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FOREWORD

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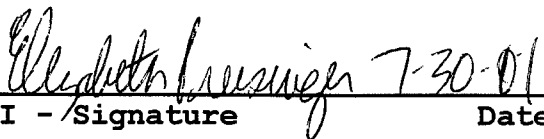

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Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	11
Reportable Outcomes.....	11

Elizabeth Preisinger

A Novel Monoallelically Expressed Candidate Tumor Suppressor Gene for Breast Carcinogenesis on Chromosome 11p15.5

Introduction

Loss of heterozygosity (LOH) at broadly defined chromosomal regions is a hallmark of tumor suppressor gene inactivation in cancer. Chromosome subband 11p15.5 has been consistently identified as a target for LOH in numerous studies in breast cancer. The basis for LOH in this region may be complicated by the superimposition of a cluster of imprinted genes. I have identified an unusual monoallelically expressed, nuclear-retained transcript from this region which morphologically resembles the Xist transcript on RNA FISH. This transcript is a candidate for the tumor suppressor locus on 11p15.5 and may offer insight into the mechanisms of differential allelic expression in imprinted chromosomal loci.

Strong evidence supports the view that a tumor suppressor locus for breast cancer is located in chromosome 11 in band 11p15.5 [1,2]. Loss of heterozygosity is also observed in a number of other tumor types, notably rhabdomyosarcoma [3,4], adrenocortical carcinoma [5], hepatocellular carcinoma [6], and Wilm's tumor [7]. A second line of evidence suggesting that 11p15.5 may play a key role in tumorigenesis is the association of a hereditary overgrowth condition, Beckwith-Weidemann syndrome (BWS) to this chromosome band. Features of BWS include organomegaly and increased predisposition to a number of embryonal tumors which include Wilms' tumor and rhabdomyosarcoma [8]. Genetic linkage demonstrates that familial BWS maps to chromosome 11p15.5.[9,10]. Sporadic BWS can be associated with chromosomal rearrangements or paternal isodisomy for this chromosomal region [11] These observations suggest that BWS may involve inactivation in this region by genomic imprinting [12].

Physical mapping and cloning, high throughput sequencing, gene identification and thorough mutational analysis in tumors has been carried out on 11p15.5, yet the basis for LOH here remains obscure. These difficulties may be explained, in part, by an extra level of complexity due to the superimposition in this region of a cluster of imprinted genes. Genomic imprinting refers to the phenomenon of differential allelic expression patterns, depending on the maternal or paternal derivation of the allele [13,14] Expression patterns of imprinted genes are established early in embryogenesis and may persist in a clonally heritable manner. Because imprinted genes occur in clusters, they are thought to be regulated in part by higher order chromatin structure in a domain-wide fashion. Imprinting is one type of epigenetic mechanism which depends on heritable transcriptional silencing. The phenomenon of X inactivation in female mammals is another paradigm for stably heritable transcriptional silencing. Transcriptional silencing on the inactive X chromosome is mediated by Xist RNA, which accumulates at the site of its transcription from the inactive X [15]. Xist RNA accumulates massively in the interphase nucleus where it appears to coat the inactive chromosome [16]. In this manner the silencing effect mediated by Xist is exerted through the entire length of the X chromosome.

I hypothesized that an Xist-like RNA might play a part in local and regional gene regulation at the imprinted domain on chromosome 11p. I carried out a screen by RNA in situ hybridization using, as a model for LOH on 11p, an embryonal rhabdomyosarcoma (ERMS) cell line. Probes from a 1MB contig of overlapping genomic clones for which genomic sequence is available were used [17]. This contig represents a putative tumor suppressor locus for breast cancer and embryonal malignancies as well as the breakpoint cluster region for chromosomal rearrangements in BWS [18] and includes the imprinted genes H19, IGF-2, p57kip2, KvLQT, and ASCL-1. Using a single P1 clone representing sequence from an intronic region between exons 10 and 11 of the imprinted gene KvLQT, I identified a novel nuclear transcript which is strikingly reminiscent of the RNA in situ signal seen with Xist (Figure 1). This RNA accumulates in the interphase nucleus at its site of transcription, is prominent in the vast majority of cells and appears to be expressed from a single allele. Like Xist, the RNA signal colocalizes with the DNA signal in two color FISH experiments (Figure 1), and thus appears to accumulate in the interphase nucleus at the presumed site of transcription in the same highly distinctive

manner as Xist RNA. It is interesting to note in light of these findings that non-coding and antisense RNAs are increasingly being identified as being transcribed from the differentially methylated CpG islands of imprinted genes [13]. To show that this strong, monoallelic RNA FISH signal is not an artefact of cell culture or an idiosyncratic feature of a single tumor cell line, I used mouse BAC clones, which correspond to the same human KvLQT intron [19], as probes in *in situ* hybridizations against disaggregated 14day mouse embryo tissue. I saw similar nuclear-retained monoallelic RNA FISH signals in all cells in all tissue types examined (skin, brain, liver, lung, whole embryo) in the normal mouse (data not shown).

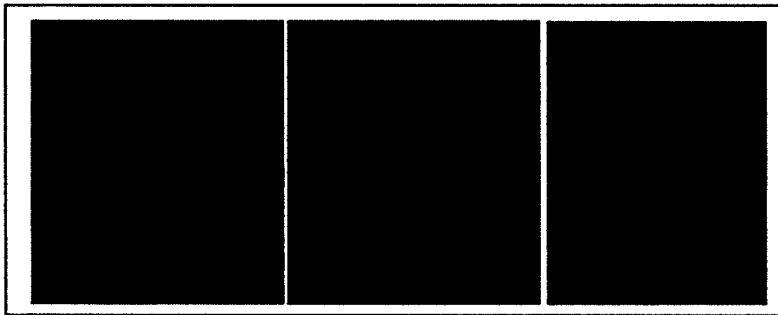


Figure 1. Left: RNA FISH signal (green) from intronic transcript within single DAPI-stained nucleus (blue) in A204 cells. Middle: RNA FISH signal from Xist transcript in A204 cells. Right: RNA FISH

followed by DNA FISH showing accumulation of the intronic transcript at the presumed site of transcription from a single chromosome. Green: RNA FISH signal. Red: DNA FISH signals.

As outlined in Task 1 of the Statement of Work in my proposal, I sought to further define the genomic extent from which this RNA is being transcribed, using subcloned and PCR-generated fragments as double-stranded FISH probes (Figure 2). We showed by *in situ* hybridization that the transcript is bounded by a CpG-rich region which is differentially maternally methylated in human and mouse [20] [21], and extends at least 40 kb toward the telomere in the human. RT-PCR experiments using sequence-specific-primed, reverse transcribed RNA from the A204 line showed that transcriptional orientation is antisense to that of the KvLQT gene from whose intron the RNA is being transcribed (Figure 3). Sequence analysis of the genomic region using computer algorithms (Genscan) to predict exon/intron boundaries yielded no open reading frame, predicted exon or canonical splice site in the entire 40 kb of genomic sequence telomeric to the CpG-rich region, indicating that this RNA may well be untranslated, again reminiscent of Xist RNA.

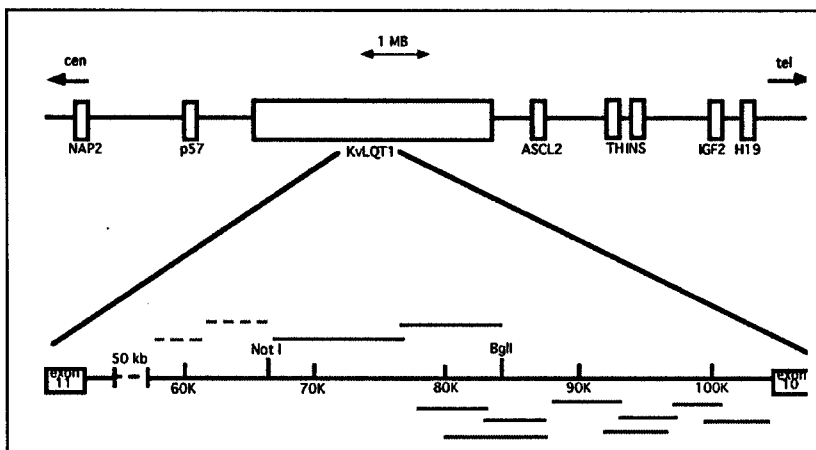


Figure 2. Schematic representation of the putative WT-2 locus on human 11p15.5. Top: position of selected genes within 1 MB contig of overlapping P1 clones [17]. Bottom: detail of

100 kb intronic region from which Xist-like nuclear transcript expressed. Red: Subcloned genomic fragments giving positive nuclear RNA FISH signal. Dashed red: Subcloned fragments giving negative FISH signal. Green and blue: PCR-based probes giving positive FISH signal. Not I: Differentially methylated Not I site within CpG island.

Task 2 consisted of creating a cDNA library from A204 RNA and screening with probes which had given positive FISH signals. Sequencing such cDNA clones and comparing them to genomic sequence should give evidence of intron/exon boundaries and splicing patterns, if 11p-xInt is indeed spliced. In order to identify possible intron/exon boundaries within this transcript which may not have been predicted by computer algorithms, I constructed a directionally cloned cDNA library from the A204 line in the LambdaZap phagemid vector (Stratagene) using both oligo-dT and random primers for first strand cDNA synthesis. Screening of this cDNA library using PCR-generated probes, representing a total of 20 kb of intronic sequence previously shown to give a strong RNA FISH signal in the A204 cell line, yielded only a small number of cDNA clones of limited length (2kb total), which confirmed a transcriptional orientation antisense to that of KvLQT. I found that up to 90% of the total 20kb sequence used to screen the library corresponded to probes which generate a strong nuclear signal in *in situ* hybridization experiments but which do not appear to be represented as cDNA. This result may indicate that standard RNA extraction procedures (guanidinium thiocyanate) may not be adequate to isolate certain portions of this unusual nuclear-retained transcript, perhaps due to sequence-specific RNA interactions with binding proteins or components of the nuclear matrix. Because 11p-xInt appears to be an unusually large RNA, and because full length cDNA of up to 60 kb could not be expected to be recovered from library screening, plans for ectopic expression of cDNA constructs as outlined in Task 3 were deferred.

Since 11p-xInt appears to be accumulating at its site of transcription and is monoallelically expressed in the A204 cell line by RNA FISH, I sought to determine whether monoallelic transcription is occurring from the paternal allele or the maternal allele. Because no parental genotypes are available for this established cell line, and because expressed polymorphisms had not been identified for this transcript, I chose to approach this question using a two-color RNA FISH approach. For this experiment I used H19, an untranslated RNA under relatively tight imprinting control which is normally expressed only from the maternal chromosome. I assayed for H19 expression in these cells by RNA FISH as a marker for the maternal chromosome in the interphase nucleus, which is possible due to its location about 500kb telomeric to our transcript in question and its demonstrated monoallelic expression by RT-PCR-RFLP in the A204 line (Figure 3). The results of two color RNA FISH for H19 and for 11p-xInt showed that the transcript appears to be transcribed from the maternal chromosome in about 50% of cells (9 of 20) and from the paternal chromosome in about 50% of cells (11 of 20) (Figure 3). To confirm this surprising result, we identified an expressed single nucleotide polymorphism within the transcript by sequence analysis. The presence of this polymorphism may be assayed by BglI digestion of PCR and RT-PCR products. Results of PCR and RT-PCR experiments in cell populations from the A204 cell line showed that expression is indeed occurring from both the paternal and the maternal chromosomes in

the cell population, although expression is monoallelic as assayed by RNA FISH in single cells.

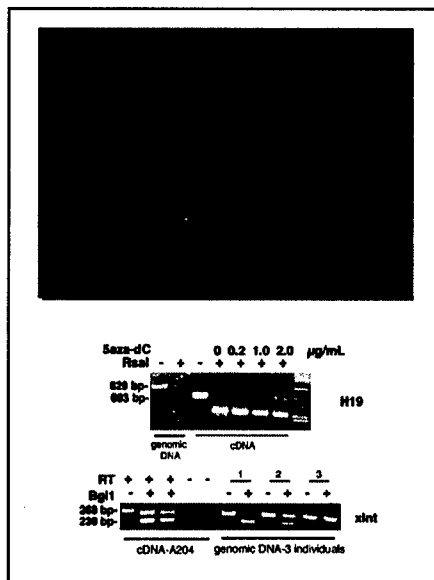


Figure 3. Top: Two color RNA FISH for the intronic transcript (green) and the maternally expressed H19 gene (red), showing random monoallelic expression of the intronic transcript in A204 cells. Middle: Rt-PCR for H19 expression followed by Rsa I digestion showing monoallelic expression of H19 in all untreated A204 cells and reactivation of the silent imprinted allele in cells treated with increasing concentrations of the methyltransferase inhibitor 5aza-dC. Bottom: Allelic expression analysis of the intronic transcript in A204 cells using a single nucleotide polymorphism detectable by Bgl I digestion. Results show expression from both maternal and paternal alleles within the population of A204 cells.

As a further confirmation of the unusual random monoallelic expression pattern of 11p-xlnt in this cell line, I developed a single-cell RT-PCR assay. For this assay, I sorted A204 cells into 96 well plates using single cell sorting by flow. I subjected these cells to reverse transcription followed by 105 cycles of a nested PCR reaction, and assayed the PCR product for the presence or absence of the Bgl I polymorphism. Results (not shown)

XLNT is biallelically expressed in A204 clones



support the random monoallelic expression pattern of 11p-xlnt in these cells.

In the process of X-inactivation, Xist expression is random with respect to maternal or paternal chromosome, and this choice is made early in development. Subsequently, the choice of chromosome for inactivation and Xist expression is fixed and clonally heritable through mitosis by the progeny of each cell. To determine whether or not the random monoallelic expression of 11p-xlnt is similarly fixed and heritable through mitosis, I plated A204 cells in 96 well plates at limiting dilution and allowed clones to expand from a single cell to about 100 cells (10 days). I then assayed by RT-PCR. Results, shown above in Figure 4, indicate that the random monoallelic expression is not in fact clonally heritable in the A204 cell line, but rather both maternal and paternal alleles are expressed in colonies grown for ten days from a single cell.

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Elizabeth Preisinger

A Novel Monoallelically Expressed Candidate Tumor Suppressor Gene for Breast Carcinogenesis on Chromosome 11p15.5

Key Research Accomplishments

- Determined size and extent of novel transcript spanning 40 kb
- Created and screened cDNA library to map intron/exon boundaries of transcript
- Observed unusual random monoallelic expression pattern of transcript in tumor cell line
- Developed single cell RT-PCR assays to confirm random monoallelic expression
- Observed unusual unstable monoallelic expression which is not clonally heritable

Reportable Outcomes

- Manuscript in preparation