.R. 1990. DNA ploidy and cell cycle analysis of colorectal carcinoma by flow cytometry: a prospective study of 137 cases using fresh whole cell suspensions. *Amer. J. Clin. Pathol.* 93: 615 - 620.

Horwich, A., Alsanjari, N., A'Hern, R., Nicholls, J., Dearnely, D.P. and Fisher, C. 1992. Surveillance following orchidectomy for stage I testicular seminoma. *Br. J Cancer* 65: 775 - 778.

Hughes, T.P., Morgan, G.J., Martiat, P. and Goldman, J M. 1991. Detection of residual leukaemia after bone marrow transplant for chronic myelogenous leukemia by retrotranscriptase polymerase chain-reaction to predict relapse. *Blood* 82: 1264 - 1269.

Humphries, M.J., Olden, K. and Yamada, K.M. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science* 233: 467 - 470.

Hynes, N.E. 1993. Amplification and overexpression of the erbB-2 gene in human tumours: it's involvement in tumour development, significance as a prognostic factor, and potential as a target for cancer therapy. *Semin. Cancer Biol.* 4:19 - 26.

Hynes, R.O. 1987. Integrins: a family of cell surface receptors. *Cell* 48: 549 - 554.

Idzerda, R.L., Carter, W.G., Nottenberg, C., Wayner, E.A., Gallatin, W.M. and St.John, T. 1989. Isolation and DNA sequence of a cDNA clone encoding a lymphocyte adhesion receptor for high endothelium. *Proc. Natl. Acad. Sci. USA*86: 4659 - 4663.

Jackson, B.W., Grund, C., Schmid, E., Burk, I. K., Franke, W.W. and Illmensee K. 1980. Formation of cytoskeletal elements during mouse embryogenesis. *Differentiation* 17: 161 - 179.

Jackson, D.G., Buckley, J. and Bell, J.I. 1992. Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain. *J. Biol. Chem.* 267: 4732 - 4739.

Jackson, D.P., Hayden, J.D. and Quirke, P. 1991. Extraction of nucleic acid from fresh and archival material. In: McPherson, M.J., Quirke, P. and Taylor, G.R. (eds.)*PCR: a practical approach.* Oxford. Oxford University Press. 29 - 50.

Jalkanen, S.T., Bargartze, R.F., Hernon, L.R. and Butcher, E.C. 1986. A lymphoid cell surface protein involved in endothelial cell resorption and lymphocyte homing in man. *Eur. J. Immunol.* 16: 1195 - 1202.

Jalkanen, S., Jalkanen, M., Bargatze, R.F., Tammi, M. and Butcher, E.C. 1988. Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. *J. Immunol.* 141: 1615 - 1623.

Jalkanen, S., Steeve, A.C., Fox, R.I. and Butcher, E.C. 1986. A distinct endothelial cell recognition system that controls lymphocyte traffic into inflamed synovium. *Science* 238: 556 - 558.

James, P.P.B. and Williams C.J. 1993. Reverse transcription polymerase chain reaction (RT-PCR) detects keratin 19 in lymph nodes. *Br. J. Cancer* 67 (suppl.): 19.

Janovski, N.A. and Paramandandham, T.L. 1973. Tumours and tumour-like conditions of the ovaries, fallopian tubes and ligaments of the uterus. In: Freedman E.A. (ed.) *Major problems in obstetrics and gynaecology* Philadelphia. W.B. Saunders. Vol. 4: 12.

Johnson, J.P., Stade, B.G., Holzmann, B., Schwable, W. and Riethmuller, G. 1989. De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis. *Proc. Natl. Acad. Sci. USA*86: 641 - 644.

Johnson, P.W.M., Joel, S.P., Love, S., Butcher, M., Pandian, M.R., Squires, L., Wrigley, P.F.M. and Slevin, M.L. 1993. Tumour markers for prediction of survival and monitoring of remission in small cell lung cancer. *Br. J. Cancer* 67: 760 - 766.

Juacaba, S.F., Horak, E., Price, J.E. and Tarin, D. 1989. Tumour cell dissemination patterns and metastasis of murine mammary carcinoma. *Cancer Res.* 49: 570 - 575.

Kacker R.K., Giovanella, B.C. and Pathak, S. 1990. Consistent karyotypic abnormalities in human malignant melanomas. *Anticancer Res.*10: 859 - 870.

Kahn, P. 1992. Adhesion protein studies provide new clue to metastasis. *Science* 257: 614.

Kainz, C., Gitsch, G., Lee, A., Danihel, L. and Breitenecker, G. 1993. Infiltrating lobular breast carcinoma: Detection of occult regional lymph node metastasis by immunohistochemistry. *Anticancer Res.* 13: 73 - 74.

Kallioniemi, O-P., Kallioniemi, A., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F. and Pinke, D. 1993. Comparative genomic hybridisation: a rapid new method for detecting and mapping DNA amplification in tumours. *Semin. Cancer Biol.* 4: 41 - 46.

Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S. III, Johnson, B.E. and Skolnick, M.H. 1994. A cell cycle regulator potentially involved in genesis of many tumour types. *Science* 264: 436 - 440.

Karovsky, P. 1990. Misuse of PCR. *Trends Biochem. Sci.* 15: 419.

Kath R., Jambrosic, J.A., Holland, L., Rodeck, U. and Herlyn, M. 1991. Tumour progression in melanoma: development of invasive and growth factor independent cell variants from primary human melanomas. *Cancer Res.* 51: 2205 - 2211.

Kawakami, Y., Eliyahu, Sakaguchi, K., Robbins, P.F., Rivoltini, L., Yanelli, J.R., Appella, E. and Rosenberg, S.A. 1994. Identification of the immunodominant peptides of MART-1 human melanoma antigen recognised by the majority of HLA-A2 restricted tumour infiltrating lymphocytes. *J. Exp. Med.* 180: 347 - 352.

Kefford, R.F. and Trent, R.J. 1989. The diagnostic applications of molecular biology. *Med. J. Aust.* 151: 467 - 481.

Kefford, R.F. 1992. Hereditary melanoma and the search for the melanoma gene. *World J. Surg.* 16: 246 - 250.

Kelly, K.A., Kirkwood, J.M., Nordlund, J.J. and Lerner, A.B. 1982. Hypopigmentation in melanoma: Favourable prognosis in a randomised controlled adjuvant study. *Proc. UICC Congress XIII*: 396192 (abstr.).

Kelly, S.L. and Meyer, T.J. 1983. Carcinoma of unknown primary site: a prudent approach. *Postgrad. Med.* 74: 269 - 280.

Kennedy, P.S. Meyer, T.J. 1979. Adenocarcinoma of unknown origin: a rational approach to a diagnostic problem. *Postgrad. Med.* 65: 151 - 160.

Kerbel, R.S. 1990 Growth dominance of the metastatic cancer cell: cellular and molecular aspects *Adv. Cancer Res.* 55: 87 - 131.

Kieran, M.W. and Longnecker, B.M. 1983. Organ specific metastasis with special reference to avian systems. *Cancer Metastasis Rev.* 2: 165 - 182.

Killion, J.J. and Fidler, I.J. 1989. The biology of tumour metastasis. *Semin. Oncol.* 16:106 - 115.

Kindt, T.J. and Robinson, M.A. 1984.Major histocompatibility complex antigens. In: Paul, W.E. (Ed.)*Fundamental Immunology* New York. Raven Press. 347 - 378.

Klein, G. and Klein, E. 1985. Evolution of tumours and the impact of molecular biology. *Nature* 315: 190 - 195.

Kleppe, K. Ohstuka, E., Kleppe, R., Molineux, L. and Khorana, H.G. 1971. Studies on polynucleotides: replication of short synthetic DNAs as catalysed by DNA polymerases. *J. Molec. Biol.* 56: 341 - 361.

Knapp, A.C. and Franke, W.W. 1989. Spontaneous losses of control of cytokeratin gene expression in transformed, non-epithelial human cells occurring at different levels of regulation. *Cell* 59: 67 - 79.

Koh, H. 1991. Cutaneous melanoma. *N. Engl. J. Med.* 325: 171 - 182.

Kohn, E.C. and Liotta, L.A. 1993. Invasion and metastasis: New approaches to an old problem. *Oncology (Williston Park)* 7: 47 - 62.

Konopka, J. Watanabe, S. and Witte, O. 1984. An alteration of the human cABL protein K562 leukaemia cells unmasks associated tyrosine kinase activity. *Cell* 37: 1035 - 1042.

Koopman, G., Griffioen, A.W., Ponta, H., Herrlich, P., van den Berg, F., Manten-Horst, E. and Pals, S.T. 1993. CD44 splice variants; expression on lymphocytes and in neoplasia. *Res. Immunol.* 144: 750 - 754.

Koscielny, S. Tubiana, M., and Valleran, A-J. 1985. A simulation model of the natural history of breast cancer. *Br. J. Cancer* 52: 515 - 524.

Krauss, S. and Franke, W.W. 1990. Organization and sequence of human gene encoding cytokeratin 8. *Gene* 86: 241 - 249.

Kulesh, D.A., Cecena, G., Darmon, Y.M., Vasseur, M. and Oshima, R.G. 1989. Psttranslational regulation of keratins: degradation of mouse and human keratins 18 and 8. *Mol. Cell. Biol.* 9: 1553 - 1565.

Kuper, S.W. and Bignall, J.R. 1964. Tritiated thymidine uptake by tumour cells in blood. *Lancet* i: 1412 - 1414.

Kurman R.J., Scardino, P.T., McIntire, K.R., Walddmann, T.A. and Javadpour, N. 1977. Cellular localisation of alpha-fetoprotein and human chorionic gonadotropin in germ cell tumour of the testis using an indirect immunoperoxidase technique. A new approach to classification utilising tumour markers. *Cancer* 40: 2136 - 2151.

Kwok, S. and Higuchi, R. 1989. Avoiding false positives with PCR. *Nature* 339: 237 - 238.

Kwon, B.S. 1993. Pigmentation genes; the tyrosinase family and the pmel 17 gene family. *J. Invest. Dermatol.* 100 (suppl.):134 - 140.

Kwon, B.S., Jaq, A.K., Pomerantz, S.H. and Halaban, R. 1987. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc. Natl. Acad. Sci. USA* 84: 7473 - 7477.

Lakhani, S., Selby, P., Bliss, J.M., Perren, T.J., Gore, M.E. and McElwain, T.J. 1990. Chemotherapy for malignant melanoma: combinations and high doses produce more responses without survival benefit. *Br. J. Cancer* 61: 330 - 334.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759 - 1764.

Lane, E.B. and Alexander, C.M. 1990. Use of keratin antibodies in tumour diagnosis. *Semin. Cancer Biol.* 1: 165 - 179.

Langenbeck, B. 1841. On the development of cancer in the veins. *Edinburgh Med. J.* 55 251 - 260.

Langer-Safer, P., Levine, M. and Ward, D. 1982. Immunological method fro mapping genes on Drosophila polytene chromosomes. *Proc. Natl. Acad. Sci. USA* 79: 4381 - 4385.

Laurie, J.A., Moertel, C.G., Fleming, T.R., Wieand, H.S., Leigh, J.E., Rubin, J., McCormack, G.W., Gerstner, J.B., Krook, J.E., Malliard, J., Twito, D.I., Morton, R.F., Tschetter, L.K. and Barlow, J.F. for the North Central Cancer Treatment Group and the Mayo Clinic. 1989. Surgical Adjuvant therapy of large bowel carcinoma: an evolution of levamisole and the combination of levamisole and fluorouracil. *J. Clin. Oncol.* 7: 1447 - 1456.

Lebowitz, P. 1983. Oncogenic genes and malignancy. *J. Biol. Med.* 56: 121 - 129.

Lee, E.Y., To, H., Shew, R.A.C. et al 1988. Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* 241: 218 - 221.

Lee, M-S., LeMaistre, A., Kanterjian, H.M., Talpez, M., Freidreich, E.J., Trujillo, J.M. and Stass, S.A.1989. Detection of two alternative BCR/ABL mRNA junctions and minimal residual disease in Philadelphia positive chronic myelogenous leukaemia by polymerase chain reaction. *Blood* 73: 2165 - 2170.

Lee, W-H., Bookstein, R., Hong, F., Young, L-J, Shew, J-Y. and Lee, Y-P. 1987. Human retinoblastoma susceptibility gene cloning, identification and sequence. *Science* 235: 1394 - 1399.

Leonard, R.J. and Nystrom, J.S. 1993. Diagnostic evaluation of patients with carcinoma of unknown primary site. *Semin. Oncol.* 20: 244 - 250.

Leterte, M., Iturbe, S. and Quackenbush, E.J. 1985. A glycoprotein of molecular weight 85,000 on human cells of B-lineage: detection with a family of monoclonal antibodies. *Mol. Immunol.* 22: 113 - 119.

Levin, V.A., Gutin, P.H. and Leibel, S. 1993. Neoplasms of the central nervous system. In: De Vita V.T., Hellman S. and Rosenberg S.A.*Cancer. Principles and Practice of Medicine* (4th edition) Philadelphia. J.B. Lippencott. 1679 - 1737.

Levine, A.J., Momand, J. and Finlay, C.A. 1991. The p53 suppressor gene*Nature* 351: 453 - 456.

Limpens, J., de Jong, D., van Krieken, J.H, Price, C.G., Young, B.D., van Ommen, G.J. and Kluin, P.M. 1991. BCL-2/JH rearrangements in benign lymphoid tissues with follicular hyperplasia. *Oncogene* 6: 2271 - 2276.

Lichter, P., Cremer, T., Tang, C-J.C. Watkins, P.C., Manuelidis, L. and Ward, D.C. 1988. Rapid detection of human chromosome aberrations by in situ hybridisation.*Proc. Natl. Acad. Sci. USA*85: 9664 - 9668.

Lindemann, F., Schlimok, G., Dirschedl, P., Witte, J. and Riethmuller, G. 1992. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. *Lancet* 340: 685 - 689.

Linnenbach, A.J., Huebner, K., Reddy, E.P., Herlyn, M., Parmiter, A.H., Nowell, P.C. and Koprowski, H. 1988. Structural alteration in the myb proto-oncogene and deletion within the gene encoding α-type protein kinase in human melanoma cell lines. *Proc. Natl. Acad. Sci. USA.* 85: 74 - 78.

Liotta, L.A. 1986. Tumour invasion and metastasis - role of the extracellular matrix. Rhoads Memorial Award lecture. *Cancer Res.* 46: 1 - 7.

Liotta, L.A., Kleinerman, J. and Saidel, G.M. 1974. Quantitative relationships of intravascular tumour cells, tumour vessels, and pulmonary metastases following tumour implantation. *Cancer Res.* 34: 997 - 1004.

Liotta, L.A. and Stetler-Stevenson W.G. 1991. Tumour invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res.* 51 (suppl): 5054 - 5059.

Liu, E.T., He, M. and Rajgopal, U. 1993. Differential polymerase chain reaction in the analysis of gene dosage. *Semin. Cancer Biol.* 4: 47 - 58.

Livingston R.B. 1994. High dose consolidation for stage IV breast cancer. *ASCO Educational Booklet.* 74 - 79.

Lurie, B.B., Lowenstein, M.S. and Zancheck, N. 1975. Elevated carcinoembryonic antigen levels and biliary tract obstruction.*J. Amer. Med. Assoc.*233: 326 - 330.

McClay, E.T., Mastrangelo, M.J., Sprandio, J.D., Bellet, R.E. and Berd, D. 1989. Importance of Tamoxifen to a cisplatin combination regimen in the treatment of metastatic melanoma. *Cancer* 63: 1292 - 1295.

Macfarlane, D.E. and Dahle, C.E. 1993. Isolating RNA from whole blood- the dawn of RNA based diagnosis? *Nature* 362: 186 - 188.

McGuire, W.L. and Clark, G.M. 1992. Prognostic factors and treatment decisions in axillary node negative breast cancer. *N. Engl. J. Med.* 236: 1756 - 1761.

Mahadevan, V. and Hart I.J. 1991. Tumour angiogenesis and metastasis. *Eur. J. Cancer* 27: 679 - 680.

Mansi, J.L., Berger, U., Wilson, P., Shearer, R. and Coombes, R.C. 1988. Detection of tumour cells in bone marrow of patients with prostatic carcinoma by immunocytochemical techniques. *J. Urol.* 139: 545 - 548.

Mansi, J.L., Easton, D., Berger, U., Gazet, J.C., Ford, H.T., Dearneley, D. and Coombes, R.C. 1991. Bone marrow micrometastases in primary breast cancer: prognostic significance after 6 years follow-up. *Eur J. Cancer* 27: 1552 -1555.

Manzotti, C., Audisio, R.A. and Pratesi, G. 1993. Importance of orthotopic implantation for human tumours as model systems: relevance to metastasis and invasion. *Clin. Exp. Metastasis* 11: 5 - 14.

Mareel, M.M. 1992. Micro-ecosystems of invasion and cancer metastasis. *Clin. Exp. Metastasis* 10 (suppl.): 9 - 10.

Marlin, S.D. and Springer, T.A. 1987. Purified intercellular adhesion molecule 1 (I CAM-1) is a ligand for lymphocyte function associated antigen 1 (LFA-1). *Cell* 51: 813 - 819.

Mason, H.S. 1948. The chemistry of melanin III: mechanism of oxidation of dihydrophenylalanine by tyrosinase. *J. Biol. Chem.* 172: 83 - 99.

Matsumura, Y. and Tarin, D. 1992. Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 340: 1053 - 58.

Mattano, L.A., Moss, T.J. and Emerson, S.G. 1992. Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. *Cancer Res.* 52: 4701 - 4705.

Maxam, A. and Gilbert, W. 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65: 499 - 560.

Mayer, R.J., Garnick, M.B. and Steele, G.D. 1978. Carcinoembryonic antigen (CEA) as a monitor of chemotherapy in disseminated colorectal cancer. *Cancer* 42: 1428 - 1433.

Meese, E., Meltzer, P.S., Witkowski, C.M. and Trent, J.M. 1989. Molecular mapping of the oncogene MYB and rearrangements in malignant melanoma. *Genes Chromosomes Cancer* 1: 88 - 94.

Meittinen, M. and Frassila, K. 1989. Immunohistochemical spectrum of malignant melanoma. *Lab. Invest.* 6: 623 - 628.

Meittinen, M. and Rapola, J. 1989. Immunocytochemical spectrum of rhabdomyosarcoma-like tumours. Expression of cytokeratin and the 68 kD neurofilament protein. *Amer. J. Surg. Pathol.* 13: 120 - 132.

Meling, G.I. Rognum, T.O., Clausen, O.P. Chen, Y., Lunde, O.C., Schlichting, E., Wiig, J.N., Hagnestad, J., Bakka, A., Hauig, O. and Bergan, A. 1991. Association between DNA ploidy pattern and cellular atypia in colorectal cancers; a new clinical application of DNA flow cytometric study? *Cancer* 67: 1642 - 1649.

Menon, R.S., Chang, Y-F., St. Clair, J. and Ham, R.G. 1991. RT-PCR artefacts from processed pseudogenes. *PCR Methods Applic.* 1: 70 - 71.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futneal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., Bell, R., Rosenthal, J., Hussey, C., Tran, T., McClure, M., Frye, C., Hattier, T., Phelps, R., Haugen-Strano, A., Katcher, H., Yakumo, K., Gholami, Z., Shaffer, D., Stone, S., Bayer, S., Wray, C., Bogden, R., Dayananth, P., Ward, J., Tonin, P., Narod, S., Bristow, P.K., Norris, F.H., Helvering, L., Morrison, P., Rostek, P., Lai, M., Barrett, C., Lewis, C., Neuhausen, S., Cannon-Albright, L., Goldgar, D., Wiseman, R., Kamb, A. and Skolnick, M.H. 1994. Isolation of BRCA1, the 17q-linked breast and ovarian cancer susceptibility gene. *Science* 266: 66 - 71.

Miyake, K., Underhill, C.B., Lesley, J. and Kincade, P.W. 1990. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 172: 69 - 75.

Moertel, C.G., Fleming, T.R., MacDonald, J.S. Haller, D.G., Laurie, J.A., Tange, C.M., Ungerleider, J.S., Emerson, W,A., Tormey, D.G., Glick, J.H., Veeder, M.H. and Mailliard, J.A. 1995. Fluorouracil plus levamisole as effective adjuvant therapy for stage III colon carcinoma: a final report. *Ann. Int. Med.* 122: 321 - 326.

Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. 1982. The catalog of human cytokeratins: patterns of expression in epithelia, tumours and cultured cells. *Cell* 31: 11 - 24.

Moll, R. Lowe, A., Laufer, J. and Franke, W.W. 1992. Cytokeratin 20 in human carcinomas. A new histodiagnostic marker detected by monoclonal antibodies. *Amer. J. Pathol.* 140: 427 - 447.

Moore, G.E. and Sandberg, A.A. 1965. Clinical aspects of cancer cells in the blood. *Acta Cytol.* 9:175 - 184.

Moore, M.R., Garrett Jr., P.R., Walton, K.N., Wadmann, T.A., McIntire, R.K., Counts, P. and Vogel, C.J. 1978. Evaluation of human chorionic gonadotrophin and alpha fetoprotein in benign and malignant disorders. *Surg. Gynaecol. Obstet.* 147: 167 - 174.

Moore, S., Townsend, D.E., Palmer, J.D. and Sigmon, H. 1962. The problem of tumour cells in the peripheral blood. *Can. J. Surg.* 5: 366 - 369.

Moreno, J.G., Croce, C.M., Fischer, R., Monne, M., Vihko, P., Mulholland, S.G. and Gomella, L.G. 1992. detection of hematogenous micrometastases in patients with prostate cancer. *Cancer Res.* 52: 6110 - 6112.

Mountain, C.F. 1977. Assessment of the role of surgery for control of lung cancer *Ann. Thoracic. Surg.* 24: 365 - 373.

Mukherji, B. and Chakraborty, N.G. 1995. Immunobiology and immunotherapy of melanoma. *Curr. Opin. Oncol.* 7: 175 - 184.

Mulder, J.W., Kryt, P.M., Sewenath, M., Oosting, C.A., Seldenrijk, C.A., Weidema, W.F., Offerhaus G.L. and Pals, S.T. 1994. Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 344: 1470 - 1472.

Mulder, J.W., Weilenga, V.J., Polak, M.M., van den Berg, F.M., Adolf, G.R., Herrlich, P., Pals, S.T. and Offerhaus, G.L. 1995. Expression of mutant p53 protein and CD44 variant proteins in colorectal tumourigenesis. *Gut* 36: 76 - 80.

Mullis, K.B. and Faloona, F.A.S. 1987. Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. *Methods Enzymol.* 155: 335 - 350.

Muss, H.B., Thor, A.D., Berry, D.A., Kute, T., Liu, E.T., Koerner, G., Cirrincione, C.T., Budman, D.R., Wood, W.C., Darcos, M. and Henderson, I.C. 1994. c-erbB-2 expression and response to adjuvant therapy in women with node positive early breast cancer. *N. Engl. J. Med.* 330: 1260 - 1266.

Nadel, E. M. 1965. Prospice- tumour cells in circulating blood. *Acta Cytol.* 9:185 - 188.

Nagle, R.P. 1988. Intermediate filaments: a review of basic biology. *Amer. J. Surg. Pathol.* 12 (suppl.): 4 - 16.

Nagy, K. P. 1965. A study of normal, atypical and neoplastic cells in the white cell concentrate of the peripheral blood. *Acta Cytol.* 9: 61 - 67.

Naito, H., Kuzumaki, N., Uchino, J., Kobayashi, R., Shikano, T., Ishikawa, Y. and Matsumoto, S. 1991. Detection of tyrosine hydroxylase mRNA and minimal neuroblastoma cells by the reverse transcription-polymerase chain reaction. *Eur. J. Cancer* 27: 762 - 765.

Naito, S., Giauazzi, R. and Fidler, I.J. 1987. Correlation between the in vitro interactions of tumour cells with an organ environment and metastatic behaviour in vivo. *Invasion Metastasis* 7: 16 - 29.

Nedelkoff, B., Christopherson, W.M. and Harter J.S. 1962. A study of abnormal cells in the peripheral circulation of patients with lung cancer. *Acta Cytol.* 6: 319 - 324.

Nicolson, G.L. 1988. Cancer metastasis: tumour cell and host organ properties important in metastasis to specific sites. *Biochim. Biophys. Acta* 48: 175 - 224.

Nicolson, G.L. and Winkelhake J.L. 1975. Organ specificity of blood borne tumour metastases determined by cell adhesion? *Nature* 255: 230 - 232.

Nicolson, G.L., Dulski, K., Bassson, C. and Welch, D.R. 1985. Preferential organ attachment and invasion by *B16* melanoma cells selected for differing metastatic colonization and invasive properties. *Invasion Metastasis* 5: 144 - 158.

Nordlund, J.J., Kirkwood, J.M., Forget, B.M., Milton, G., Albert, D.M., and Lerner, A.B. 1983. Vitiligo in patients with metastatic melanoma: A good prognostic sign. *J. Amer. Acad. Dermatol.* 9: 689 - 696.

Norris, M.D., Gilbert, J., Marshall, G.M. and Haber, M. 1994. Detection of minimal residual neuroblastoma by reverse transcription polymerase chain reaction. *Proc. Amer. Soc. Clin. Oncol.* 35: 1218 (abstr.).

Oesterling, J.E. 1991. Prostate-specific antigen: A valuable clinical tool. *Oncology* 5: 107 - 126.

Oh, J.H. and MacLean, L.D. 1977. Prognostic use of preoperative and immediate post-operative carcinoembryonic antigen expression determination in colonic cancer. *Can. J. Surg.* 20: 64 - 67.

Old, L.J. 1981. Cancer immunology: the search for specificity. GHA Clowes Memorial Lecture. *Cancer Res.* 41: 361 - 375.

O'Reilly, S.M., Camplejohn, R.S., Barnes, D.M., Millis, R.R., Rubens, R.D. and Richards, M.A. 1990. Node negative breast cancer; prognostic subgroups defined by tumour size and flow cytometry. *J. Clin. Oncol.* 8: 2040 - 2046.

Osborne, C.K. 1991. Receptors. In: Harris, J.R., Hellman, S., Henderson, I.C. and Kinne, D.W. (eds.) *Breast diseases* (2nd ed.) Philadelphia. J.B. Lippencott 301 - 325.

Padua, R.A., Barrass, N.C. and Currie, G.A. 1985. Activation of N-ras in human melanoma cell line. *Mol. Cell. Biol.* 5: 582 - 585.

Paget, S. 1889. Distribution of secondary growths in cancer of the breast. *Lancet* 1: 571 - 573.

Pantel, K., Izbicki, J.R., Angstwurm, M., Braun, S., Passlick, B., Karg, O., Thetter, O., and Riethmuller, G. 1993. Immunocytological detection of bone marrow micrometastasis in operable non-small cell lung cancer. *Cancer Res.* 53:1027-1031.

Perren, T.J. 1991. *c-erbB-2* oncogene as a prognostic marker in breast cancer. *Br. J. Cancer* 63: 328 - 332.

Perry-O'Keefe, H. and Kissenger, C.M.. 1991. Enzymatic manipulation of DNA and RNA. In: Asubal, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) *Current protocols in molecular biology.* Vol.1. New York. John Wiley and Sons Inc. 3.0.1-3.19.8.

Pinhorn, A. 1993. Tumour markers and primary site of cancer *Br. Med. J.* 306: 651.

Pittman, K., Southgate, J., Plumb, M., Trejdosiewicz, L. and Selby, P. 1993. Reverse transcriptase and polymerase chain reaction utilising cytokeratin 8 to detect metastatic carcinoma cells in peripheral blood. *Br. J. Cancer* 67 (suppl.): 18 (abstr.).

Pittman, K., Burchill, S., Smith, B., Southgate, J., Joffe, J., Gore, M. and Selby, P. Reverse transcriptase-polymerase chain reaction for expression of tyrosinase to identify malignant melanoma cells in peripheral blood. (submitted).

Poste, G. and Fidler, I.J. 1979. The pathogenesis of cancer invasion and metastasis. *Nature* 283: 139 - 146.

Potter, M. N., Steward, C.G. and Oakhill, A. 1993. The significance of detection of minimal residual disease in childhood acute lymphoblastic leukaemia. *Br. J. Haematol.* 83: 412 - 418.

Proctor, J.W. 1976. Rat sarcoma model supports the 'soil seed' and 'mechanical' theories of metastatic spread. *Br. J. Cancer* 34: 652 - 654.

Prossart, P., Keilholz, U., Willhack, M., Scheibenbogen, C., Mohler, T. and Hunstein, W. 1993. Hematogenous spread of malignant melanoma cells in different stages of disease. *J. Invest. Dermatol.* 101: 887 - 889.

Pruitt, J.C., Powell, R.V. and Prater T.F.K. 1965. A quantitative comparison of processing techniques for study of circulating malignant cells. *Acta Cytol.* 9:116 - 122.

Purves, L.R., Bersohn, I. and Geddes, E.W. 1970. Serum alpha fetoprotein and primary cancer of the liver in man. *Cancer* 25: 1261 - 1276.

Quinlan, R.A. and Franke, W.W. 1982. Heteropolymer filaments of vimentin and desmin in vascular smooth muscle and cultured baby hamster kidney cells demonstrated by cross-linking. *Proc. Natl. Acad.* Sci. *USA* 70: 3452 - 3456.

Radinsky, R., Weisberg, H.Z., Staroselsky, A.N. and Fidler I.J. 1992. Expression level of the nm23 gene in clonal populations of metastatic murine and human neoplasms. *Cancer Res.* 52: 5808 - 5814.

Rappolee, D.A., Wang, A., Mark, D. and Werb, Z. 1989. Novel method for studying mRNA phenotypes in single or small numbers of cells. *J. Cell. Biochem.* 39:1 - 11.

Rawles M.E. 1948. Origin of melanophores and their role in development of color patterns in vertebrates. *Physiol. Rev.* 28: 383 - 408.

Raynal, F. Michot, B. and Bachellerie, J-P. 1984. Complete nucleotide sequence of mouse $^{18}$s rRNA gene: comparisons with other available homology. *FEBS lett.* 167: 263 - 268.

Redding, W.H., Monaghan, P., Imrie, S.F., Ormerod, M.G., Gazet, J.C., Coombes, R.C., Dearnaley, D.P., Sloane J.P., Powles, T.J. and Neville, A.M. 1983. Detection of micrometastases in patients with primary breast cancer. *Lancet* ii:1271 - 1273.

Riethmuller, G., Schneider-Gadicke, ER., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Pichlmaier, H., Hirche, H., Pichlmayr, R., Buggisch, P., Witte, J. and the German Cancer Aid 17 - 1A Study Group. 1994. Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. *Lancet* 343: 1177 - 1183.

Roberts, S., Jonasson, O., Long, L., McGrew, E.A., McGrath, R. and Cole, W.H. 1962. Relationship of cancer cells in circulating blood to operation. *Cancer* 15: 232 - 240.

Romsdahl, M.M., Chu, E.W., Hume, R. and Smith, R.R. 1961. The time of metastasis and release of circulating tumour cells as determined by an experimental system. *Cancer* 15: 883 - 888.

Romsdahl, M.M., McGrath, R.G., Hoppe, E. and McGrew, E.A. 1965. Experimental model for the study of tumour cells in the blood. *Acta Cytol.* 9:141 - 145.

Romsdahl, M.M., Valaitis, J., McGrath, R.G. and McGrew, E.A. 1965. Circulating tumour cells in patients with carcinoma. method and recent studies. *J. Amer. Med. Assoc.* 193: 1087 - 1090.

Rosenberg, S.A. 1994. Classification of lymphoid neoplasms. *Blood* 84: 1359 - 1360.

Safi, F., Kohler, I., Rottinger, E. and Beger, H. 1991. The value of the tumour marker CA 15-3 in diagnosing and monitoring breast cancer. A comparison study with carcinoembryonic antigen. *Cancer* 68: 574 - 582.

Saiki, R. K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K. and Erlich, H. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487 - 491.

Sainsbury J.R.C., Farndon J.R., Needham G.K., Malcolm, A.J. and Harris, A.L. 1987. Epidermal growth factor receptor status as a predictor of early recurrence and death from breast cancer. *Lancet* i: 1398 - 1402.

Salisbury, A.J. 1975. The significance of the circulating cancer cell. *Cancer Treat. Rev.* 2: 55 - 72.

Sarker, G. and Sommer, S.S. 1990. Shedding light on PCR contamination. *Nature* 343: 27.

Savtchenko, E.S., Schiff, T.A., Jiang, GK., Freedberg, I.M. and Blumenberg, M. 1988. Embryonic expression of the human 40-kD keratin: evidence from a processed pseudogene sequence. *Amer. J. Hum. Genet.* 43: 630 - 637.

Schirrmacher, V., Cheingsong-Popov, R. and Arnheiter H. 1980. Hepatocyte - tumour cell interaction in vitro. Conditions for rosette formation and inhibition by *Anti-H-2* antibody. *J. Exp. Med.* 151: 984 - 989.

Schirrmacher, V. 1985. Cancer metastasis: experimental approaches, theoretical concepts, and impacts for treatment strategies. *Adv. Cancer Res.* 43:1 - 73.

Schlimok, G., Funke, I., Bock, B., Schweiberer, B., Witte, J. and Riethmuller, G. 1990. Epithelial tumour cells in bone marrow of patients with colorectal cancer: immunocytochemical detection, phenotypical characterisation and prognostic significance.
*J. Clin. Oncol.* 8: 831 - 837.

Schlimok, G. and Riethmuller, G. 1990. Detection, characterisation and tumourigenicity of disseminated tumor cells in human bone marrow. *Semin. Cancer Biol.* 1: 207 - 215.

Schoenfeld, A., Luqmani, E., Smith, D., O'Reilly, S., Shousha, S., Sinnett, H.D. and Coombes, R.C. 1994. Detection of breast cancer micrometastases in axillary nodes by using polymerase chain reaction. *Cancer Res.* 544: 2986 - 2990.

Schwartz, D.C. and Cantor, C.R. 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37: 67 - 75.

Schwartz, M.A. and Ingber, D.E. 1994. Integrating with integrins. *Molec. Biol. Cell* 5: 389 - 393.

Seiter, S., Arch, R., Reber, S., Komitowski, D., Hofmann, M., Ponta, H., Herrlich, P., Matzku, S. and Zoller, M. 1993. Prevention of tumour metastasis formation by antivariant CD44. *J. Exp. Med.* 177: 443 - 455.

Sekiya, T., Mirohaashi, S., Nishimara, S. and Sugimura, T. 1983. Transforming activity of human melanoma DNA. *Gann* 74: 794 - 797.

Selby, P. 1980. M.D. Thesis. University of Cambridge.

Sellner, L.N., Coelen, R.J. and Mackenzie, J.S. 1992. Reverse transcriptase inhibits Taq polymerase activity. *Nucl. Acids Res.* 20: 1487 - 1490.

Sellwood, R.A., Kuper, S.W.A., Burn, J.I. and Wallace, E.N. 1964. Circulating tumour cells. *Br. Med. J.*1: 1683 - 1686.

Sewell, H.F., Thompson, W.D. and King, D.J. 1986. IgD myeloma/immunoblastic lymphoma cells expressing cytokeratin. *Br. J. Cancer* 53: 695 - 696.

Shimoyama, Y. and Hiroshashi, S. 1991. Cadherin intercellular adhesion molecule in hepatocellular carcinomas: loss of E-cadherin expression in an undifferentiated carcinoma. *Cancer Lett.* 57: 131 -135.

Shih, I-M. and Herlyn, M. 1993. Role of growth factors and their receptors in the development and progression of melanoma. *J. Invest. Dermatol.* 100 (suppl.): 196 - 203.

Shuldiner, A.R., Tanner, K., Moore, C.A., and Roth, J. 1991. RNA template-specific PCR: an improved method that dramatically reduces false positives in RT-PCR. *Biotechniques* 11: 760 - 763.

Sidransky, D., von Eschenbach, A., Tsai, Y.C., Jones, P., Summerhayes, I., Marshall, F., Paul, M., Green, P., Hamilton, S.R., Frost, P. and Vogelstein, B. 1991. Identification of p53 gene mutations in bladder cancers and urine samples. *Science* 252: 706 - 709.

Siebert, P.D. and Larrick, J.W. 1993. PCR MIMICS: competitive DNA fragments for use as internal standards in quantitative PCR. *Biotechniques* 14: 244 - 249.

Sklar, J. 1993. Principles of molecular cell biology of cancer: molecular approaches to cancer diagnosis. In: De Vita, V.T., Hellman, S., and Rosenberg, S.A., (eds.) *Cancer: Principles and practice of Oncology* (4th edition) Philadelphia. J.B. Lippencott. 92 - 113.

Sklar, J. and Weiss, L.M. 1988. Applications of antigen receptor gene rearrangements to the diagnosis and characterisation of lymphoid neoplasms. *Annu. Rev. Med.* 39: 315 - 334.

Sklar, J., Weiss, L.M. and Cleary, M.L. 1987. Diagnostic molecular biology of non-Hodgkin's lymphomas. In: Bernard C.W. and Dorfman R.F. (eds.) *Malignant Lymphoma* Baltimore. Williams and Wilkins. 204 - 224.

Smith, B., Selby, P., Southgate, J., Pittman, K., Bradley, C. and Blair, G.E. 1991. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 338: 1227 - 1229.

South Australian Cancer Registry 1994. Epidemiology of cancer in South Australia 1977 - 1993. *South Australian Cancer Registry.* Adelaide.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98: 503 - 517.

Springer, T.A. 1990. Adhesion receptors of the immune system. *Nature* 65: 13 - 24.

Stahel, R.A., Mabry, M. Skarin, A.T., Speck, J. and Bernal, S.D. 1985. Detection of bone marrow metastases in small cell lung cancer by monoclonal antibody. *J.Clin Oncol.* 3: 455 - 461.

Stamenkovic, I., Amiot, M., Pesando, J.M. and Seed, B. 1989. A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. *Cell* 56: 1057 - 1062.

Stamenkovic, I., Aruffo, A., Amiot, M., and Seed, B. 1991. the hematopoietic and epithelial forms of CD44 are distinct polypeptides with different adhesion potentials for hyaluronate-bearing cells. *EMBO J.* 10: 343 - 348.

Stasiak, P.C. and Lane, E.B. 1987. Sequence of cDNA coding for human keratin 19. *Nucl. Acids Res.* 15: 10058.

Steinberg, W., Gelfand, R., Anderson, K. *et al* 1986. Comparison of the sensitivity and specificity of the CA 19-9 and the CEA assays in detecting cancer of the pancreas. *Gastroenterology* 90: 343 - 349.

Steinert, P.M., Idler, W.W., Cabra, I.F., Gottesman, M.M. and Goldman, R.D. 1981. In vitro assembly of homopolymer and coploymer filaments from intermediate filament subunits of muscle and fibroblastic cells. *Proc. Natl. Acad. Sci. USA* 78: 3692 - 3696.

Steinert, P.M. and Roop, D.R. 1988. Molecular and cellular biology of intermediate filaments. *Ann. Rev. Biochem.* 57: 593 - 625.

Steinert, P.M., Steven, A.C. and Roop, D.R. 1985. The molecular biology of intermediate filaments. *Cell* 42: 411 - 419.

Stevenson, T.D. and von Haam, E. 1965. Cancer cell detection in the peripheral blood II. Comparison of techniques using seeded specimens. *Acta Cytol.* 9: 111 - 115.

Sun, T-T., Tseng, S.C.G., Huang, A.J.W., Cooper, D., Schermer, A., Lynch, M., Weiss R. and Eichner, R. 1985. Monoclonal antibody studies of mammalian epithelial keratins: a review. *Ann. New York Acad. Sci.* 307 - 329.

Sugarbaker, E.V. 1979a. Some characteristics of metastasis in man. *Amer. J. Pathol* 97: 623 - 632.

Sugarbaker, E.V. 1979b. Cancer metastasis: a product of tumour host interactions. *Curr. Prob. Cancer* 3: 1 - 59.

Sugarbaker, E.V., Weingrad, D.N. and Roseman, J.M. 1982. Observations on cancer metastases. In : Liotta, L.A. and Hart, I.R. (eds.) *Tumour invasion and metastasis.* The Hague. Martinus Nijhoff 427 - 465.

Takiechi, M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* 251: 1451 - 1455.

Tandon, A.K., Clark, G.M., Chamness, G.C., Chirgwin, J.M. and McGuire, W.L. 1990. Cathepsin D and prognosis in breast cancer. *N. Engl. J. Med.* 322: 297 - 302.

Tarin, D. and Matsumura, Y. 1993. Deranged activity of the CD44 gene and other loci as biomarkers for progression to metastatic malignancy. *J. Cell. Biochem.* 17 (suppl.): 173 - 185.

Tarin, D. and Price, J.E. 1981. Influence of microenvironment and vascular anatomy on "metastatic" colonisation potential of mammary tumours. *Cancer Res.* 41: 3604 - 3609.

Tarin, D., Price, J.E., Kettlewell, M.G., Souter, R.G., Vass, A.C. and Crossley, B. 1984. Mechanisms of human tumour metastases studied in patients with peritoneo-venous shunts. *Cancer Res.* 44: 3584 - 3592.

Taylor, G.R. 1991. Polymerase chain reaction: basic principles and automation. In: McPherson, M.J., Quirk, P. and Taylor, G.R. (eds.)*PCR: a practical approach* Oxford. Oxford University Press. 1 - 14.

Telen, M.J., Shehata, H. and Haynes, B.F. 1986. Human medullary thymocyte p80 antigen and In (Lu)-related p80 antigen reside on the same protein. *Hum. Immunol.* 17: 311 - 324.

Tillman, D.M., Aitchinson, T., Watt, D.C. and Mackie R.M. 1991. Stage II melanoma in the West of Scotland, 1976 - 1985: prognostic factors for survival. *Eur. J. Cancer* 27: 870 - 876.

Thomas, P.S. 1980. Hybridisation of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA*77: 5201 - 5205.

Thomson, T., Mattes, M.J., Roux, L., Old, L.J. and Lloyd, K. 1985. Pigmentation-associated glycoprotein of human melanomas and melanocytes. definition with a mouse monoclonal antibody. *J. Invest. Dermatol.* 85: 169 - 174.

Thor, A.D., Moore, D.H., Edgerton, S.M. Kawasaki, E.S., Reihsaus, E., Lynch, H.T., Marcus, J.N., Schwartz, L., Chen, L.C., Mayall, B.H. and Smith, H.S. 1992. Accumulation of p53 tumour suppressor gene protein: an independent marker of progression in breast cancer. *J. Natl. Cancer Inst.* 84: 845 - 855.

Tobal, K., Shermon, L.S., Foss, A.J. and Lightman, S.L. 1993. Detection of melanocytes from uveal melanoma in peripheral blood using the polymerase chain reaction. *Invest. Ophthalmol. Vis. Sci.* 34: 2622 - 2625.

Todd, J.H., Dowle, C., Williams, M.R., Elston, E.I.O., Hinton, C.P., Blamey, R.W. and Haybittle, J.L. 1987. Confirmation of a prognostic index in primary breast cancer *Br. J. Cancer* 56: 489 - 492.

Tomita, Y., Takeda, A., Okinaga, S., Tagami, H. and Shibahara, S. 1989. Human oculocutaneous albinism caused by single base insertion in the tyrosinase gene. *Biochem. Biophys. Res. Commun* 164: 990 - 996.

Trask, D.K., Band, V., Zajchowski, D.A., Yaswen, P., Suh, T. and Sager, R. 1990. Keratins as markers that distinguish normal and tumour-derived mammary epithelial cells. *Proc. Natl. Acad. Sci. USA*87: 2319 - 2323.

Traweek, S.Y., Liu, J. and Battiford, H. 1993. Keratin gene expression in non-epithelial tissues. *Amer. J. Pathol.* 142: 1111 - 1118.

Trejdosiewicz, L.K., Southgate, J., Kemshead, J.T. and Hodges, G. 1986. Phenotypic analysis of cultured melanoma cells. *Exp. Cell Res.* 164: 388 - 398.

Trent, J.M., Rosenfeld, S.B. and Meyskens, F.L. 1983. Chromosome 6q involvement in human malignant melanoma. *Cancer Genet. Cytogenet.* 9: 177 - 180.

Trent, J.M., Thomson, F.H. and Meyskins, F.L. 1989. Identification of a recurring translocation site involving chromosome 6 in human malignant melanoma. *Cancer Res.* 49: 420 - 423.

Urbach, F. 1983. Sunlight and melanoma. *J. Dermatol. Surg. Oncol.* 9. 679 - 680.

Valagussa, P., Bonnadonna, G. and Veronesi, J. 1978. Patterns of relapse and survival following radical mastectomy. *Cancer* 41: 1170 - 1178.

Vanin, E.F. 1985. Processed pseudogenes: characteristics and evolution. *Annu. Rev. Biochem.* 19: 253 - 272.

Van den Eynde, B., Hainat, P., Herin, M., Knuth, A., Lemoine, C., Weynants, P., van der Bruggen, P., Fauchet, R. and Boon, T. 1989. Presence on a human melanoma of multiple antigens recognised by autologous CTL. *Int. J. Cancer* 44: 634 - 640

van Muijan, G.N.P., Ruiter, D.J., and Warnaar, S.O. 1987. Coexpression of intermediate filament polypeptides in human fetal and adult tissues. *Lab. Invest.* 57: 359 - 369.

Veronesi, U., Adamus, J. Aubert, C., Bajetta, E., Beretta, G., Bonnadonna, G., Bufalino, R., Cascinelli, N., Cocconi, G., Durand, J., De Marillac, J., Ikonopisov, R.L., Kiss, B., Lejeune, F., MacKie, R., Madej, G., Mulder, H., Mechl, Z., Milton, G.W., Morabito, A., Peter, H. Priario, J., Paul, E., Rumke, P., Sertoli, R. and Tomin, R. 1982. A randomised trial of adjuvant chemotherapy and immunotherapy in cutaneous melanoma. *N. Engl. J. Med.* 307: 913 - 916.

Vlemincx, K., Vakaet, L., Mareel, H., Fiers, W. and van Roy, F. 1991 Genetic manipulation of E-Cadherin expression by epithelial tumour cells reveals an invasion suppression role. *Cell* 66: 107 -119.

Vormwald-Dogen, V., Nickel., P., Willemse, M., Thome, M. and Tilgen, W. 1994. Prevalence of melanoma cells in peripheral blood of patients with malignant melanoma stage i-iv - detection by polymerase chain reaction. *J. Invest. Dermatol.* 103: 405 (abstr.).

Watne, A.L. Sandberg, A.A. and Moore, G.E. 1960. The vascular dissemination of cancer. *Quart. Rev. Surg. Obstet. Gynaecol.* 17: 203 - 218.

Weiner, A.M., Deininger, P.F. and Efstratiadis, A. 1986. Non-viral retroposons: genes, pseudogenes and transposable elements generated by the reverse flow of genetic information. *Annu. Rev. Biochem.* 55: 631 - 661.

Weiss, L. 1986. Metastatic inefficiency: causes and consequences.*Cancer Rev.* 3: 1 - 24.

Weiss, L. 1992. Comments on hematogenous metastatic patterns in humans as revealed by autopsy. *Clin. Exp. Metastasis* 10:191-199.

Weiss, L. 1993. Analysis of the incidence of intraocular metastasis.*Br. J. Ophthalmol.* 77:.149.-.151.

Weiss, R. 1991. Hot prospect for new gene amplifier.*Science* 254:.1292 - 1293.

West, J.T., Malmgren, R.A. and Chu, E.W. 1964. An unsuccessful attempt to demonstrate diurnal patterns of tumour cell embolisation. *Surg. Gynaecol. Obstet.* 119: 83 - 91.

Wewer, V. Liotta, L.A., Jaye, M., Ricca, G.A., Drohan, W.N., Claysmith, A.P., Rao, C.N., Wirth, P., Coligan, J.E., Albrechtsen, R., Mudryj, M. and Sobel, M.E. 1986. Altered levels of laminin receptor mRNA in various human carcinoma cells that have different abilities to bind laminin.*Proc. Natl. Acad. Sci. USA*83: 7137 - 7141.

Wielenga, V.J., Heider, K.H., Offerhaus, G.J., Adolf, G.R., van den Berg, F.M., Ponta, H., Herrlich, P. and Pals, S.T. 1993. Expression of CD44 variant proteins in human colorectal cancer is related to tumour progression.*Cancer Res.* 53: 4754 - 4756.

Winstanley, J., Cooke, T., Murray, G.D., Platt-Higgins, A., George, W.D., Holt, S., Mysov, M., Spedding, A., Barraclough, B.R. and Rudland, P.S. 1991. The long term prognostic significance of c-erbB-2 in primary breast cancer. *Br. J. Cancer* 63: 447 - 450.

Wolfel, T., Van Pel, A., Brichard, V., Schneider, J., Seliger, B., Meyer zun Buschenfelde, K.H. and Boon, T. 1994. Two tyrosinase nonapeptides recognised on HLA-A2 melanomas by autologous cytolytic T lymphocytes.*Eur. J. Immunol.* 24: 759 - 764.

Wolmark, N., Rockette, H., Fisher, B., Wickerham, D.L., Redmond, C., Fisher, E.R., Jones, J., Mamounas, E.P., One, L., Petrelli, N.J., Spurr, C.L., Dimitrov, N., Romond, E.H., Sutherland, C.M., Kardinal, C.G., DeFusco, P.A. and Jochimsen, P. 1993. The benefit of leucovorin -modulated fluorouracil as post operative adjuvant therapy in primary colon cancer. results from National Surgical Adjuvant Breast and Bowel Project protocol C-03. *J. Clin. Oncol.* 11: 1879 - 1887.

Wright, C., Angus, B., Nicholson, S., Sainsbury, R.C., Cairns, J., Gullick, W.J., Kelly, P., Harris, A.L. and Horne, C.H.W. 1989. Expression of c-erbB-2 oncoprotein. A prognostic indicator in human breast cancer. *Cancer Res.* 49: 2087 - 2090.

Wu, Y-J. and Rheinwald, J.G. 1981. Keratin filament protein made by some cultured human squamous cell carcinomas. *Cell* 25: 627 -635.

Yakota, J. ,Tsunetsugu-Yokota, Y., Battifora, H., Le Fevre, C. and Cline, M.J. 1986. Alterations of myc, myb and ras proto-oncogenes in cancers are frequent and show clinical correlation. *Science* 231: 261 - 267.

Yamaguchi, M., Dieffenbach, C.W., Connolly, R., Cruess, D.F., Baur, W. and Sharefkin, J.B. 1992. Effect of different laboratory techniques for guanidinium-phenol-chloroform RNA extraction A260/A280 and on accuracy of mRNA quantitation by reverse transcriptase-PCR. *PCR Methods Applic.* 1: 286 - 290.

York, R.M. and Foltz, A.T. 1988. Bleomycin, vincristine, lomustine and DTIC chemotherapy in advanced malignant melanoma. *Cancer* 55: 2183 - 2188.

Yoshida, T., Sakamoto, H. and Terada, M. 1993. Amplified genes in cancer in upper digestive tract. *Semin. Cancer Biol.* 4:33 - 40.

Zeidman, I. 1965. The fate of circulating tumour cells *Acta Cytol.* 9: 136 - 140.

Zucman, J., Delattre, O., Desmaze, C., Plougastel, B., Joubert, I., Melot, T., Peter, M., Dejong, P., Rouleau, G. and Aurias, A. 1992. Cloning and characterisation of the Ewing's sarcoma and peripheral neuroepithelioma t(11 22) translocation breakpoints. *Genes Chromosomes Cancer* 5: 271 - 277.

## APPENDIX A.

# COMMONLY USED ABBREVIATIONS IN THE TEXT

| | |
|---|---|
| AMPPD | 3-(2'-spiroadamantane)-4-methoxy-4-(3'phosphoryl)-phenyl-1,2-dioxetane |
| dATP | Deoxy-adenine triphosphate |
| dCTP | Deoxy-cytosine triphosphate |
| DEPC | Diethylpyrocarbonate |
| dGTP | Deoxy-guanine triphosphate |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| DNAse | Deoxyribonuclease |
| dNTP | Deoxynucleotide triphosphate |
| dTTP | Deoxy-thymidine triphosphate |
| EDTA | Ethylene-diamine-tetra-acetate |
| FCS | Fetal calf serum |
| FITC | Fluorescein isothiocyanate |
| HPLC | High performance liquid chromatography |
| KCl | Potassium chloride |
| mg. | Milligram |
| $MgCl_2$ | Magnesium chloride |
| ml. | Millilitre |
| MOPS | 3-(N-morpholino)-propanesulphonic acid |
| NaAc | Sodium acetate |
| NaCl | Sodium chloride |
| $^{32}P$ | Radioactive phosphorous |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| RNAse | Ribonuclease |
| RNAsin | Ribonuclease inhibitor |
| RPMI | Roswell Park Memorial Institute |
| RT | Reverse transcription / reverse transcriptase |
| SDS | Sodium dodecyl sulphate |
| SSC | Saline sodium citrate buffer |
| ssDNA | Salmon sperm deoxyribonucleic acid |
| TAE | Tris. acetate EDTA buffer |
| TBE | Tris. borate EDTA buffer |
| TE | Tris. EDTA buffer |
| Temed | N,N,N',N-tetramethylethylenediamine |
| Tris. | Tris (hydroxymethyl) aminomethane |
| Tween 20 | Polyoxyethylene sorbitan monolaurate |
| ug. | Microgram |
| ul. | Microlitre |
| UV | Ultraviolet |

## APPENDIX B.

**Papers arising from this thesis, that have already been published or presented at national meetings or that have been prepared or submitted for publication.**

Burchill, S.A., Bradbury, M.F., **Pittman, K**, Southgate, J., Smith, B. and Selby, P. 1995. Detection of epithelial cancer cells in peripheral blood by reverse-transcriptase-polymerase chain reaction. *Br. J. Cancer* 71: 287 - 291.

**Pittman, K**, Southgate, J., Plumb, M., Trejdosiewicz, L. and Selby, P. 1993. Reverse transcriptase and polymerase chain reaction utilising cytokeratin 8 to detect metastatic carcinoma cells in peripheral blood. *Br. J. Cancer* 67 (suppl.): 18 (abstr.).

**Pittman, K**, Burchill, S., Smith, B., Southgate, J., Joffe, J., Gore, M. and Selby, P. Reverse transcriptase-polymerase chain reaction for expression of tyrosinase to identify malignant melanoma cells in peripheral blood. (submitted).

Smith, B., Selby, P., Southgate, J., **Pittman, K**, Bradley, C. and Blair, G.E. 1991. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 338: 1227 - 1229.

B-CODE

# ERRATUM

| page | paragraph | line | |
|---|---|---|---|
| vi | | 11 | chemiluminescent |
| xvii | | 3 | luminescent |
| 2 | 2 | 16 | "... manipulation are based ..." |
| 7 | 2 | 19 | warranted |
| 8 | 1 | 5 | warranted |
| 10 | 2 | 9 | burden |
| 15 | 2 | 9 | tendency |
| 18 | 1 | 1 | "... The management of metastatic disease is paramount to the management of cancer ..." |
| 21 | 3 | 11 | "... Chronic myeloid leukaemia (CML) :- ..." |
| 23 | 4 | 17 | appear |
| 28 | 1 | 8 | "... theory that tumour cells ..." |
| 28 | 1 | 9 | "... examining at several ..." |
| 28 | 3 | 22 | "...potentially lose tumour cells and distort normal blood cells..." |
| 29 | 2 | 9 | "... criteria tend to decrease ... also eliminate the ..." |
| 29 | 3 | 12 | "... prognosis upon detection ..." |
| 30 | 2 | 11/12 | "... 28 % of breast cancer patients ..." |
| 31 | 2 | 7 | "... achieved with a combination ..." |
| 37 | 2 | 7 | eluent |
| 48 | 2 | 6 | temperature |
| 49 | 1 | 5 | vacuum |
| 49 | 3 | 17 | was |
| 50 | 2 | 9 | DMSO |
| 59 | 2 | 18 | "... the *c-myb* proto-oncogene ..." |
| 60 | 3 | 11 | "... sequences predispose to ..." |
| 62 | 3 | 15 | expressing |
| 63 | 4 | 21 | its |
| 79 | 2 | 9 | comparative |
| 90 | 3 | 15 | "... technique have been ..." |
| 104 | | 1 | fluorescent |
| 104 | | 4 | fluorescent |
| 140 | | 8 | and |
| 145 | 2 | 11 | immunofluorescence |
| 145 | 2 | 12 | autofluorescence |
| 149 | 2 | 12 | its |
| 149 | 3 | 21 | consistent |
| 151 | 1 | 6 | intermittent |
| 152 | 3 | 18 | "... levels given the long ..." |
| 153 | 2 | 21 | not |
| 159 | 2 | 4 | "... a CD44 ..." |
| 165 | | 3 | the |
| 167 | | 8 | prominent |
| 167 | | 9 | prominent |
| 168 | | 4 | prominent |
| 174 | 1 | 17 | "... treatment, so that..." |
| 175 | 2 | 10 | PSA |
| 177 | 2 | 10 | "... now are studies |
| 182 | 1 | 3 | "... techniques is yet ..." |
| 184 | 1 | 1 | "... inferred from these ..." |
| 186 | 2 | 5 | its |
| 187 | 2 | 13 | "... have been readily ..." |