The demonstration of Estrogen Receptors in various tumours: A study using immunohistochemistry and in situ hybridisation

Anthony F Henwood

Master of Science Student
Faculty of Science
THE UNIVERSITY OF ADELAIDE.

Laboratory Manager and Senior Science Officer,
Histopathology Department,
The Children’s Hospital, Westmead
New South Wales

Formerly: Principal Science Officer,
Histopathology Department,
Repatriation General Hospital,
DAW PARK, SA.
# CONTENTS

Abstract ................................................................. 4  
Declaration ................................................................ 6  
Acknowledgements .................................................... 7  

Section I: Background ................................................ 8  
  Introduction .................................................................. 8  
  The Histological assessment of Estrogen Receptors .......... 10  
  Studies of estrogen receptor in non-breast tumours .......... 14  
  Estrogen receptors in Melanoma ..................................... 16  
  Heat Induced Antigen Retrieval (HIAR) ......................... 19  
  In Situ Hybridisation Methodology: ............................... 22  
  Choice of probe .......................................................... 25  
  Labelling Systems for ISH ............................................ 27  
  Hybridisation Conditions ............................................. 31  
  Tissue Fixation ........................................................... 35  
  In situ hybridisation pre-treatments ................................ 38  
  Quality Control ........................................................... 40  

Section II: Materials and Methods ................................ 45  
  Probes .......................................................................... 45  
  Immunohistochemical Demonstration Procedure ............ 46  
  Series 1: Optimum Conditions for ISH on Frozen Sections ........................................... 47  
    1.3. Biotinylated probe demonstration: .................................. 50  
    1.4. The effect of formamide on working probe solution shelf life: ............................. 50  
    1.5 Optimum temperature of hybridisation: ................................................. 51  
    1.6 Post hybridisation stringency washes: ....................................................... 51  
    1.7. Study on the expression of estrogen receptor mRNA and total mRNA in frozen sections of breast and lung carcinoma......................................................... 52  
  Series 2: ISH development for formalin fixed paraffin embedded sections ........... 52  
    2.1. Development of Paraffin Section ISH ................................................. 53  
    2.2. Influence of endogenous biotin on specificity of ISH. .................................. 54  
    2.3. Expression of poly A (total mRNA) in formalin fixed, paraffin embedded sections of various tissues. ................................................................. 54  
    2.4. Study into the expression of Estrogen Receptor mRNA in formalin fixed paraffin embedded sections of breast carcinoma ........................................ 55  
    2.5. Study into the expression of Estrogen Receptor mRNA in formalin fixed paraffin embedded sections of melanoma .............................................. 55  
  Series 3: Development of immunohistochemistry .................. 56  
    3.1. H222 staining on frozen sections of breast and lung carcinoma: .................... 56  
    3.2. Estrogen Receptor staining on formalin fixed, paraffin embedded sections of breast carcinoma and melanoma: .......................................... 57  

Section III: Results ......................................................... 59  
  Optimum conditions for ISH on frozen sections .......... 59  
  Fixation Effect ............................................................ 59  
  Comparison between demonstration techniques.................. 62  
  Effect of Formamide on ISH ............................................ 65  
  Hybridisation temperatures and Stringency washes .............. 65
Results of optimum ISH staining for Estrogen Receptor and H222 staining on frozen sections of breast and lung carcinoma: ............................................................... 66
Results of ISH on Formalin Fixed Paraffin Sections: ........................................ 68
  Influence of endogenous biotin ...................................................................... 71
  Distribution of Poly A in various tissues .......................................................... 73
Presence of Estrogen Receptor mRNA in paraffin sections of breast carcinoma. 74
Presence of Estrogen Receptor mRNA in paraffin sections of melanoma .......... 74
Results of ER1D5 and D5 protein immunohistochemistry in paraffin sections of
Breast carcinoma and correlation with Estrogen Receptor mRNA-ISH ............ 77
Results of ER1D5 and D5 immunohistochemistry on paraffin sections of melanoma and correlation with Estrogen Receptor mRNA-ISH .............................. 78
Section IV – Discussion..................................................................................... 80
Conclusions...................................................................................................... 90
References: .................................................................................................. 91
Abstract

In order to study the incidence of Estrogen Receptors (ER) in breast carcinoma, lung carcinoma and melanoma, an in situ hybridisation technique for ER mRNA (ER mRNA-ISH) was developed. Various technical aspects of the procedure including tissue fixation, hybridisation conditions, and demonstration technique were investigated in order to obtain an optimum technique for routine use. ISH results were compared with ER immunohistochemistry using the monoclonal antibodies ER1D5 and D5.

Commercially available biotin labelled antisense oligonucleotides to ER, Poly A (total mRNA), and sense chromogranin (negative control) were applied to frozen and formalin-fixed paraffin sections of breast carcinomas. For frozen sections, various fixatives including formalin, alcohol, Schoobridge, Zamboni’s and acetic-alcohol were compared. A direct streptavidin-peroxidase and an indirect demonstration method using anti-biotin were also compared. The effect of differing formamide concentrations and post hybridisation stringency washings were analysed. An optimised ISH technique was then applied to frozen sections of 21 cases of breast carcinoma and 11 cases of lung carcinoma. Results were compared to H222 staining on adjacent sections.

The ISH technique was also optimised for use on formalin-fixed, paraffin-embedded sections of 28 breast carcinomas and 17 melanomas. The results were compared with ER1D5 and D5 immunohistochemistry done on adjacent sections. The occurrence of endogenous biotin was also studied on a range of normal tissues.

Consistent ISH results were obtained when formamide was omitted from the hybridisation cocktail, high stringency post hybridisation washes were discarded, room temperature hybridisations and an indirect demonstration method were used. Fixation of frozen sections in acetic/ethanol gave more consistent results with good morphology and resulted in positive nucleolar staining in 90% of breast and 45% of lung carcinomas. Positive nucleolar staining was also present in frozen sections of one metastatic melanoma.
In formalin fixed paraffin sections, acid hydrolysis and pronase treatment were required prior to ISH. Cytoplasmic and/or nucleolar ER mRNA-ISH staining was seen in 87% of breast carcinoma and 97% of melanoma studied. ER1D5 was present in 54% of breast carcinomas but was absent in all melanomas. D5, on the other hand, was found in 88% of the melanomas.

In conclusion, ER mRNA-ISH can be successfully done on acetic/alcohol fixed frozen sections and formalin fixed paraffin sections. Formamide, high stringency washes and elevated hybridisation temperatures are detrimental to a successful ISH reaction and an indirect demonstration method (using anti-biotin) is preferred. Unfortunately, endogenous biotin can cause false positive ISH reactions and needs to be considered during interpretation.

Results show that the localisation of ER mRNA in the nucleolus is specific. Both ER mRNA-ISH and ER immunohistochemistry indicate that melanomas and some lung carcinomas contain a receptor possibly similar to that in breast carcinomas.
Declaration

I declare that:

1. This thesis does not contain any material that has been accepted for the award of any other degree or diploma in any University or other tertiary institutions.
2. 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.
3. I consent to the thesis being made available for photocopying and loan if applicable if accepted for the award of the degree.

Anthony F Henwood JP, BAppSc, Grad Dip Sys Analys, CT(ASC).
7 October 2004
Acknowledgements

I would like to acknowledge the support and guidance of Dr R Barbour (Adelaide University), Professor A Leong (Hunter Area Pathology Service), Professor APB Ng (Retired) and Dr J Kumaratilake (Adelaide University). I also recognise the support of the management and staff of the following laboratories: The Histopathology Department at the Repatriation General Hospital Daw Park, South Australia and the Anatomical Pathology Department at the Royal Prince Alfred Hospital, Sydney.