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Genomic Instability with Non- Homologues Chromosomes in Aneuploid Cell Increase Risk Factor for the Development of Breast Cancer Patients

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ABSTRACT

Chromosome aberrations (CAs) play an important role in tumor initiation, followed by metastasis. Short-term peripheral blood lymphocyte (PBL) cultures using RPMI-1640 media with 5 % FBS were used to evaluate the frequency of structural and numerical chromosome aberrations in breast cancer patients. Highest frequency of aneuploidy (11.53%) were observed followed by trisomy -21(8.93%) in karyotypes. De-novo mutation involving 12q21 with loss of 16.38 Mbp DNA fragment is the most relevant finding in breast cancer (BC) patients and has not been reported earlier. Secondly, role of trisomy-21 (8.93%) might have increase genetic susceptibility of disease because of "giant satellites", are the active sites of rRNA sub unit of 18S and 28S. These active sites might have increase gene-expression of truncated protein during tumor Significant increase in the frequency of aneuploidy with increased number of non-homologous chromosome was the striking feature due to increase of non-disjunction event followed by unequal crossing-over and synapse formation by known environmental factors like arsenic. However, the present study is small, but interesting to explore the etiopathology of BC patients associated chromosome instability. However, further study is required to confirm these changes, whether these mutations are either familial (inherited) or spontaneous in nature.

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Introduction

Breast cancer (BC) is a complex heterogeneous group of genetic disorder and chromosome aberrations (CA) play a relevant role to increase risk for the development of disease. Globally, the incidence of 2.26 million new cases of BC registered world-wide and is tremendous increase 24.00% in Asia including China, Indonesia and Japan perhaps due to changes in life - style that lead endocrine dysfunction [1,2]. CAs are significantly associated with activation of proto oncogenes and inactivation of tumor suppressor gene (p53) during progression of disease [3]. The knowledge of specific genetic loci and their correlation with different stages of BC becomes relevant to understand the etiopathology for better management. Peripheral blood lymphocytes (PBL) technique is a valuable tool for cytogenetics analysis to understand the knowledge of genetic damage during chemotherapeutic regime in pre/postoperative cancer patients. Spontaneous chromosome aberrations (SCAs) to form complex chromosome rearrangements (CCRs) in Bloom's syndrome, Fanconi anaemia, and ataxia telangiectasia for developing risk in variety of tumor. The predisposition of tumor gene and their loci develop curiosity to known mechanism of chromosome instability that increase genetic susceptibility during tumorigenesis. During tumor progression several predisposition gene involved in metabolic pathways to makes the mechanism more complex like tumor suppressor gene (p53), heat shock protein (Hsp72), tumor necrosis factor alpha, (TNF-α), transforming growth factor-beta (TGF-β) and folate-metabolism. These signalling modulates based on protein kinases, growth factors including endocrine gene regulation (receptors) and cytokines are responsible for epigenetic modifications during pre or post metastasis events during disease progression and enhance genomic instability [4-6].

Molecular biology of BC are quite interesting due to variation in the frequency of gene mutation between somatic and germ cells. Genetic heterogenicity is the characteristic feature in tumor biology and somatic gene mutation arises from single cell is differs from germ line mutation. The germ-line mutation in BC are highly complex because of variable allele frequency in different population based on age and ethnic groups. High frequency of BRCA gene mutation (>70%) in women were observed, who attain the age of 70 years. BRACI and BRACII gene are mapped on 17q21 and 13q12-13 locus and their mutation frequency varies between 2-6 % but, BRCAI frequency is higher (25%) in African American population. In Jewish population the allele frequency varies shows three mutation BRCA1185delAG, BRCA1538inC and BRACII6174 del [7,8]. The BRCAI gene is a frame shift mutation has also been reported in colon, endometrial, pancreatic and prostrate, while, BRCAII gene mutation increased "risk factor" for gall bladder, prostrate and stomach because of hereditary in nature [9]. Recent study of our group shows that 3-5 methylene tetrahydrofolate reductase (MTHFR C677T)

gene polymorphism increase risk of BC in heterozygous (CT genotype) condition. MTHFR, an important enzyme to regulate folate-metabolism during DNA methylation and increased genetic susceptibility after substitution of nucleotide from cytosine to thymidine (C→T) followed by change an amino acid from alanine to valine, resulting disequilibrium of intracellular folate-pool with increased frequency of chromosome aberrations in tumor cells [10]. Recent study is based on circulating tumor cells (CTC) shows variable expression of epithelial - mesenchymal transition (EMT) markers cytokeratin (CK19), epithelial cell adhesive molecule (EpCAM), Sox4 (Sry-transition transcription factor 4) and Vimentin during progression of disease in BC patients [11,12]. Present study has been designed to evaluate the frequency of structural and numerical chromosome (karyotype) variations using whole chromosome painting to differentiate homologous and non- homologous chromosomes in different age- groups of BC patients and to find out specific end-point as genetic biomarkers for metastasis during progression of disease.

Materials and Methods

Peripheral blood samples (1.0 ml) were collected under sterile conditions from clinically diagnosed cases (n=34) in heparinized vial from OPD of the department of surgery to the cytogenetics and molecular genetics laboratory, department of pathology/ laboratory medicine to set up short term lymphocytes cultures. Family history were also recorded to find out the mode of inheritance and environmental exposure towards carcinogen (pesticide) during agriculture farming or exposure with radiation. The study is approved by IEC (Institute ethical committee) and after informed patient written consent were carried out successfully to maintain secrecy with ethnically of the genetic data.

Cytogenetics Analysis

Karyotypes was developed from BC patient using short term peripheral blood lymphocytes (PBL) cultures with RPMI 1640 media containing phytohemagglutinin, (5.0%) FBS and 1.0 % antibiotics solution (streptomycin-penicillin) for 72 hours at 37°C. Before harvesting the cultures, colchicine was added two hours prior to arrest the dividing cells. Pre warmed KCl solution was used as hypotonic and cells were fixed in 3:1 methanol: acetic acid solution. At least ten well spread metaphases were selected for karyotypes after GTG banding of chromosomes. Both structural and numerical chromosome aberrations karyotypes were prepared according to the recommendations of the International System for Chromosome Nomenclature (ISCN 2016) using applied spectral imaging software (Genesis USA) [13]. Fluorescence in situ hybridization (FISH) analysis is highly sensitive technique for confirmation of chromosome aberrations used for evaluate extra copy of chromosome-21 in interphase cell using DNA probe labeled with spectrum orange of 220 kb and their cytogenetic location is assigned between 21q22.13-q22.21, obtained from Abbott-Vysis, Inc. (USA). All procedure details concerning hybridization were carried out according to the instructions provided in the kit. Cells were counterstained with DAPI and viewed under fluorescence microscope (Olympus Japan) and more than 10-20 interphase were recorded for analysis.

Cell-Cycle Analysis

Flowcytometric based analysis was carried out (n=5) for understanding of cell -kinetic during in-vitro tumor cells (PBL) proliferation and their correlation with chromosome aberrations (CAs) in BC patients using kit BD CycleTestTM Plus DNA Reagent Kit (USA). First the cells (1x106) were fixed in buffer solution, centrifuge at 1600 rpm for 5 minutes, saved the pellet and dissolved in solution A (250 ul). Cells were incubate for 10 minute at room temperature followed by adding solution B and C (200 ul) as prescribe by kit and samples becomes ready for cell-kinetics assay using flowcytometery [14,15].

Statistical Analysis

X2-test was used to find out level of significance difference (p-value) between the normal and abnormal karyotypes in the BC patients.

Results

Breast cancer is a heterogeneous group of cell population and shows variety of structural and numerical chromosome aberrations with different percentage (%) frequency using peripheral blood lymphocytes (PBL) culture techniques to confirm end-point for chromosome instability during progression of disease as detailed data of karyotypes are documented in Table 1.

Table 1: Karyotypes and Their (%) Frequency in Breast Cancer Patients

S. No	Total structural and numerical variation in karyotypes	Numbers & their (%) frequency
1	Aneuploidy	9(11.53)**
2	45,XO (Turner Syndrome)	2(02.56)
3.	45,XX, Monosomy 20	1(01.28)
4	47,XX ,Trisomy 10	1(01.28)
5.	47,XX ,Trisomy13 (Patau Syndrome)	1(01.28)
6.	47,XX ,Trisomy15	1(01.28)
7.	47,XX ,Trisomy 18 (Edward's Syndrome)	1(01.28)
8	47,XX ,Trisomy 19	1(01.28)
9	47,XX ,Trisomy 20	1(01.28)
10	47,XX ,Trisomy 21(Down Syndrome)	7(08.97)**
11.	47,XX ,Trisomy 22	1(01.28)
12.	46XX , Chromatid break (12q12)	1(01.28)
13	46,XX , Dicentrics	4 (05.12)
14	46,XX , Ring chromosome	1 (01.28)
15	46, XX ,Robertsonian translocation	1 (03. 80)

Cell Kinetics Analysis

Cell-cycle, is an important tool for evaluation of cell-kinetic during proliferation in tumor biology and to correlate with disease progression. Present study showing the highest frequency (%) was observed in G1G0 (76.69) phase and subsequently decreasing trend was observed in S (15.61) and G2M (5.38) phase of the cell-division as shown in figure-1.

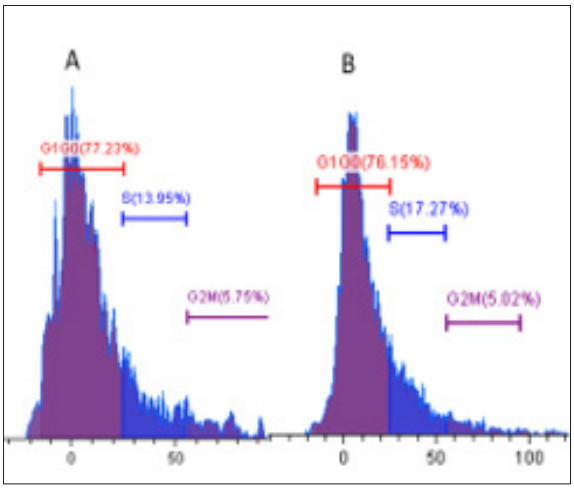

Figure 1: Cell-cell cycle kinetics analysis of circulating tumor cells after staining with promidium iodide, a fluorescence dye showing different phases of the cell division in histograms (fig,A&B) followed by decreasing trends were observed in S phase (blue) and G2M phase (purple),respectively in BC patients.

Cytogenetics Analysis

The frequency of numerical chromosome aberrations (aneuploidy) is 11.53% and to distinguish between homologous and non-homologous chromosomes and confirm the aneuploidy, the whole chromosome painting techniques was used to visualize individual chromosome on the basis of different colours intensity using in build software tool to minimise chances of error as shown in Figure 2a (metaphase) and karyotype (Figure 2b). The trisomy-21 (8.97%) followed by decreasing trend were observed in trisomy of chromosomes-10, 13, 15, 18, 19, 20 and 22 with frequency (01.28 each) as shown in Figure 3. Interestingly, two karyotype showing 45, XO, the loss of sex-chromosome having frequency (0.2.56%), while one karyotype showing monosomy of chromosome -20 (01.28%). Statistical analysis were carried out between total chromosome aberrations and aneuploidy or trisomy-21, the calculated values of confidence interval (C.I) at 95% varies 249.17-279.55 with odd ratio (44.52) showing significant differences ($p<0.001$).Giemsa stain is also used simultaneously to confirm the findings. The whole chromosome painting is a highly sensitive, reliable technique for tumor bearing cases for confirmation of diagnosis to understand the metastatic events. The karyotypes showing high structural chromosome aberrations with dicentrics (05.12%) followed by decreasing of Robertsonian translocation (0.3.80%, chromatid break (12q12) and ring chromosome with 01.28 % frequency in BC patients. Statistical analysis were carried out between total chromosome aberrations and total structural chromosome aberrations with calculated values of confidence interval (C.I) at 95% varies from 0.7927-2.5427 with odd-ratio (1.4) showing lack of significant differences ($p=0.2386$) using x2-test.The structural de-novo mutation involving chromosome-12 (del12q21) showing loss of 16.38 Mbp DNA fragment is the most relevant and new finding in BC has not been reported earlier.

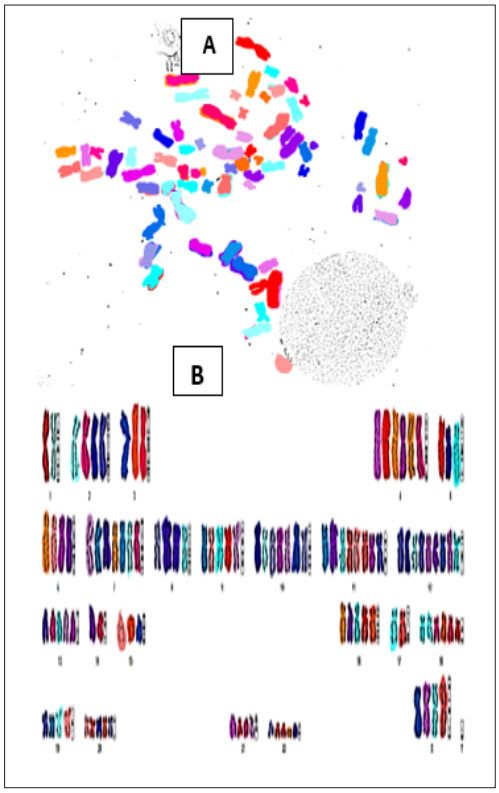

Figure 2A&B: Differentiating homologous and non homologous chromosomes in aneuploid cell after whole chromosome painting in metaphase (fig.-2A) and karyotype (fig-/2B) in BC patients

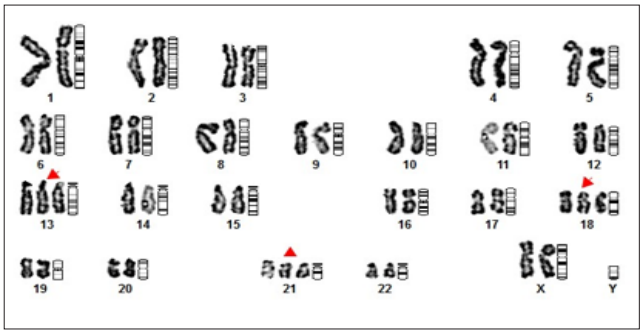

Figure 3: Karyotype Showing Trisomy of Chromosome-13,18 and 21 after using Giemsa (1.0%) Staining in BC Patients (Mark by arrow)

Discussion

There is positive correlations were observed between aneuploidy and proliferating tumor cells (PBL) during progression of breast cancer. Earlier study shown that significant increase of frequency of dicentrics in total chromosome aberration might be the inducing factor in BC patients [16]. In the present study, individual structural chromosomal aberrations showing decreasing trend dicentrics (5.0%) followed by in D/G association or Robertsonian translocation (3.80%), chromatid breakage and ring (1.28% each) with lack of significant difference either due to unstable in nature or may lost in next phase (G2M) of the cell-cycle. The mechanism behind the origin of dicentrics, ring and translocation of chromosomes involves breakage of two ends of the same chromosomes and further reunion to form chromatid fusion bridge (CFB), followed by disposition of genes resulting activation of oncogene. Cell-cycle analysis showing that most of the cells exist in G1/G0 phase (76.69%) of the cell-division and curiosity has

been generated to evaluate the cytogenetic end-point (chromosome aberrations) to confirm the role of chromosome instability followed by increase of genetic susceptibility to explore the etiopathology of disease. The intra-chromosomal rearrangement such as Robertsonian translocation and ring chromosome becomes important factor to understand the primary event due to disposition of tumor inducing gene(s) during progression of disease. Although, the Sox4, act as transcription factor is associated significantly during the onset of disease either alone or synergistically with other molecules like EpCAM, CK19 and vimentin in BC patients [12,17]. Further, the data was analyzed to concludes genetic basis for confirmation of disease i.e. the origin of stable and unstable chromosomes configurations during cancer progression, suggesting de-novo mutation involving chromosome (12q21) after mapping and loss of 16.38 Mbp DNA play an important role either in cell-signaling such as tumor suppressor gene (p53) or may activate proto-oncogenes becomes relevant to understand the etiopathology of BC patients reporting first time in population of Bihar. Besides, these structural variations, numerical chromosome play a significant role due in stable mutations and makes the tumor cell unbalanced that increase chromosome instability. The high frequency (8.93%) of extra copy of chromosome- 21 is the characteristic feature of Down syndrome patients due to poor development of cerebral cortex region resulting fall under the category of mental retardation and their association in BC patients might have raise the question ? does tumor inducing gene(s) linked to "giant satellites", the characteristic feature of D/G group chromosomes and active sites of rRNA sub unit of 18S and 28S followed by increase expression of B24 & C 23 protein. However, the correlation between two diseases is regulating by the same chromosome-21, but certainly by two different gene(s), and further studies are required to confirm the role of genetic instability [18]. In-vitro demethylation agent like 5-azacytidine induces break points (fragile-sites), premature condensation of chromosomes (PMC) also increase chromosome instability in cancer patients [19]. Earlier study also shows that significantly higher expression of GIMAPs act as tumor-suppressive in women with trisomy-21 other than BC patients [20,21]. Furthermore, the karyotypes showing trisomy of chromosome-13 and 18 categorically known as Patau and Edward syndrome, respectively, and their linkage with BC patients at present is difficult to correlates, until further studies are required in different labs. The etiopathology of loss of sex-chromosome is not clear but it might be associated with either due to heterochromatization or inactivation of one X-chromosome in BC patient The, another interesting significant observation is the increase frequency of non-homologous chromosome in aneuploid cells due to non-disjunction event might be due to the environmental exposure like arsenic. Earlier study of the same group shows that arsenic down regulates the expression of epithelial-mesenchymal transition markers- Sox4, EpCAM and CK19 in circulating tumor cells (CTC) of BC followed by increase genetic heterogenicity of MTHFRC677T polymorphism because of point-mutation [10,11,22]. Hence, aneuploidy may be use a potential biomarker for early prognosis and diagnosis due to stabile in nature and becomes relevant for the clinicians during management of BC patients.

Conclusion

Genetic diversity is based on complex chromosome rearrangement (CCRs) in BC patients reporting first time in Bihar and genetic heterogenicity of MTHFR C677T polymorphism increased risk factor for disease. Present study is small, but interesting to include novel de-novo mutation (12q21) and loss of 16.38 Mbp DNA fragment reporting first time that increase chromosome instability. Non– homologous chromosome increase the frequency of aneuploidy that further confirm the events of pre or post metastasis and as may use as marker for progression and diagnosis of disease like cancer.

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Conflict of Interest

The authors declare no conflict of interest

Author Contributions

Ajit Kumar Saxena: Design and writing the manuscript; Shalini: Cell cycle analysis; Vipul Vaibhava: Proof reading; Pritanjali Singh: Clinical diagnosis of the patients.

Ethics Approval And Consent to Participate

Study was approved by Institute ethical committee of AIIMS Patna (AIIMS/Pat/IRC/2020/610), and informed consent form was dually signed by patients.

Patient Consent For Publication

Patients consent form was dually signed by patients involved in study.

Consent for Publication

Not applicable

Availability of Data

Data used in this work are available from the corresponding author upon request.

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