
# *Analyses Of Candidate Tumour Suppressor Genes Mapping To The 16q24.3 Breast Cancer Loss Of Heterozygosity Region.*

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***Summary***

Breast cancer is the second largest cause of cancer related deaths and the most common cancer occurring in women of the western world. Loss of heterozygosity (LOH) of chromosome 16q24.3 is a common genetic alteration observed in invasive ductal and lobular breast carcinomas. Chromosomal regions of frequent LOH are believed to represent the location of candidate tumour suppressor genes. Refined LOH analysis by collaborating laboratories was successful in narrowing down the candidate region to approximately 3 Mb, demarked by the 16q telomere and the marker *D16S498*.

The main aim of this thesis was to isolate the gene(s) responsible for breast cancer using a positional cloning strategy. At the commencement of this study a cosmid contig of this LOH region had already been constructed and stretched 800 kb from the 16q telomere to the gene *CDH15*. This physical map was extended another 1.8 Mb to totally encompass the 16q24.3 LOH interval. The resulting physical map consisted of 63 PAC and 102 BAC clones, with an average of 4.5X coverage. The individual restriction fragments from each clone were ordered and orientated, enabling precise clone overlaps to be established. From this detailed physical map, a minimal tiling path of 26 clones was selected and used as template DNA for large scale sequencing analyses. Each clone was shotgun sequenced, assembled and finished. The sequence data generated was used to anchor the sequence available from the first draft of the human genome. Detailed *in silico* analysis of the genomic sequence data enabled the identification of 116 predicted genes. Several Unigene EST clusters were linked together into single genes and numerous singleton ESTs were rejected as database contaminants. These *in silico* predicted genes were tested for their transcription status by RT-PCR. 104 of the 116 predicted genes amplified from pooled tissue RNA and therefore represent real genes.

Seven of theses genes have been previously screened for mutation in breast tumour DNA: SPG7, BBC1, CPNE7, CDK10, FANCA, GAS11, and C16ORF3. None of these genes were found to harbor any mutations in sporadic breast cancer DNA samples. It was impractical to use this labor-intensive procedure to screen all the remaining candidates and furthermore, tumour DNA stocks were limited.

To identify a possible involvement of any 16q24.3 genes in breast cancer, quantitative mRNA expression analyses of all the corresponding transcripts were performed. It was hypothesized that the tumour suppressor genes associated with the 16q24.3 LOH may be aberrantly expresses due to the presence of mutations or promoter hypermethylation. Using the expression value of each gene in normal breast tissue as the baseline, the expression variability of each gene was examined in a panel of human breast cancer cell lines. Two known tumour suppressor genes, *SYK* and *CDKN2A*, exhibited high variability across the panel of breast cancer cell lines and consequently, validated the screen. Of the 104 genes studied, 3 exhibited considerable expression variability across the panel of breast cancer cell lines, *CBFA2T3, CYBA* and Hs.7970. The notion of these genes being breast cancer tumour suppressors is compatible with what is known about their respective function.

Of the remaining transcripts, several still presented as good candidates based on additional functional evidence. For example, microcell mediated chromosome transfer experiments identified a 16q24 YAC clone capable of inducing cellular senescence in breast cancer cell lines (Reddy *et al*, 2000). The YAC 792E1 was mapped to the 16q24.3 LOH interval and shown to contain two genes, Hs.118944 and Hs.7970. The genomic structure and expression profile of these uncharacterized Unigene EST clusters was established. These candidates were subsequently screened for mutation in breast tumour DNA and finally, the strongest candidate, Hs.7970, was subjected to a cell-based assay for assessment of potential tumour suppressor function. Neither gene exhibited any disease specific mutation and furthermore, Hs.7970 failed to suppress the formation of colonies in soft agar and on plastic, a hallmark of known tumour suppressors. These results suggest that these genes are not involved in breast cancer.

Additional genes presented as candidates based on their homologies to other known human or mouse proteins. For example, TSG16 exhibited significant homology to the ankyrin (ANK) repeat motif of the BRCA1-associated RING domain protein 1 (BARD1). As ankyrin repeat domains function as sites for protein-protein interactions, it was hypothesized that TSG16 shares common protein partners with BRCA1 and therefore may itself function in breast carcinogenesis. *In silico* analysis identified

matching ESTs with reported down regulation in nasopharyngeal carcinoma (NPC). Interestingly, NPC also exhibits 16q LOH further supporting a possible tumour suppressor function for TSG16. Comprehensive RT-PCR and expression analysis identified TSG16 as a 9307 bp transcript, expressed in all tissues examined. The TSG16 ORF of 7995 nucleotides encodes a 2,664 amino acid protein with a predicted molecular weight of 298 kDa. Mutational analysis in sporadic breast tumour DNA identified one tumour specific missense mutation. The biological significance of the mutations awaits functional complementation. Furthermore, the screening of non-BRCA1 and BRCA2 familial breast cancer patients uncovered a nucleotide change present in the blood of three affected individuals from the same family. However, DNA analysis of one tumour revealed the wild type pattern and therefore it is unlikely that this nucleotide change is related to the risk of breast cancer. Subsequent experiments examined this large novel gene in detail.

The ANK repeat domain of TSG16 was used as bait to screen a human breast cDNA library and this domain identified several members of the PIAS (potent inhibitors of STAT transcription) protein family. PIAS proteins perform an increasing repertoire of functions including the inhibition of the STAT signaling. TSG16, the ANK repeat domain, PIAS1 and PIAS3 were all amplified and cloned into mammalian expression vectors for transient transfection and subsequent immunoprecipitation experiments. All exogenously expressed proteins were stability expressed in 293T cells, but failed to immunoprecipitate with each other. A STAT3 transcriptional reporter assay was performed to access the effect of TSG16 on PIAS mediated inhibition of STAT transcription. PIAS3 inhibited LIF stimulated STAT3 transcription, confirming earlier reports. The affect of TSG16 could not be assessed as this gene was found to activate the *Renilla* luciferase reporter construct, originally included for transfection efficiency normalization. It is possible that TSG16 may act as a generalized transcriptional activator and future experiments should investigate this possibility.

In summary, 104 candidate genes have been identified in the 16q24.3 LOH interval. Quantitative mRNA expression analysis in breast cancer cell lines served as an

initial screen for potential tumour suppressors. Additional genes presented as candidates and subsequent experiments examined these genes in detail. The precise mapping and sequencing of the human genome has greatly assisted positional cloning strategies for candidate disease gene isolation.