Deciphering the Role of the Barr Body in Malignancy
An insight into head and neck cancer
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ABSTRACT: X chromosome inactivation is the epitome of epigenetic regulation and long non-coding ribonucleic acid function. The differentiation status of cells has been ascribed to X chromosome activity, with two active X chromosomes generally only observed in undifferentiated or poorly differentiated cells. Recently, several studies have indicated that the reactivation of an inactive X chromosome or X chromosome multiplication correlates with the development of malignancy; however, this concept is still controversial. This review sought to shed light on the role of the X chromosome in cancer development. In particular, there is a need for further exploration of the expression patterns of X-linked genes in cancer cells, especially those in head and neck squamous cell carcinoma (HNSCC), in order to identify different prognostic subpopulations with distinct clinical implications. This article proposes a functional relationship between the loss of the Barr body and the disproportional expression of X-linked genes in HNSCC development.

Keywords: Sex Chromatin; X Chromosome; Lyonization; X-Linked Genes; Cell Differentiation; Cancer; Squamous Cell Carcinoma, Head And Neck.

GENETIC AND EPIGENETIC PROCESSES RESULT in heritable changes in the expression of cancer cells; consequently, the molecular targets of malignancy include critical tumour-associated genes—such as tumour suppressor genes (TSGs) or oncogenes—along with their mutations, amplifications, deletions, loss of heterozygosity or other epigenetic modifications.1 Recently, researchers have confirmed the role of DNA methylation and histone modification of the cytosine-guanine (CpG) site in malignancy as well as the interrelation between nuclear architecture, chromatin packaging, heterochromatin organisation, epigenome and non-coding ribonucleic acid (RNA).2 Many X-linked potential TSGs and oncogenes have been attributed to the distinctive biology of the X chromosome and its specific implications in malignancy.3 The exclusivity of TSGs to the X chromosome can be attributed to their inactivation by a single referred loss of function mutation (i.e. hit); in other words, if a tumour suppressor gene is localised on the X chromosome, one hit is sufficient to induce tumorigenesis because the other allele on the X chromosome is inactivated by epigenetic modification. Moreover, the reactivation of the inactive X chromosome (Xi) could ultimately lead to oncogene overexpression.4

In female somatic cells, an Xi is referred to as the Barr body. In malignant cells, the disappearance of the

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Barr body results in misregulation of the centromere-associated satellite heterochromatin and the peripheral heterochromatic compartment, potentially causing broad epigenetic instability. As such, the Barr body is considered an epigenetic nuclear landmark in cancer development. Recent interest in exploring the loss of the Barr body in different malignancies has been encouraged by the high frequency of this phenomenon in aggressive breast cancers. Nevertheless, the association between Barr body disappearance and genetic loss, epigenetic instability or transcriptional reactivation is still ambiguous.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and has been associated with conventional aetiological factors including tobacco and alcohol consumption. However, the growing incidence of oropharyngeal squamous cell carcinoma in Western countries in the absence of a corresponding rise in smoking and alcohol consumption points towards the involvement of additional behavioural and environmental factors, such as human papilloma virus (HPV) infection and epigenetic instability. Strong evidence exists that altered DNA methylation profiles in HNSCC cases reflect the aberrant epigenetic regulation of TSGs and oncogenes. As such, it is imperative that researchers concentrate on epigenetic pathways because of their reversible nature when seeking new approaches to the molecular diagnosis and targeted treatment of cancer.

While X chromosome perturbations have been reported in breast, uterine, cervical, ovarian, renal and colon cancers, they are rarely documented in HNSCC cases. This article focuses on reviewing variations in Barr body frequency in different malignancies and proposing its hypothetical involvement in HNSCC development. There is a need to further explore the role of sex chromosomes in HNSCC development in order to determine potential clinical implications.

**The Barr Body and X Inactivation**

During early embryonic development in females, the random inactivation of one of the two X chromosomes occurs and is maintained subsequently throughout further cell division. The term Barr body was first used to describe this transcriptionally inert, heterochromatic and late-replicating chromatin mass by Barr et al. in 1949. This inactivation of an X chromosome results in equivalent gene dosage (i.e. XX and XY) between the sexes by the synchronised transcriptional silencing of genes; thus, both sexes have one copy of an active X chromosome (Xa), which is necessary for the embryo to survive.

Critically, X inactivation represents numerous epigenetic mechanisms that result in the formation and maintenance of facultative heterochromatin in mammals. The X-inactive specific transcript (XIST) gene is the linchpin of X inactivation, whereby heterochromatin silencing is mediated via XIST expression and stabilisation of its non-coding RNA transcript. The XIST gene is located in the X inactivation centre and belongs to a class of RNA molecules known as non-coding transcripts. With the exception of 3–15%, 1,500 genes located on the human X chromosome undergo transcriptional silencing due to X inactivation.

**Distinct X Chromosome Perturbations in Malignancy**

The differentiation status of cells is determined by X chromosome activity, whereby undifferentiated or poorly differentiated cells have been ascribed to the presence of two Xa. Variations in Xi frequency have been reported with age, pregnancy, the use of oral contraceptives, fluctuations in menstrual cycle and neoplasia. Moore et al. found that the frequency of the sex chromatin in the nuclei of female hosts was low in malignant tissues, appearing in only about one-third of tumours in comparison to non-malignant tissues; this finding was attributed to the diverse chromosomal abnormalities that occur in malignancy. Straub et al. suggested that there was apparent reversion to the early embryonic state and loss of the Barr body in some female mammalian tumours wherein the condensed X chromosome may become partially or fully extended, altering its genetic activity. Thus, X chromosomes could be considered as existing in a dynamic state rather than a permanent or invariant one. It therefore appears that cancer is linked to an unusual escape from X inactivation. However, the extent of Xi perturbations and disruptions to the epigenetic state in cancer have not yet been systematically explored. Barring head and neck cancers, there is no dearth of literature reporting Xi reactivation in malignant tumours.

Sirchia et al. noted that the lack of X inactivation in breast oncogenesis occurs independently from breast cancer 1 gene status and XIST expression and is due to the loss of the Xi and replication of the Xa, without the reactivation of the native Xi (i.e. the X chromosome predestined to be inactivated from the beginning), which results in the gain of an additional Xa together with the lack of an Xi. According to Kaur et al., Barr body frequency in buccal mucosal cells was significantly lower among menstruating patients with cervical cancer as compared to those who were
cancer-free; however, the findings were non-significant among breast cancer patients. This suggests that low Barr body frequency occurs only in the tissue directly involved with the change. In breast cancer patients, a significantly low incidence of inactive X chromatins has been observed among menstruating as well as menopausal women, indicating that this low incidence is due to Xi reactivation.

Jäger et al. observed that DNA replication stress during oncogenesis led to Xi hypermutations in aberrantly proliferating cells; moreover, mutation rates were comparatively higher in late replicating regions due to the lack of transcription-coupled DNA repair. Furthermore, Vijay Kumar et al. reported a significant association between sex chromatin status and the histopathological grading of breast carcinomas, in which there was a lower frequency of sex chromatins in tumours with a higher microscopic grade. Another study found increased expression of cancer/testis antigens and loss of X inactivation in endometrial carcinoma cases which was attributed to global hypomethylation and a high number of copy number variations (CNVs); this might indicate that other cancers with a high degree of CNV, such as colorectal cancer, non-small-cell lung cancer and HNSCC could also present with loss of X inactivation. Moreover, Kobayashi et al. reported that XIST expression could be used to predict the survival rate and prognosis of patients with cervical squamous cell carcinoma.

Overall chromatin state is determined by DNA and histone modifications which maintain whether genes are transcriptionally active or inactive. The structure and function of chromatins and subsequent X inactivation can potentially become disrupted by environmental, toxicological and/or disease conditions; for example, recent research has indicated that XIST function may be severely affected by defects in heterochromatin stability and epigenetic modifications. The misexpression of XIST may potentially be a mechanism underlying oncogenesis and low XIST levels may reduce X inactivation with continuous X reactivation. However, the molecular cascade that alters X inactivation and X chromosome copy numbers in both female and male cancer cells remains undetermined. Several plausible explanations for Xi reactivation are proposed in Figure 1. Weakley et al. described three patterns of Xi loss in that certain cells lose Xi without Xa, others lose Xi and undergo Xa multiplication and a few undergo Xi reactivation. Significant epigenetic changes could also be caused by viral oncoproteins, which potentially lead to abnormal cellular growth, transformation and, in some cases, oncogenesis. Thus, virus-mediated transformation could be another explanation which has yet to be completely understood.

**X-Linked Genes in Head and Neck Squamous Cell Carcinoma**

The role of various X-linked genes in different cancers has been previously documented. Consistent genetic abnormalities have been found to be associated with the development and/or progression of HNSCC in various karyotyping and molecular analyses. Martin et al. previously published a thorough description of specific genetic changes involving autosomes in oral squamous cell carcinoma cases, which included the loss of chromosomal segments 3p, 5q, 7q, 8p, 9p, 11q and 18q in addition to the gain of 3q, 5p, 7p, 8q and 11q.
Table 1: X-linked genes involved in the development of head and neck squamous cell carcinoma and other malignancies

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Function</th>
<th>Locus</th>
<th>Role in HNSCC and other malignancies</th>
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</table>
| FHL1         | TSG           | Regulates muscle development, structural maintenance and signalling       | Xq26   | • FHL1 mRNA and protein expression are frequently decreased in HNSCC cases, with FHL1 modulating HNSCC proliferation via the downregulated expression of cyclin D1, cyclin E1 and p27.41  
|              |               |                                                                          |        | • FHL1 silencing notably enhances the proliferation of HNSCC cells, whereas forced FHL1 expression dramatically represses HNSCC cell growth.41  
|              |               |                                                                          |        | • The DNA hypermethylation of FHL1 has been detected in certain types of cancer.41                      |
| BEX genes    | TSGs          | Potential regulators of the cell cycle and apoptotic signalling           | Xq22   | • BEX4 controls OSCC proliferation and growth.46  
| (including BEX1, BEX2, BEX3, BEX4 and BEX5) |               |                                                                          |        | • Reduced BEX4 expression occurs early on in OSCC development.45  
|              |               |                                                                          |        | • BEX genes are epigenetically silenced in OSCC cases.45  
|              |               |                                                                          |        | • BEX1 and BEX3 are involved in modulating the NF-kB signalling pathway and have been implicated in cell death and the cell cycle.44 |
| FOXP3        | HNSCC oncogene| Involved in immune system responses and the development and function of regulatory T cells | Xp11.23| • The FOXP3 gene modulates the expression of various other genes implicated in cancer development (i.e. TSGs and oncogenes).45  
|              |               |                                                                          |        | • Immune evasion via FOXP3 expression in tumour cells may represent the main mechanism of cancer progression.45  
|              |               |                                                                          |        | • High FOXP3 expression in tumours has been found to be significantly associated with poor prognosis in OSCC cases (e.g. decreased survival and lymph node metastasis).45 |
| ATRX         | Chromatin regulator gene | Involved in transcriptional regulation and chromatin remodelling | Xq21.1 | • ATRX is one of the most frequently mutated chromatin factors in cancers.45  
|              |               |                                                                          |        | • ATRX mutations promote telomere lengthening, increased genomic instability and cellular proliferation.45  
|              |               |                                                                          |        | • ATRX loss-of-function mutations have been associated with cancers that exhibit ALT phenotypes, including oesophageal SCC.47  
|              |               |                                                                          |        | • ATRX mutations have also been associated with abnormal DNA methylation patterns.45 |
| MECP2        | Oncogene      | Acts as a transcriptional activator, likely by binding to another epigenetic DNA modifier, and induces the MAPK and PI3K growth factor signalling pathways | Xq28   | • MECP2 amplification/overexpression has been linked to cancer.46 |
| DDX3X*       | TSG           | Implicated in cell cycle regulation, cell differentiation, cell survival and apoptosis | Xp11.4 | • DDX3X expression has been evaluated in breast, lung, colon, oral and liver cancers and a positive correlation has been recently reported between high DDX3X levels and poor prognosis in human tumours.46  
|              |               |                                                                          |        | • DDX3X inhibits apoptosis by reducing caspase 3 activation.46  
|              |               |                                                                          |        | • An inverse relation between cytoplasmic DDX3X expression and survival rate has been found in smokers with OSCC.46  
|              |               |                                                                          |        | • Missense DDX3X mutations have been reported in HNSCC and HPV patients.46 |
| MAGE genes   | Oncogenes     | Encode certain tumour-associated antigens recognised by cytotoxic T lymphocytes | Xq26–28Xp21 | • Several MAGEA subgroups contribute to malignancy.  
| 50, 51, 52   |               |                                                                          |        | • One study found that 71% of HNSCC cases expressed at least one of six different MAGE genes.48  
| and 53       |               |                                                                          |        | • MAGE1 and MAGE4 were the most frequently expressed genes in poorly differentiated SCC cases.48  
|              |               |                                                                          |        | • The transcription of MAGE genes may be linked to a transformation event; various viruses (such as HPV and EBV) have easy access to the head and neck region, which might influence cell transformation.51  
|              |               |                                                                          |        | • In HNSCC cases, MAGEA2 expression is regulated by promoter demethylation, which interacts with the p53 pathway by increasing cellular proliferation and decreasing cell cycle arrest.51  
|              |               |                                                                          |        | • As a result of promoter demethylation, MAGEB2 overexpression was reported almost exclusively in tumours, with growth-promoting effects.51 |
| ARAF1        | Proto-oncogene | Potentially involved in cell growth and development                        | Xp11.3 | • ARAF1 may be involved in malignancy as a component gene of the MAPK pathway.46 |
FANCB TSG Involved in DNA repair Xp22.2

- Patients with Fanconi’s anaemia have been reported to have an increased susceptibility to early-onset HNSCC.35
- FANCB hypermethylation has been observed sporadically in HNSCC tumours.35

COLA4A6 and COLA4A5 genes Collagen genes Involved in synthesising COLA, an important protective component against invasion and metastasis Xq22

- In carcinogenesis, COLA is gradually fragmented, collapsed or even dissolved completely, thus providing channels for cancer cells to invade the lamina propria.29
- As they become less differentiated, SCC cells were found to lose their ability to form basement membrane components.29

ELK1† Transcription activator gene Involved in determining the cellular response to extracellular signals and controlling the expression of genes involved in cell cycle progression, differentiation and apoptosis³ Xp11.23

- ELK1 proteins are a nuclear target for the Ras-Raf-MAPK signalling cascade which is important for the control of growth signals, differentiation and cell survival.27
- ELK1 is involved in the hypoxic induction of HIF2α-dependent genes, which can facilitate tumour cell survival by making them more resistant to therapeutic intervention.28

G6PD Oncogene Encodes the G6PD enzyme which produces NADPH and pentoses involved in reductive biosynthetic activity Xq28

- Increased G6PD activity has been found in cancer cells.29
- G6PD inhibition has been reported to decrease cancer cell survival and NADPH levels and increase ROS production.24
- Some researchers consider high G6PD activity to be an independent negative prognostic marker in cancer.99
- In breast cancer patients, G6PD overexpression is considered a predictor of high risk of recurrent metastasis.41
- G6PD becomes hyperactive in tumours with p53 inactivation, such as HNSCC.98
- G6PD activity ensures a steady supply of pentoses and stabilisation of the NADPH equilibrium which is an essential prerequisite for uncontrolled cell growth and proliferation, particularly for tumour cells.84

LDOC1 TSG Able to induce apoptosis in various kinds of human cancer cells Xq27

- LDOC1 downregulation due to epigenetic silencing by promoter hypermethylation has been observed in oral, cervical and ovarian cancers.85

SSX genes§ Oncogenes Expression of these genes is restricted to malignant tumours Xp11.1–11.2

- Expression of at least one SSX subfamily member was most frequently observed in head and neck cancer (75%), followed by ovarian cancer (50%), malignant melanomas (43%), lymphomas (36%), colorectal cancer (27%) and breast cancer (23%).52

XIAP Oncogene Encodes a protein that belongs to a family of apoptotic suppressor proteins/caspase inhibitors Xq25

- The elevated expression of potent apoptotic inhibitor XIAP is a significant biomarker for HNSCC, with high XIAP expression predicting poor prognosis.41
- XIAP overexpression in tumour cells has been shown to inhibit cell death induced by a variety of apoptotic stimuli and induce resistance to chemotherapy.51
- The XIAP gene was found to be hypomethylated in oral tumours.62

KDM6A and KDM6B genes TSGs Act as the only enzymes displaying histone di- and tri-demethylase activity and are required for the reactivation of epigenetically silenced genes Xp11.3

- HPV type 16 E7 expression has been reported to cause KDM6A and KDM6B upregulation, resulting in epigenetic reprogramming as evidenced by the aberrant expression of homeobox genes which are frequently dysregulated during carcinogenesis.77,86
- These genes have been found to be mutated in >10% of HNSCC cell lines, although not in human HNSCC tumours.85

APEX2 and TREX2 genes DNA repair genes DNA repair DNA repair Xp11.21

- APEX2 and TREX2 genes are hypomethylated in cancer tissues.4
- Screening suggests DNA repair genes, which are located on the X chromosome, have a propensity for aberrant methylation.4

FHL1 = four-and-a-half LIM domains; TSG = tumour suppressor gene; mRNA = messenger ribonucleic acid; HNSCC = head and neck and squamous cell carcinoma; BEX = brain-expressed X-linked; OSCC = oral squamous cell carcinoma; NF-xB = nuclear factor kappa B; FOXP3 = forkhead box P3; ATRX = α-thalassemia mental retardation syndrome, X-linked; AL T = alternative lengthening of telomeres; SCC = squamous cell carcinoma; MECP2 = methyl cytosine guanine dinucleotide-binding protein 2; MAPK = mitogen-activated protein kinase; PERK = phosphorosostide 3-kinase; DDX3X = DEAD-box helicase 3, X-linked; HPV = human papillomavirus; FANCB = Fanconi’s anaemia complementation group B; COLA = collagen type IV; ELK1 = E26 transformation-specific domain-containing protein E1K-1; HIF2α = hypoxia-inducible factor 2α; G6PD = glucose-6-phosphate dehydrogenase; NADPH = nicotinamide adenine dinucleotide phosphate; ROS = reactive oxygen species; LDOC1 = lactuin zipper downregulated in cancer I; SSX = synovial sarcoma, X chromosome-related; XIAP = X-linked inhibitor of apoptosis; KDM6 = lysine-specific demethylase 6; APEX2 = apurinic/apyrimidinic endodeoxyribonuclease 2; TREX2 = three prime repair exonuclease 2.

†DDX3X is a highly conserved subfamily of DEAD-box proteins, the largest group of RNA helicases.3 Type I MAGE genes in the MageA, MageB and MageC subfamilies are clustered on chromosome X. Type II MAGE genes in the MageD, MageE, MageF, MageG, MageH, MageI and MageL and nucleotide protein subfamilies are clustered on chromosome X as well as a few autosomes.3 A member of the E2F transformations-specific oncogene family.3 NF-xB regulates vascular endothelial growth factor expression through ELK1 and activator protein 1 transcription factors.3 SSX genes comprise six members of the recently described cancer/testis antigen class.
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Table 2: Hypothesised mechanisms leading to X reactivation in the development of head and neck squamous cell carcinoma

<table>
<thead>
<tr>
<th>Mechanism</th>
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<tr>
<td>Loss of Xi via deletion</td>
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<tr>
<td>Chromosomal segregation errors</td>
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<tr>
<td>Reactivation of Xi through epigenetic changes (i.e. hypomethylation or heterochromatin instability)</td>
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<tr>
<td>HPV oncoproteins influencing XIST expression</td>
</tr>
<tr>
<td>DNA replicating stress in proliferating malignant cells</td>
</tr>
<tr>
<td>Translocations involving regions of the X chromosome to autosomes and vice versa</td>
</tr>
</tbody>
</table>

HPV = human papilloma virus; Xi = inactive X chromosome; XIST = X-inactive specific transcript.

segments. However, a sex link was dubious; the loss of the short arm of the Xi was a common observation in females and Y loss was observed in about 50% of males.

In HNSCC cases, Xi reactivation can potentially be considered a marker of heterochromatin instability associated with poor prognosis as, much like cervical cancer, the disease may be associated with epigenetic modifications as well as oncoviruses that could alter the X-linked genes. Thus, the destabilised genomic repertoire in HNSCC appears to be further undermined by epigenetic events. However, before considering an association between the Barr body and HNSCC, a causal relationship between X-linked TSGs and HNSCC development must be established. A summary of the X-linked genes involved in HNSCC development and their various loci, functions and mechanisms can be found in Table 2. The involvement of X-linked genes in HNSCC, which bears similarities to the molecular pathogenesis of cervical carcinomas and other epithelial malignancies, indicate that there is a potential association between altered Barr body frequency and HNSCC development. Probable contributors leading to Xi reactivation in HNSCC cases are documented in Table 2. However, these hypothetical conclusions can only be confirmed or negated by experimental research.

Major disruptions in the DNA methylation profiles of malignant cells—including the hypermethylation of gene promoters, global hypomethylation and increased mutation rates at methylated CpG dinucleotides—have been observed in both HPV-positive and -negative patients with HNSCC. Additionally, Fang et al. found that individual genes and gene expression programmes are regulated by various long non-coding RNAs by either implicating epigenetic control or altering basal transcriptional machinery. According to Goedert et al., long non-coding RNAs induced by viral oncoproteins play critical roles in

tumour initiation and progression. As previously mentioned, increased XIST expression—which contains a long non-coding RNA transcript—has been found to predict a favourable prognosis in cases of cervical squamous cell carcinoma. Since the transcriptional capacity of host cell chromatins can be regulated by HPV E6 and E7 oncoproteins, further research is needed to fully comprehend HPV-induced modulation of long non-coding RNAs.

Clinical Implications

Xi reactivation is an emerging topic of interest with potential clinically relevant applications which may pave the way for further understanding of chromatin changes and other drivers of tumour development. X-linked genes can serve as potential targets for the genetic and epigenetic alterations observed in malignant cells. Therefore, considering heterochromatin defects and the involvement of epigenetic processes in switching on or off transcriptional cell machinery in malignancy, attempts have been made to delineate specific drug targets. Epimutations could potentially be reversed via chemical agents known as epirugs, such as DNA methyltransferase or histone deacetylase inhibitors which help to re-establish the expression of tumour suppressors that have been suppressed by hypermethylation or repressive chromatin marks. The upregulation of oncogenes and cancer/testis antigens located on the X chromosome are induced by the loss of X inactivation, which leads to increased tumour aggressiveness; this could therefore be a susceptible target for immunotherapy. Other options for reactivating X-linked TSGs in cancer therapy also deserve further investigation. Advanced genomic techniques, single-cell profiling and other highly specific tools could be utilised to explore epigenetic changes and X inactivation, thus opening new horizons for HNSCC treatment.

Conclusion

Previous research has elucidated in detail the physiological phenomenon of X inactivation and subsequent reactivation in various malignancies, particularly breast, ovarian and cervical cancers in females. This article reviewed the distinct perturbations of the X chromosome in various malignancies and suggested a similar hypothesis for HNSCC development. The careful profiling of X-linked gene expression in tumour cells could help to elucidate the X chromosome-related events which lead to oncogenesis.
References


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