Sex Chromatin Frequency Variation among Breast Cancer Patients and Normal Females of Two Reproductive Stages from Bengalee Hindu Females

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ABSTRACT

X chromosome inactivation refers to the developmentally regulated process of silencing gene expression from all but one X chromosome per cell in female mammals in order to equalize the levels of X chromosome derived gene expression between the sexes. In females, X chromosome inactivation (XCI) begins with the expression of the XIST gene from the X chromosome destined to be inactivated (Xi) and the coating of XIST RNA in cis. The apparent cytological overlap between BRCA1 and XIST RNA across the Xi raised the possibility of a direct role of BRCA1 in localizing XIST. The present study was conducted to evaluate the comparison of prevalence of sex chromatin in Bengalee Hindu Breast Cancer patients with Normal Bengalee Hindu Caste females, belong to two different reproductive stages viz. Menstruating females and Menopausal females. Materials for the present study consisted of the samples of buccal smears of 75 normal females (40 individuals from menstruating stage and 35 individuals from Menopausal stage) and 68 females of carcinoma of breast belonging to stage II, III, and IV. One hundred cells from each individual will be studied to ascertain the modal rate of incidences of sex chromatin. Our results revealed a significant difference between mean prevalence of Sex Chromatin among Breast Cancer patients, Menstruating normal and Menopausal normal females. Further, One-way ANOVA revealed a significant difference between the mean on Sex Chromatin frequencies among different stages of Breast cancer patients. This result is indicating reactivation of inactive X chromosome in case of malignancy. This result suggested the prognostic value of prevalence of sex chromatin in Breast Cancer patients.

1. Introduction

In all female mammals one or other of the two X chromosomes is inactive in all somatic cells [1,2]. The presence of an inactive X (Xi) chromosome in female cells was first observed at the cytological level by Barr and Bertram in 1949 as a 1µ heteropycnotic body mass that was often found at the nuclear periphery or within the perinuclear region[3,4]. In mammalian females; dosage compensation of X-linked genes is achieved by random inactivation of one of the two X chromosomes in somatic cells. The major genetic locus proposed to control the X chromosome inactivation process is the X inactivation center (XIC). XIC is defined as a region of the X chromosome from which a currently ill-defined inactivation signal exerts its effect in cis along the chromosome; derivative X chromosomes lacking this XIC are unable to become inactivated [5].

In search of X inactivation, the mechanism by which X-linked gene expression is equalized between XX females and XY males revealed the antisense gene Tsix determines X chromosome choice and represses the noncoding silencer, Xist [6,7,8]. This process of X chromosome inactivation (XCI) is a remarkable example of long range, mono-allelic gene silencing and facultative heterochromatin formation [9,10,11,12] even in female human embryonic stem cells [13]. Xist provides to functionally define epigenetic transitions in development, to understand cell identity, pluripotency and stem cell differentiation [14].

The study of X inactivation may also provide insight into cancer biology, as two active Xs have been found in many human breast and ovarian tumors [15]. Additionally, the X-inactivation process can extend beyond the scope of X-linked genes and be applied to many human disorders involving imprinted genes—genes expressed from only one of two parental chromosomes that are...
apparently also regulated by noncoding RNAs [16]. Without a doubt, X inactivation represents a great model system with which to study a broad range of developmental and epigenetic processes—those involving stable gene expression without changes to the underlying DNA sequence.

It is initially believed that the X chromosome inactivation is stably maintained in all progeny cells. However, various studies showing alteration of X chromatin frequency during age changes, different phases of menstrual cycle, pregnancy [17] and neoplasia [18] suggested skewed X inactivation (non-random) and reactivation of inactive X chromosome. Skewing of X inactivation has strong implication in biological consequences for female individual. Because XCI is stable once established, clonal expansion of somatic cells as occur in the cancer- result in a cell population with extremely skewed X inactivation [19]. This is often used to assess tumor clonality [20]. It is known that the two X chromosomes are active in oocytes [21,22,23], indicating that the inactive X chromosome must be reactivated during germ cell development [24]. Some recent findings indicate that reactivation of the inactive X chromosome occurs at least twice during mammalian development, once in the epiblast cell lineage at the peri-implantation stage and once in the Primordial Germ Cells at the midgestation stage, and that the reactivation of the inactive X chromosome appears to be tightly correlated with major genomic reprogramming events occurring during mammalian development [25,26]. The reactivation of inactive X chromosome was also observed whenever the body was under physiological stress especially in neoplasia [18]. Because female mammalian cells only have one active X chromosome, either loss of heterozygosity at the active X chromosome or skewed X chromosome inactivation may result in the loss of the function of an X-linked tumor suppressor gene and may lead to the cancer predisposition [27].

In the view of the above, the present study is an attempt to compare the prevalence of sex chromatin changes, if any, in breast cancer patients with normal healthy females of two reproductive stages.

**Materials and Methods**

A total of 143 females were included in the study, out of which 75 of normal females and 68 were diagnosed as carcinoma breast cancer patients belongs to Bengalee Hindu community. Further, normal females was categorised in to Menstruating females and Menopausal females and carcinoma breast cancer patients categorised in to different stages viz. Stage II, III and IV. The diagnosis of these breast cancer patients was confirmed by histopathologic biopsy and for both the group one hundred (100) cells from each individual was studied to ascertain the modal rate of incidences of sex chromatin.

A structured schedule was used to collect the data on the demography, life style pattern, reproductive history and clinical details from breast cancer patients.

Buccal smear samples have been collected with the help of foam-tip buccal cell collection swab. The scrapped material was spread quickly over the glass slide. Fixation and staining (CarbolFuchsin) of slides have done by using standard technique [28]. For each individual 100 cells will be considered at random. Slides were scanned through 10×40 resolution. Appropriate statistical tests were carried on using in SPSS software (version 16.0).

**Results**

A total of 143 samples were analysed for the present study. Frequency of sex chromatin distribution indicated that a majority of participants (44%) were showing presence of Sex chromatin in 21-30 cells, 21% of them showing in 41-50 cells, 18% of them showing in 11-20 cells, 13% of them showing in 31-40 cell and only 5% of subjects showing in >50 cell. Group (Breast cancer and Normal females) wise distribution indicated that in breast cancer patients, the maximum participants were showing the Sex chromatin in 21-30 cells followed by 11-20 cells and only 4% of them showing in 31-40 cells. In case of Normal females, the maximum participants showing the presence of Sex chromatin in 41-50 cells followed by 21-30 cells, 31-40 cells, and >50 cells. In case of breast cancer none of them were showing the presence of Sex chromatin in more than 40 cells and in case of normal females none of them were showing presence of Sex chromatin in less than 20 cells. Further, contingency table analysis revealed a significant association between frequency of sex chromatin distribution and Groups (Breast cancer patients and Normal females) (CC=0.573; P=0.000). In other words the presence of Sex chromatin in breast cancer patients is comparatively lower than the normal females (Table 1).

The frequency distribution of sex chromatin with menstruating and menopausal females also found to be significant (CC=0.672; P=0.000) and it is found that in menstruating females the frequency of Sex chromatin were more and in Menopausal females the frequency was less, none of the menopausal females were shown the presence of Sex chromatin in more than 40 cells. However, the non significant result was observed in case of frequency distribution of sex chromatin with different stages of breast cancer.

The frequency distribution of sex chromatin with different stages of breast cancer (CC=0.500; P=0.004). It is clear from the table, as the stages of the breast cancer increases the frequency of sex chromatin decreases.
The average frequency sex chromatin of breast cancer subject and normal subject were found to be 22.57 and 37.39 respectively. Student ‘t’ test revealed a significant difference between these average frequency sex chromatin with t value of 12.251 at significance of 0.000 level. In other words the average frequency of sex chromatin in normal females was significantly increased in 14.82 cells than breast cancer patients.

Further, ‘t’ test revealed a significant difference in the frequency of sex chromatin between menstruating and menopausal females (t=16.458; P=0.000). The average frequency of sex chromatin significantly decreased in menopausal with 15.73 cells (Table 3).

<table>
<thead>
<tr>
<th>Table: 1. Frequency Distribution of Sex chromatin in different groups</th>
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<tr>
<td><strong>Group</strong></td>
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<tr>
<td><strong>Breast Cancer</strong></td>
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<tr>
<td>11-20 cells</td>
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<td>21-30 cells</td>
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<tr>
<td>21-40 cells</td>
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<td>31-40 cells</td>
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<td>41-50 cells</td>
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<td>50</td>
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<tr>
<td>&gt;50 cells</td>
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<td><strong>Total</strong></td>
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Note: S= Stage, MNS= Menstruating Females, MNP= Menopausal Females, CC=Contingency Coefficient, HS=Highly Significant, NS= Non Significant

<table>
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<th>Table: 2. Frequency Distribution of Sex chromatin in different stages of Breast Cancer</th>
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<tr>
<td><strong>Group</strong></td>
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<tr>
<td><strong>Breast Cancer</strong></td>
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<tr>
<td>11-15 cells</td>
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<td>16-20 cells</td>
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<td>CC (Breast Cancer Stages)=0.500; P=0.004 (HS)</td>
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Note: S= Stage, MNS= Menstruating Females, MNP= Menopausal Females, CC=Contingency Coefficient HS=Highly Significant, NS= Non Significant

<table>
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<th>Table: 3. Descriptive statistics of Mean prevalence of Sex chromatin in different groups and sub groups</th>
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<td><strong>Group</strong></td>
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<tr>
<td><strong>Breast Cancer</strong></td>
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<td><strong>Normal Females</strong></td>
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Note HS=Highly Significant, NS= Non Significants

The average frequency sex chromatin of breast cancer subject and normal subject were found to be 22.57 and 37.39 respectively. Student ‘t’ test revealed a significant difference between these average frequency sex chromatin with t value of 12.251 at significance of 0.000 level. In other words the average frequency of sex chromatin in normal females was significantly increased in 14.82 cells than breast cancer patients. Further, ‘t’ test revealed a significant difference in the frequency of sex chromatin between menstruating and menopausal females (t=16.458; P=0.000). The average frequency of sex chromatin significantly decreased in menopausal with 15.73 cells (Table 3).
One-way ANOVA revealed a significant difference between average frequency sex chromatin among breast cancer, menstruating and menopausal females of Bengalee Hindu population. The average frequency of sex chromatin varies from 22.44 to 44.73 among different groups of female participants and which is found to be significant (F=307.015; p=0.000). The average frequency chromatin of breast cancer, menstruating and menopausal females were 22.44, 44.73 and 29.00 cells respectively. Further, Scheffe's post hoc test revealed that each mean difference between different groups of female subjects was significantly different. Further, average frequency sex chromatin frequency varies from 20.13 to 25.60 among different stages of breast cancer, which is found to be highly significant. One-way ANOVA revealed a significant difference between these average sex chromatin frequencies with F value of 9.829 and significance level of 0.000 level. Further, Scheffe's post hoc test mentioned at superscript in alphabets which revealed that different alphabets are statistically significant in other words mean difference between tribes was significantly different and same alphabets are found to be significantly no different (Table 3).

Discussion

Although higher prevalence of sex chromatin has been reported in breast cancer patients, again a study indicated no significant change in X chromatin frequency in cases of breast cancer patients. Apart from these, earlier studies have shown significantly lower prevalence of sex chromatin in different malignancies (1,31,32,33,34,35,36). The similar results indicating lower prevalence of sex chromatin have been revealed in the studies on carcinoma for example, esophageal cancer (37), breast cancer (38,39).

In the context, the present study also revealed comparatively lower prevalence of sex chromatin in breast cancer patients than normal females which corroborates the earlier studies (38,40,39). In our present study the prevalence of sex chromatin in breast cancer patients have been shown significantly lower value in menstruating as well as menopausal patients as compared to the normal menstruating and menopausal females. The reason behind the comparatively lower prevalence of sex chromatin in breast cancer patients may be the reactivation of inactive X chromosome.

In eutherian mammals, the inactive X chromosome (Xi) differs from its active homologue (Xa) in a number of ways, including increased methylation of selected CpGs, replication late in S-phase, expression of the Xist gene with binding of Xist RNA and underacetylation of core histones (41). DNA methylation and histone acetylation plays an important role in the stability and maintenance of gene silencing in inactive X chromosome (42,43). A strong correlation between DNA hypermethylation, transcriptional silence and tightly compacted chromatin has been established in many different research works (44,45,46). Similarly, many research works had shown that inactive X in female individuals contains underacetylated H4 because histone under acetylation plays an important role in the stabilization of inactive state of a gene (47,41).

But alteration in DNA methylation and Acetylation are common in various types of tumors as well as development (47). Hypomethylation of DNAs causes transcriptional activation and has been hypothesized to contribute to oncogenesis by activation of oncogenes, found in various kinds of cancers such as breast cancer, cervical cancer, brain cancer (48,49). The DNA methylation is correlated with deacetylation of histone H3 and H4, along with shifts in histone methylation pattern (50,51). In this way, the pattern of histone modification modulate a landscape that organizes and maintains the integrity of the nuclear architecture while establishing and enabling the expression pattern of specific genes within chromatin regions (52). In the work done by Roh et al. in the year of 2005 and 2007, high resolution genome wide mapping has revealed high levels of histone H3 acetylation in carcinogenic cells.[53,54]

Recently, research studies have demonstrated quantitative variation in prevalence of sex chromatin can be used as a prognostic tool in case of breast cancer. Ghosh et al. have shown a positive significant correlation between sex chromatin incidence and a five year survival time or disease free interval of distant metastasis (55). Longitudinal studies on cancer patients, however, demonstrated initial higher prevalence of sex chromatin and gradual lower frequency of sex chromatin at the final stage and in addition to that, the study by Perry in 2005 on evaluation of breast tumor sex chromatin (barr body) as an index of survival and response to pituitary ablation reported that the high tumor sex chromatin counts were associated with long survival after treatment, and low counts with short survival (40). Another work done by Wacker & Charles in 2006 revealed a higher frequency of sex chromatin in patients who survived longer than 8 years than who expired earlier (4). The quantitative features of chromatin structure in the prognosis of breast cancer and the results demonstrated that the criteria used enable prediction of prognosis with higher accuracy (92%) (56). The significance of sex chromatin test consists in the fact that one may judge the rate of tumor growth by the sex chromatin content in a small volume of biopsy material. Therefore, the sex chromatin test is an index of the growth rate (proliferative activity) of the examined tumor. By this test it is possible to determine the degree of the tumor progression, to assess the mitotic activity in the small pieces of the biopsy material. The sex chromatin test may be an additional method for differential diagnosis of malignant tumors (57).

Conclusion

In our present study, it has been revealed that sex chromatin prevalence in breast cancer patients is comparatively lower than apparently normal healthy female individual. Furthermore the frequency of sex chromatin varies among the stages of breast cancer patients also. Then the findings of the present study signifies that the alteration in the pattern of DNA methylation and histone acetylation in cancer cells may be the cause behind lower prevalence of sex chromatin in breast cancer females than normal females. Furthermore, the present study is being the first attempt from Bengalee Hindu Caste females that whether the prevalence of sex chromatin has any prognostic value for Bengalee Hindu Caste breast cancer patients or not.
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