Chromosomal Disorders and Aberrant DNA Methylation as Early Biomarkers of Breast Cancer Risk in Young Women

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ABSTRACT
Genetic instability is an early and constant characteristic of tumor cells. Chromosomal aberrations and epigenetic anomalies are the factors leading to genomic instability. This study aimed to investigate the relationship between chromosomal disorders and aberrant DNA methylation as strong biomarkers in early diagnostics of breast cancer in young women. The research was conducted in 20 patients, and involved 15 young females with breast cancer (BC) at stages T2-4N0-3MO (histologically confirmed). Cytogenetic tests of lymphocytes of females with breast cancer (BC) revealed chromosomal abnormalities expressing as deletions, isochromosome deletions of chromosomes and gaps. Activity of the DNA methyltransferase (DNA MTase) in BC is shown to rise up to 58%, in comparison with the normal indexes. Conclusion: Cytogenetic analysis of lymphocytes in BC women has revealed chromosomal abnormalities in the form of deletions, isochromosome deletions and gaps. It has been shown that in BC, the activity of DNA methyltransferase is increased by 58%, compared with the normal indexes.

INTRODUCTION
Breast cancer (BC) is the most common form of malignancy in women and the leading cause of female cancer death. Despite the fact that BC is more common at the age of 55-65 years, recently the worldwide trend has developed towards BC higher incidence in young women. The highest morbidity is reported at the age of 32-38 years, i.e. during the active reproductive period [1-4].

The problem of early diagnosis of the tumor development, primary BC prevention, i.e., anticipation of conditions that lead to functional and morphological prerequisites to the onset of dysplasia viz mammary gland precancer, remains an urgent one. To solve the problem of early diagnosis, some reliable and simple methods of the tumor detection at preclinical stages are needed.

Genetic instability is an early and permanent hallmark of tumor cells [5]. Such disorders of the genetic apparatus of cells as chromosomal aberrations and epigenetic abnormalities are the factors leading to genomic
instability. Chromosomal abnormality is one of early genetic disorders resulting in the induction of the cell genome instability and, as a consequence, its malignant transformation. The pattern of neoplastic cells methylation changes significantly in comparison with normal cells; total demethylation is accompanied by an increase in the activity of DNA methyltransferase DNA MTase and local hypermethylation of CpG islands. The mechanism of local hypermethylation is not completely clear. Apparently, the important role in this process is played by an increase in methyltransferase activity [6, 7]. The interrelation of genes, chromosomal and epigenetic disorders in induction of genomic instability in the development of BC is of great importance [8].

MATERIAL AND METHODS

During the research, DNA samples obtained from peripheral blood leukocytes of BC patients (15 females) were used. The blood of BC patients was received at the Department of Mammalogy of the National Center for Cancer Research of the Ministry of Health (MoH) of Uzbekistan. As a control, DNA from peripheral blood leukocytes taken from healthy donors was used (10 donors). Generally accepted clinical and morphological prognostic criteria were evaluated: the tumor histological type, tumor receptor status, HER2/neu expression. All of them were studied using the biopsy material.

Ethical approval

The review board and ethics committee of National Center for Cancer Research under the MoH Tashkent, Uzbekistan AND Bioorganic Chemistry Institute named after A.S. Sadyko. Tashkent, Uzbekistan. Academy of Sciences, Tashkent, Uzbekistan approved the study protocol and gave permission.

Extraction of eDNA from serum / plasma

One ml of peripheral blood taken from the ulnar vein was transferred to plastic tubes with Na2-EDTA sprayed. The blood was centrifuged at 40 °C sequentially at 1500 rpm for 10 minutes, at 3000 rpm for 15 minutes, at 5000 rpm for 15 minutes. After centrifugation, 400 μl of blood serum were taken from the tubes and transferred to new sterile tubes. The serum was pretreated with the RNA (100 μg/ml), incubated at 37 °C for 1 h, then resuspended with proteinase K (50 μg/ml), incubated for 1 hour at 37 °C. After enzymatic treatment, the blood serum was added to 200 μl of lysis buffer (100 mM Tris- HCl, pH 8.0; 25 mM EDTA, pH 8.0; 0.15 M NaCl; 0.7 M β-Mercaptoethanol; SDS) to a final concentration of 2%. The lysis was carried out in the cold for 3 minutes (on ice). The aliquots were deproteinized for 15 minutes in 1.5 ml phenol / chloroform mixture (1:2) followed by 15 min. centrifugation at 5000 rpm at 40 °C. The supernatant was transferred to new test-tubes, 1/10 volume of 3M sodium acetate pH 5.2 was added, as well as 2.5-volume of cooled 96% ethanol, and the tubes were left overnight at -200 °C. The denatured eDNA preparations were centrifuged at 5000 rpm for 30 min at 40°C. The eDNA precipitate was washed in 1 ml with cooled 70% ethanol for 15 minutes, and then centrifuged at 13,000 rpm for 15 min at 40 °C; then it was dried in vacuum desiccator for 15 minutes and dissolved in 300 μl of TE buffer, pH 8.0 and stored at -200 °C. The eDNA aliquots were analyzed in 2% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was conducted for 1 hour at 100V; the gel was photographed in UV rays.

Cultivation of lymphocytes

The culture medium consisting of (per vial): 6 ml of RPMI 1640 medium with glutamine (PANECO, Russia), 1 ml of fetal bovine serum (produced in France-Germany), 40 μg/ml gentamicin and 20 μg/ml mitogen - phytohemagglutinin (PHA DifcoP) was added to 0.8 ml of whole blood. The prepared cell culture was incubated in thermostat at 370°C and was periodically shaken gently (1-2 times per day). This procedure prevents excessive agglutination of erythrocytes. The cultivation time under the experimental conditions was 72 hours. The cells were fixed for 72 hours after the initiation of cultivation. Two hours prior to fixation, colchicine (0.4 μg/ml) was injected into the culture medium that destroyed the spindle microtubules and prevented chromosome divergence. In consequence, the cellular mitosis stopped at the metaphase stage. The cultured cells suspension was poured into centrifuge tubes, centrifuged at 1000 rpm for 7 minutes. Then, the supernatant was removed, the precipitate was shaken, 7 ml of hypotonic solution 0.075 1M KCl pre-heated to 37 °C were added. After that the tubes were again placed in thermostat for 20 minutes. Hypotonic treatment is carried out for the best spread of chromosomes of lymphocytes. After the end of hypotonic treatment of the cells, they were fixed in three stages. At the first stage, the cell suspension, after treatment in KCl hypotonic
solution in thermostat, was centrifuged at 1000 rpm for 7 minutes, and then the supernatant was removed, leaving about 0.5 ml of hypotonic solution above the cell precipitate. After precipitate shaking, 8 ml of freshly prepared cold fixative solution, consisting of 3 parts of ethanol and 1 part of glacial acetic acid, were added to the cell suspension, and then placed into refrigerator (at 60 °C) for 20 min. Then, in the same way, the second stage of fixation was carried out. After this, the third and last stage of fixation was carried out similarly to the second stage. After the last fixation, the cells were pelleted by centrifugation (1000 rpm p/m) for 7 minutes and re-suspended in a small volume of the fresh fixative (0.5 ml). The suspension of the cultured cells was applied on wet cold glass slides by dropping. To do this, the cell suspension was dropped from 35 to 40 cm height with a Pasteur pipette onto the surface of the slides, which were then dried in the air. The preparations were stained with 4% Romanovsky-Giemsa stain. The analysis was carried out at the metaphase stage.

**Determination of the methylating enzyme activity**

The incubation mixture (130 μl) contained 5 μg of DNA, 20 μl of 3HSAM, 5 μl of methyltransferase, 50 μl of phosphate buffer containing 10 mM Na2HPO4, pH 7.5. The samples were incubated for 18 hours at 37 °C. The DNA samples were precipitated in 10% ice-cold TCA and applied to pre-moisten 5% TCA CF/C filters. The filters were washed with 50 ml of 5% TCA and 40 ml of ethanol. Radioactivity of precipitates on the filters was counted in Mark III liquid scintillation counter. With an increase in the amount of the enzyme in the incubation mixture, the amount of DNA proportionally increased. To control the maximum level of DNA methylation, the reaction was carried out till reaching the plateau. The methylated DNA was incubated for 40 min. at 600 °C in 0.5 NaOH solution, the DNA was centrifuged for 20 min. in “Beckman” centrifuge, and the DNA was precipitated.

**RESULTS AND DISCUSSION**

The research has revealed some specific features of cytogenetic disorders causing the development of genomic instability in BC. For this purpose, cytogenetic analysis of peripheral blood lymphocytes of young women with BC was performed. Cultivation of lymphocytes was carried out by the modified method of McGregor and Marley [9].

In the cell cultures at the metaphase stage in healthy women, the rearrangement was found in 0.47 - 0.56%. A slight increase in the incidence of these disorders was observed in two patients (0.66 and 0.88%, respectively). Other patients showed from 1.06 to 3.44% (almost 7 times higher than the controls).

The following types of aberrations were found: terminal single deletions occurred in 7 of 8 females (it did not occur in healthy women); isolation of chromosome deletions occurred in 4 of 8 patients; gaps were found in 3 patients.

Table 1 presents the results of cytogenetic analysis of peripheral blood lymphocytes of women with BC. They include single deletions, isolation of chromosome deletions, and gaps. The table demonstrates that the number of common chromosomal rearrangements in women suffering from BC is much higher than in healthy women.

Summarizing the results of cytogenetic analysis, it can be assumed that the occurrence of cells with chromosomal aberrations, i.e. cells with stable disorders in chromosomes and insertions, is considered a sign of tumor formation process. Genetic disorders result in genomic instability that leads to acceleration of malignant processes and development of a neoplasia process.

In order to understand the cause of simultaneous hypermethylation and hypomethylation of DNA in breast cancer, we have studied the activity of DNA methyltransferase in DNA of normal and tumor cells. To that end in view, eDNA was isolated from the blood plasma of healthy women and women with BC.

For methylation, the eDNA molecules were treated with DNA methyltransferase enzyme. The change in the activity of methyltransferase in BC was revealed (Figure 2) shows the curves reflecting changes in normal activity and those ones in BC. The Figure demonstrates that in BC, methyltransferase activity increases by 58% compared with the norm. Molecular mechanisms of enhanced expression of DNA methyltransferase in tumor cells have not been elucidated. Apparently, this can be a compensatory response of the cell to general demethylation. The increase in methyltransferase activity significantly affects both the profile of DNA methylation and local hypermethylation.
Table 1. Types of chromosomal aberrations in young women with breast cancer

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number of metaphases studied</th>
<th>Metaphases with rearrangements %</th>
<th>Types of chromosomal aberrations</th>
<th>Gaps, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Metaphases with rearrangements</td>
<td>Single terminal deletions, %</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isolation of deletions, %</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gaps, %</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>196</td>
<td>2.55 ± 1.01</td>
<td>1.53 ± 0.69</td>
<td>1.02 ± 0.72</td>
</tr>
<tr>
<td>2.</td>
<td>250</td>
<td>0.8 ± 0.56</td>
<td>0.8 ± 0.56</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>174</td>
<td>3.44 ± 1.4</td>
<td>2.87 ± 1.2</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>4.</td>
<td>156</td>
<td>2.56 ± 1.2</td>
<td>1.28 ± 0.9</td>
<td>0.64 ± 0.06</td>
</tr>
<tr>
<td>5.</td>
<td>151</td>
<td>0.66 ± 0.07</td>
<td>-</td>
<td>0.66 ± 0.07</td>
</tr>
<tr>
<td>6.</td>
<td>166</td>
<td>3.01 ± 1.34</td>
<td>2.41 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>188</td>
<td>1.06 ± 0.75</td>
<td>1.06 ± 0.75</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>193</td>
<td>2.6 ± 1.1</td>
<td>2.7 ± 1.03</td>
<td>-</td>
</tr>
<tr>
<td>9. (control)</td>
<td>212</td>
<td>0.47 ± 0.05</td>
<td>-</td>
<td>0.47 ± 0.047</td>
</tr>
<tr>
<td>10. (control)</td>
<td>178</td>
<td>0.56 ± 0.06</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 1. Cytogenetic analysis of peripheral blood lymphocytes in BC women (Chromatid breaks or deletion)

Figure 2 (A and B). Activity of DNA methyltransferase in healthy donors and BC women
CONCLUSION AND RECOMMENDATION

Cytogenetic analysis of lymphocytes in BC women has revealed chromosomal abnormalities in the form of deletions, isolation of chromosome deletions and gaps. It has been shown that in BC, the activity of DNA methyltransferase is increased by 58%, compared with the norm.

Determination of molecular markers allows us to identify a group of patients with an increased risk of suffering, early BC. But, it is not subject to preventive chemotherapy, and to assess the sensitivity to a particular type of systemic therapy, and for the purpose of individualization. Also, the importance of molecular markers can be used to develop new modern drugs, acting as a target for these molecules. Molecular-biological markers, determined in tumor tissue, make it possible to characterize the tumor with respect to: sensitivity to hormone therapy and targeted therapy, as well as a tendency to invasion and metastasis.

DECLARATIONS

Authors’ Contributions
All authors contributed equally to this work.

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Competing interests
The authors declare that they have no competing interests.

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