Published in final edited form as:

Trends Genet. 2010 June ; 26(6): 260–265. doi:10.1016/j.tig.2010.03.004.

X-linked Tumor Suppressors: Perplexing Inheritance, a unique Therapeutic Opportunity

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Abstract

Unlike autosomal genes, the majority of X-linked genes are subject to dosage compensation. As a result, female tissues are comprised of cells exclusively expressing X-linked genes from one or the other parent. The implication of having only one allele of active X-linked genes in cancer pathogenesis, i.e. somatic single-hit inactivation and dominant inheritance has not been explored extensively. Recent studies identified *FOXP3* and *WTX* as two X-linked tumor suppressor genes that are somatically inactivated by single genetic hits. Because the predicted dominant inheritance of cancer risk has not been demonstrated in human, we discuss possible conditions that might prevent such dominant inheritance. We also argue that the existence of a genetically intact allele in cancer cells in women, together with apparent abnormal X-inactivation in cancer cells, might provide an opportunity to selectively reactivate tumor suppressor genes for cancer therapy.

Unique properties of X-linked tumor suppressor genes

Unlike autosomal genes, expression levels of X-linked genes are dosage-compensated to achieve two goals: to equalize the gene dosage between males and females through X-inactivation¹ (Box 1) and to achieve grossly similar levels of transcripts between X-linked and autosomal genes through up-regulation of X-linked genes². As a result of X-inactivation, female tissues are chimeras of operatively hemizygous cells with active X chromosomes originating from either parent. Here, we will discuss two implications of this feature in the context of tumor suppressor genes. First, whereas autosomal tumor suppressor genes require two genetic hits to be inactivated³, X-linked tumor suppressors should be inactivated by a single genetic hit. One would therefore expect single-hit somatic inactivation and dominant inheritance of X-linked tumor suppressor genes. Although the single-hit inactivation has been validated, no data from studies in human have confirmed the dominant inheritance. Second, because one allele of X-linked genes has not undergone selection during carcinogenesis, it might be feasible to reactivate the X-linked tumor suppressor genes for cancer therapy.

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X-linked Cancer Risk

The first hint that X-linked genes might be involved in cancer emerged more than five decades ago ⁴ in a study aimed at understanding possible genetic contributions to prostate cancer-associated death. Death certificates indicated that patients who died from prostate cancer were more likely to have a brother than a father who died of the same disease ⁴. More recently, two analyses ^{5,6} indicated that those with an affected brother have almost twice as high risk of prostate cancer than those who have an affected father. A similar risk pattern was also reported in bladder cancer ⁷. Strikingly, the risk that brothers of a man with testicular germ-cell tumors also develop cancer is 10 times as high as that of those who do not have affected first-degree relatives. Moreover, the risks of man with affected brothers are approximately twice as high as those with either an affected father or son ^{8,9}. The extent of autosomal genes sharing between father and son should be similar to that of brothers. In contrast, X-linked genes can be shared among brothers, but are not shared between father and son. Therefore, this pattern of inheritance points to a potential role for X-linked genes in cancer risk.

These epidemiological studies have been supported by several independent genetic analyses. For prostate cancer, the strongest evidence emerged from a study of 360 pedigrees consisting of samples collected from the US (Johns Hopkins Medical Center and the Mayo Clinic), Finland and Sweden ¹⁰. Pooled data from these linkage analyses suggested that the risk is linked to the microsatellite marker DXS1113 in Xq27 while independent linkage analyses ¹¹⁻¹⁴ also supported a susceptibility locus in the Xq27 region, called hereditary prostate cancer, X-linked (*HPCX*). Further mapping using linkage disequilibrium by two independent groups allowed the identification of haplotypes within a small region of less than 200 kb ^{15,16}. However, sequencing the coding region of the genes within the region has failed to identify *HPCX* ¹⁶.

Recent advances in high density genome-wide association studies allowed the analysis of a large sample cohort of prostate cancer. Two reports indicated strong associations of two single nucleotide polymorphisms (SNPs) on the X chromosome ^{17,18}. One overlaps with the Xq27 locus identified by previous linkage analyses, whereas the other implicated a locus distinct from the putative *HPCX* in Xq27.

An X-linked locus was also suggested from a comprehensive study on testicular germ-cell tumors, utilizing samples from men with affected siblings, but unaffected fathers ¹⁹. Based on allele sharing among two families with recombination, the authors placed the putative locus called testicular germ-cell tumor gene 1 (*TGCT1*) at 175.2 cM in Xq27; the identity of *TGCT1*, however, remains elusive. The difficulties associated with the identification of these X-linked genes can potentially be attributed to either relatively weak effect of the genes involved or to the possibility that the putative loci might not encode proteins. The latter possibility deserves more attention, as the importance of non-coding RNA in cancer is now well established ²⁰.

Somatic Single-hit Inactivation of X-linked Tumor Suppressor Genes

Most X-linked genes are hemizygous and are postulated to be susceptible to single-hit genetic inactivation ²¹. As such, the analysis of somatic mutations in tumor samples could reveal X-linked tumor suppressor genes. Because operational hemizygosity is found in cells from both males and females, the somatic inactivation should be observed in patients of both sexes. Recently, clear evidence for an X-linked tumor suppressor gene emerged in prostate, breast and Wilms tumor.

FOXP3

Forkhead box P3 (FOXP3) was identified because its mutations cause X-linked lethal autoimmune disease in both mouse and human ²²⁻²⁵. A subsequent study indicated that it is subject to X-inactivation ²⁶. Two lines of evidence obtained in mice indicate that *Foxp3* might be a potential tumor suppressor gene ²⁷. First, female mice harboring a heterozygous dinucleotide insertion in *Foxp3* (*Foxp3^{sf/+}*) have high incidences of spontaneous mammary cancer and are more susceptible to chemically induced tumor development in the mammary gland. Second, exclusive expression of the mutant allele in the tumor cells and co-expression of both wild type (WT) and mutant alleles in normal epithelial cells indicate that inactivation of the WT allele might be necessary for tumor development.

The significance of *FOXP3* as X-linked tumor suppressor gene in humans is supported by the widespread prevalence of somatic mutations (36%), gene deletion (13%) and in the majority of the breast cancer samples, lack of nuclear FOXP3 ²⁷. Interestingly, an overwhelming majority of the mutations are heterozygous and the deletion of the gene is heterozygous in all cases. These data reaffirm the notion that a single hit is sufficient to inactivate X-linked genes.

As in breast cancer, somatic mutation, gene deletion or a lack of nuclear FOXP3 are observed in prostate cancer samples. Consistent with the tumor suppressor function of FOXP3, prostate specific deletion of *Foxp3* in mice cause both hyperplasia and prostate intraepithelial neoplasia (PIN) ²⁸. A significant portion of human tumor samples with *FOXP3* deletion have multiple X chromosomes; Interestingly, in these samples the *FOXP3* deletion is observed on all X chromosomes ²⁸. *FOXP3* deletion therefore likely occurs prior to X chromosome duplication.

FOXP3 is a transcription factor and normally resides in the nucleus, but the majority of FOXP3 mutant proteins are located in the cytoplasm ²⁸. Disruption of nuclear localization could represent the predominant mechanism of FOXP3 inactivation in cancer. This hypothesis might help to reconcile reported differences in FOXP3 expression in breast cancer samples by immunohistochemistry. One study ²⁹ that did not discriminate cytoplasmic from nuclear forms reported a 2-fold higher rate of FOXP3 expression in tumor samples than another study which only considered nuclear FOXP3 ²⁷. Furthermore, the study that made no distinction between cytoplasmic and nuclear FOXP3 concluded that FOXP3 expression in tumors was associated with a worse overall survival ²⁹. If the cytoplasmic FOXP3 correlates with poor prognosis, it might have a dominant oncogenic function.

As a transcription factor, FOXP3 directly represses several oncogenes, including *ERBB2*, *SKP2* in mammary epithelial cells and *cMYC* in prostate epithelial cells ^{27,28,30}. The functional relevance of the repression was demonstrated in large cohorts of human samples and in mice harboring mutations in *FOXP3* or *Foxp3*, respectively. Moreover, ectopic expression of the target genes substantially bypasses FOXP3-mediated tumor growth inhibition ^{27,28,30}. Therefore, repression of oncogene expression is a major mechanism by which FOXP3 acts as a tumor suppressor. At the same time, FOXP3 can also induce the expression of multiple tumor suppressor genes, including *p21CIP1* and *p18INK4C*. The induction of *p21CIP1* depends on locus-specific alteration of the chromatin structure ³¹. By inducing expression of tumor suppressors and repressing the expression of oncogenes, *FOXP3* functions as a significant tumor suppressor gene and is an example of a X-linked tumor suppressor gene.

WTX

Wilms tumor is the most frequent childhood solid tumor. In a high density copy number analysis, Rivera et al. ³² observed microdeletions in Xq11.1 in 5 out of 26 male Wilms tumor patients tested. The minimal overlapping region identified a previously uncharacterized gene, which is called *WTX* for “Wilms Tumor gene on the X chromosome”. A similar frequency of

heterozygous deletions (6 out of 25) was found in female Wilms tumor samples. Four cases were analyzed to determine whether deletions occurred in active or inactive X chromosomes, based on detection of the X (inactive)-specific transcript (*Xist*). It is clear that only inactive chromosomes retained *WTX*. Furthermore, somatic mutations were also identified at a significant frequency (6 out of 82 samples), all but one of which encoded truncated proteins (the remaining one is a missense mutation). In female samples, all mutations were heterozygous. Sequencing of *WTX* transcripts from a heterozygous sample indicated that only the mutant allele was expressed. These data are consistent with the notion that somatic single hits inactivate *WTX*. The tumor suppressor activity of *WTX* is demonstrated by *WTX*-mediated inhibition of tumor cell growth. *WTX* likely inhibit tumor growth by promoting the ubiquitination and degradation of β -catenin³³, which is critical for tumorigenesis³⁴.

Subsequent studies confirmed mutations and deletion of *WTX* in Wilms tumor,^{35,36}. Surprisingly, Perotti et al. reported that in heterozygous females, the retained X chromosome can have either a methylated or unmethylated CpG island at the 5' of *WTX*³⁵. However, because gene expression from the locus was not assessed, it is unclear whether the methylation status sufficiently indicates X-inactivation in the tumor tissues.

A critical issue is whether germline mutations of *WTX* cause Wilms tumors. In analyzing the genetic basis of X-linked sclerosing bone dysplasia, Jenkins et al. identified deletion and mutations of *WTX* in 19 cases³⁷. Although the mutations were predicted to inactivate the locus, none of the 19 patients developed Wilms tumors, thus failing to verify the tumor suppressor function of *WTX*. This surprising observation is quite interesting when considered in the context of other X-linked genes that affect cancer risk.

X-linked recessive inheritance of cancer risk

The studies on *FOXP3* and *WTX* offer clear evidence for single-hit inactivation of tumor suppressor genes and illustrate the difference between X-linked and autosomal tumor suppressor genes in sporadic cancers. However, it is unclear whether germline mutations of these genes are involved in familial cancers. As a result, it is not yet possible to determine the inheritance pattern of these tumor suppressor genes. Nevertheless, because the X-inactivated genes are hemizygous, one would expect a dominant inheritance.

Surprisingly, inactivating mutations of known X-linked cancer susceptibility genes appear to be recessive. One such example is *FANCB*³⁸, which is responsible for Fanconi anemia complementation group B. *FANCB* encodes an essential component of the nuclear protein “core complex” responsible for monoubiquitination of FANCD2. Because the inheritance is known to be recessive, one might conclude that this locus should be either autosomal or not subject to X-inactivation. Unexpectedly, it was found to be localized on the X chromosome (Xp22.31) and subject to normal X-inactivation. Interestingly, heterozygotes comprise predominantly cells expressing the WT allele. It is possible that the competitive disadvantage of cells expressing the mutant allele protect the heterozygotes against tumorigenesis.

A second example is the X-linked lymphoproliferative syndrome gene (*XLP*) whose mutations cause lymphoproliferative disease and dramatically increases the risk of lymphoma³⁹⁻⁴¹. Because *XLP* defects cause immune deficiencies, the increased cancer risk is likely due to defects in immune surveillance. Because immune response would be intact unless all cells express the mutant alleles, *XLP* mutations would have to be X-link recessive.

Another example is provided by glypican 3 (*GPC3*), whose deletional translocation causes Simpson-Golabi-Behmel over-growth syndrome⁴², including high risk of embryonic tumors such as Wilms tumor and neuroblastoma⁴³. Because the gene encodes an extracellular proteoglycan⁴², the cells expressing the WT allele will be able to produce the proteoglycan

for adjacent cells that do not express the gene. Therefore, the WT allele would be dominant in a tissue comprising two cell types expressing either the WT or the mutant allele.

Therefore, despite their normal X-inactivation, X-linked cancer susceptibility genes can be recessive for different reasons. Among them, *XLP* and *GPC3* are non cell-autonomous tumor suppressor genes, whereas studies of *FANCB* function suggest that it could be a *bona fide* tumor suppressor. Perhaps due to a growth advantage of normal cells expressing WT allele over those expressing the mutant allele, most normal cells from *FANCB* heterozygotes expressed WT allele³⁸. The paucity of mutant-expressing cells might thus reduce the penetrance of the mutation in heterozygous carriers. Because growth competition is not an issue in homozygotes, the impact of mutations can be observed. Of course, if the X-linked genes also happen to be homozygous lethal early in life, as is the case for *FOXP3*, the chance of identifying its involvement in familial cancer would depend on analysis of heterozygotes, which in turn will be significantly affected by growth disadvantage of the cells expressing the mutant allele.

Reactivation of X-linked tumor suppressor for cancer therapy

One of the most difficult challenges in cancer therapy is to restore the function of inactivated tumor suppressors. Deletions and/or mutations identified for known X-linked tumor suppressor genes, including *FOXP3* and *WTX*, are often heterozygous in female cancer cells. It is therefore of interest to consider whether the X-inactivated tumor suppressor gene can be reactivated for cancer therapy. Although the mechanism for the induction of X-inactivation is well understood, how X-inactivation is maintained in normal cells is less clear (Box 1). This presents considerable obstacles for any rational approach to reactivate X-linked tumor suppressor genes. Nevertheless, a serendipitous observation reported by us suggests that this might be possible. In brief, we found that anisomycin, a cellular stress inducer, induced *FOXP3* expression in both mouse and human breast cancer cell lines by activating c-Jun and ATF2 heterodimers. Such induction resulted in increased apoptosis of cancer cells and reduced growth of established mouse mammary tumors⁴⁴. Our observation raises the intriguing possibility of restoring the function of X-linked tumor suppressor function.

Several issues deserve consideration. First, the paramount concern is the potential side effects of reactivating X-linked genes. X-inactivation is critical for the development of female mice as those with a deletion of *Xist* in the paternal X chromosome are growth-retarded and embryonic lethal⁴⁵. The consequence of partial X-reactivation and/or reactivation in mature animals is less clear. Apart from the clusters of genes that normally escape X-inactivation, a global analysis of X-inactivation suggests that an additional 10% of X-linked genes show variable patterns of inactivation and are expressed from some 'inactive' X chromosomes⁴⁶. This finding suggests that variations in X-inactivation of at least 10% of the genes are tolerated in human. Still, because pharmaceutical reactivation of X-linked genes has not yet been attempted, both prudence and an openmind are advisable.

Obviously, any potential side effects would be reduced if one could selectively reactivate X-linked tumor suppressors in cancer. We believe this is possible because many cancers have partially dismantled their machineries for maintenance of X-inactivation, as discussed below. Indeed, Richardson et al.⁴⁷ showed that non-heterochromatinized X chromosomes are primarily observed in basal-like cancers, most of which harbors *BRCA1* mutations and have poor prognosis. This finding is consistent with a previous report which showed that loss of the heterochromatinized X chromosome is associated with poor prognosis⁴⁸. As expected, the markers of inactive X chromosomes, such as H3mK27 and *Xist* are reduced or absent; DNA methylation is lost in many, although not all, genes⁴⁷. Surprisingly, bi-allelic expression of a normally X-inactivated locus was shown only in one case. Moreover, array analysis indicates

that only 3% of the 1200 X chromosome genes are over-expressed⁴⁷. Because most of the over-expressed genes are localized at or near Xp22 and Xq26-28 loci, which are rich in genes that normally escape X-inactivation, the increased expression of these genes might not be due to reactivation of X-linked genes⁴⁷.

Xist, DNA methylation and histone hypoacetylation work in concert to maintain inactivation of X-linked genes (Box 1); therefore, loss of *Xist* and DNA methylation should reduce the steps needed to reactivate X-linked genes. Moreover, because methylation is altered unevenly among different genes⁴⁷, it is conceivable that reactivation might not be equally efficient for all genes. This might allow some selectivity of reactivation and thus further reduce side effects.

It is also important to consider whether reactivating X-linked tumor suppressors would provide a therapeutic effect. Elegant studies demonstrated that cancers can become “addicted” to the loss of the autosomal tumor suppressor gene *Trp53* (which encodes p53)⁴⁹. Although similar studies have not been performed with the two known X-linked tumor suppressors, it is clear that essentially all tumor cell types tested are inhibited by ectopic expression of FOXP3^{27, 28,30,31,50}. The therapeutic effect of inducing FOXP3 expression in both mouse and human tumor cells has also been demonstrated recently using anisomycin⁴⁴. Although *WTX*-mediated growth inhibition has been tested only in a limited number of cell lines³², the pathways targeted by *WTX*³³ and FOXP3, including *Wnt*, *cMyc*, *p21CIP1* and *ERBB2*^{27,28,30,31} strongly suggest that reactivating these genes can be therapeutic for a large spectrum of cancer.

Finally, it is worth considering whether the inactive allele of X-linked tumor suppressors is indeed genetically intact. Because mutations in the inactive allele are not selected against during tumorigenesis, one might be concerned that they drift away into pseudogenes. Although it is challenging to refute this hypothesis, we do not consider accumulation of neutral mutations likely during tumorigenesis. Available data also indicate that mutations and deletions occurred only in the expressed allele³². Moreover, because all but one mutations uncovered in the *WTX* gene are non-sense mutations, they are unlikely neutral³². Additional studies are needed to firmly establish that an inactive WT allele is maintained in the tumor cells that have a genetically disrupted active allele.

Concluding remarks

Recent identification of two X-linked tumor suppressor genes demonstrate clear exceptions to the classic two-hits model of tumor suppressor genes³. However, although high rates of spontaneous mammary tumors in heterozygous mice with germline mutation of *Foxp3* suggest this possibility, dominant X-linked inheritance has not been identified in human. Future studies are needed to resolve this paradox (Box 2). The existence of X-linked tumor suppressors that are subject to X-inactivation suggest a unique opportunity to restore the function of tumor suppressor genes. Regardless of whether the approach is successful, consideration of reactivating X-linked tumor suppressor genes for cancer therapy has already raised many interesting issues that deserve further investigations. A better understanding of the mechanism that maintains X-inactivation in normal and cancer cells should provide more rational approaches.

Box 1

X-inactivation: induction, maintenance and reactivation

X-inactivation is a dynamic process that occurs during embryogenesis. At the two-cell stage, both paternal and maternal X chromosomes are active. The progressive silencing of the imprinted X chromosome begins during early embryogenesis. The inactivation of the imprinted X must be “erased” in the embryo proper prior to random X-inactivation. Random

X-inactivation appears to initiate after implantation, coinciding with the lineage-specific differentiation of the epiblast. This is believed to be maintained in all but germ cells in fetus and adults. Reactivation of X-linked genes has been demonstrated in some cancers⁴⁷, but global reactivation has not been reported.

The initiation of X-inactivation (Figure I) involves pairing of two active X chromosomes, which somehow allows the cells to enumerate the number of X chromosomes in relation to that of autosomes (counting). Here, *Xist* is activated and mark the X chromosome to be inactivated. *Xist* RNA initiates the recruitment of the polycomb repressive complexes, PRC1 and PRC2. This recruitment also coincides with the formation of a silencing compartment to exclude the transcriptional machinery.

Once established, X-inactivation is maintained throughout cellular division in all but germ cells. The mechanism for maintaining X-inactivation is unclear, but seems to involve redundant mechanisms, including *Xist* expression, DNA methylation, PRC1/2 repressive complexes, structural protein maintenance and nuclear compartmentalization of the inactive X chromosome. Removal of any of these mechanisms might be insufficient for reactivation of X-linked genes. However, gross abnormality of these components observed in cancer cells might make the X-inactivated genes more susceptible to reactivation, thus offering a glimpse of hope for selective reactivation of X-linked tumor suppressor genes in cancer.

Box 1 Figure I.

X-inactivation and reactivation in normal and cancer cells. X-inactivation involves counting, choice and execution. Once established, X-inactivation is maintained in normal cells throughout cell divisions. In some cancers, various aspects of X-inactivation maintenance are dysregulated; global reactivation, however, has not yet been reported. The unique state of X-inactivation in cancer makes the selective reactivation of X-linked tumor suppressor genes in cancer plausible. Xa: activated X chromosome, Xi: inactivated X chromosome. The green bar indicates active X chromosome; red bar indicates inactive X

chromosome. The yellow bar indicates the inactive X chromosome that has lost some markers of X-inactivation.

Box 2

Outstanding questions

Because research on X-linked tumor suppressor genes is still at a very early stage, many basic questions remain. In our opinion, two of them are the most interesting.

1. Can X-linked tumor suppressor genes contribute to familial cancer in human? If so, would these genes be dominant or recessive?
2. Is it possible to selectively reactivate X-linked tumor suppressor in cancer but not in normal tissues?

Acknowledgments

We thank our colleagues, Drs. Tao Zuo, Runhua Liu, Yan Liu, Yin Wang, Carl Morrison, Richard Love, Xing Chang, Huiming Zhang, Hiroto Katoh and Kathleen A Cooney for their contribution to the original studies. We thank Dr. Yuan Zhu Ms Molly Liu and Ms Darla Kroft for critical reading, discussion and editorial advice. This work is supported by grants from National Institute of Health and the Department of Defense Breast and Prostate Cancer Research Programs. The authors have no conflict of financial interest.

Glossary

Imprinted X chromosome	The parental X chromosome which is preferentially inactivated prior to random X-inactivation. This is usually the paternal X chromosome.
Operative chimerism	Although genetically homogenous, essentially all tissues and organs in the female consist of cells that express genes from one or the other X chromosome.
Operative hemizygosity	Even though the female has two X chromosomes, only one allele of most X-linked genes is functional in a given cell.
Skewed X-inactivation	Imbalanced expression of the two alleles of X-linked genes.
Wilms tumor	Is the most frequent childhood solid tumor. The malignant cells are found in the kidney, and might spread to the lungs, liver, or nearby lymph nodes. Wilms tumor usually occurs in children younger than 5 years old.
X-inactivation	A dynamic process leading to the inactivation of most genes in one of the X chromosomes in all but germ cells in the female.
X-linked tumor suppressor genes	Tumor suppressor gene in the X chromosome.

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