Investigation of Microsatellite Instability BAT 25 and BAT 26 in Breast Cancer Patients by Conventional PCR

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Abstract

Investigate the existence of microsatellite instability (MSI) in patients with invasive ductal breast carcinoma (IDC). Investigate the existence of microsatellite instability (MSI) in breast cancer of patients. DNA had been extracted from frozen tissue samples of breast cancer. This protocol done according to the kit manufacture’s manual of QIAamp DNA Mini kit from Qiagen – USA. All samples tested for MSI by singleplex PCR reactions using two microsatellite markers BAT 25 and BAT 26. PCR achieved in a final volume of 50 µl and after thermal cycles, gel visualization performed. PCR demonstrating microsatellite instability in 13 (26%) of the 50 breast cancer sample. Eight (16%) of 50 breast cancer sample were BAT 25 positive with a PCR product size of 124 bp, while 5 (10%) of 50 breast cancer sample were BAT 26 positive with a PCR product size 121bp. The result suggests strong evidence that microsatellite instability (MSI) at the BAT 25 and BAT 26 and have involved in the pathogenesis of the great majority of breast cancers.

Keywords: Breast Cancer, Microsatellite Instability, PCR

Introduction

The development of breast and other cancers is a multistep process [1, 2]. At least six genetic changes may be required to convert normal breast epithelium to malignant breast cancer [1, 3], with each alter action presumably increasing proliferative or survival capacity. In contrast, many of the types of changes commonly observed in cancer cell genomes develop at immeasurably low frequencies in normal

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cells. This is consistent with previous proposals that one or more changes occurring during cancer evolution increase the endogenous mutation rate beyond the normal repair capacity or decrease the ability to detect and/or repair mutations, resulting in a mutator phenotype in affected cells [4, 5].

Microsatellite instability (MSI) is defined as the type of genomic instability related with defective DNA mismatch repair in tumors. MSI provides an indication of the presence of genetic instability in a given tumor by comparing the size of a subset of simple repeated sequences occurring throughout the genome (mono-, di-, tri-, and, less frequently, tetranucleotide repeats) between normal and tumor DNA from the same individual [6].

The most frequent errors associated with microsatellites are base–base mismatches that escape the intrinsic proofreading activity of DNA polymerases, and insertion–deletion loops, which are extra helical nucleotides that form DNA hairpins. Insertions or deletions in microsatellites located in DNA coding regions generate frame shift mutations, which can lead to protein truncations [6, 7].

Microsatellite instability (MSI) is a situation in which a germ line microsatellite allele has gained or lost repeated units and has thus undergone a somatic change in length. Because this type of alteration can be detected only if many cells are affected by the same change, it is an indicator of the clonal expansion that is typical of a neoplasm [8].

Initially, some authors used the term replication errors (RER), though in 1998 the National Cancer Institute Workshop on HNPCC recommended the use of the term MSI and established “MSI golden standards” that are currently employed in research and diagnostic laboratories worldwide [9].

The mononucleotide MSI loci, BAT25 and BAT26, have the highest accuracy in predicting MSI-H tumors, with sensitivity and specificity approaching 94%–98% for both markers [10]. The quasimonomorphic [11] feature of these markers, defined as little or no polymorphism in these loci across all ethnic populations, allows the testing of tumor tissue without the need for a corresponding normal control. However, some unstable tumors may have stable BAT26 loci due to a large intragenic MSH2 deletion, causing complete absence of the BAT26 loci in the tumor tissue [12]. Other MSI loci are generally added to correctly detect these cases.

The aim of this study was to investigate the frequency and presence of microsatellite instability MSI in the BAT25 and BAT26 loci of DNA samples extracted from invasive ductal breast carcinoma tissue sample by single plex PCR and its correlation with clinical and pathological parameters.
Methods

A- Specimens

This study include 50 female patients with invasive ductal breast carcinoma and 20 female with benign breast tumor as negative control were chosen from Al-Imamin Al-Kadhimin Teaching Hospital and from Dijlah Private Hospital during February to July 2013. Inclusion criteria of subjects include female patients with diagnostic feature of breast cancer and age range 26-42 years old untreated with chemotherapy or hormonal therapy. The samples introduced in this study included 50 tumors tissue sample from invasive ductal breast carcinoma. The tissue samples were preserved in normal saline until delivered to the working laboratory. Each tumor mass preserved in normal saline and freezed at -20°C for DNA extraction and PCR.

B- Ethical Approval

This research underwent to the terms of ethical considerations and in accordance with the form prepared for this purpose by the Iraqi Ministry of Health also got the approval of the research by the Committee of ethical standards in the Faculty of Medicine, Al-Nahrain University, one of the colleges affiliated to the Ministry of Higher Education and Scientific Research, Iraq.

C- DNA Extraction

Preparation of tissue homogenate: Twenty-five grams of the breast tissue was homogenized with 180 μl of Buffer ATL homogenate centrifuged for about 15 min. at 5000 rpm in (2-8) °C. The supernatant then collected carefully DNA had been extracted according to the kit manufacture’s manual of QIAamp DNA Mini kit from Qiagen – USA

DNA evaluation by Nano drop for an A260/A280 value of 1.5, the percentage of protein in the DNA preparation, the sections of tumor tissue should contain more than 50% of neoplastic cells in order to avoid false negatives [13]. Microsatellite marker amplifications are performed as single plex PCR reactions using DNA from tumor tissue. In accordance to the recommendations by the NCI.

Internal Control

Since DNA extracted from freezing tissue sample can be variably degraded and may contain PCR inhibitors, we suggest performing a preliminary quality control to test if DNA sample is suitable for MSI and to determine the optimal quantity for amplification.
For this purpose, a 167 bp fragment of the b-globin gene is amplified. Since b-globin gene is present in all the cells (it never undergoes deletions) and is not polymorphic, it is a suitable target for the control PCR [14]. DNA from normal human lymphocytes were extracted and used positive control for b-globin (50 ng/ml). Amplification Thermal cycling for b-globin gene as follows: 94°C 10” + 5 × (94°C 60”, 52°C 60”, 72°C 60”) + 35 × (94°C 30” 52°C 30” 72°C 30”) + 72°C 5’. [15]

**D-PCR for determination of BAT 25 & BAT 26**

The specific oligonucleotide primer sequences were used in conventional PCR to detect the presence of BAT 25 and BAT 26 gene as shown in table (1). To produce a DNA fragment of 124 base pair for BAT 25 and 121bp for BAT 26, primers were diluted by adding nuclease free water according to the manufacturer instructions. The master mix contents were thawed at room temperature before use, and the PCR master mix was made on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination. For each reaction within each single pre-mixed PCR reaction tube, 0.5µl from forward and reverse primers were added. Five µl of DNA template was added for each reaction tube. Twelve and a half µl of GoTaq® Green Master Mix was added for each reaction tube, the volume was completed to 50µl with Deionized Nuclease–Free distal water and tubes were then spun down with a mini centrifuge to ensure adequate mixing of the reaction components.

Thermal cycling: 94°C 10min’ + 5 × (94°C 60S”, 55°C 60S”, 72°C 60S”) + 35 × (94°C 30S” 55°C 30S” 72°C 30S”) + 72°C 5min’

**Table 1: Primers Sequences for Microsatellites instability.**

<table>
<thead>
<tr>
<th>No</th>
<th>Microsatellite marker</th>
<th>Gene near marker, gene bank number</th>
<th>Location of repeated</th>
<th>Repeated motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BAT25</td>
<td>e-kit</td>
<td>4q12</td>
<td>TTTT.TTTT.(T)7.A(T)25</td>
</tr>
<tr>
<td>2.</td>
<td>BAT26</td>
<td>hMSH2</td>
<td>2p16.3-p21</td>
<td>(T5)…..(A)26</td>
</tr>
</tbody>
</table>

**E- Gel visualization:**

Mix 8 µl of PCR product with 2 µl of 6× loading buffer; load on a 2% agarose gel prepared with 1× TBE containing 0.5 mg/ml ethidium bromide. Five µl of the 100
bp DNA ladder is specially designed for determining the size of double strand DNA were mixed with one µl of blue/orange 6X loading dye and subjected to electrophoresis. Served as marker during PCR products electrophoresis amplicon visualization was performed using an UV light transilluminator and then photographed using digital camera (Sony-Japan).

F- Statistical analysis:

The significance of differences in proportions analyzed by Fisher’s exact test. Entry of data into the computer and the Fisher exact tests performed with SPSS version 20 and p values equal or less than 0.001 considered statistically significant.

Results

Fifty tissue samples from female patients with invasive ductal breast carcinoma (IDC) enrolled in this study, the age ranged from 26-42 years old. In addition to 20 females with benign breast tumor used in this study as negative control.

All samples tested for MSI by singleplex PCR reactions using two microsatellite markers BAT 25 and BAT 26. The internal control B-globin appeared in the region 167 bp as shown in (figure 1) while BAT26 in 121 bp and BAT25 in 124 bp.

PCR demonstrating microsatellite instability in 13 (26%) of the 50 breast cancer sample. Eight (16%) of 50 breast cancer sample were BAT 25 positive with a PCR product size of 124 bp as shown in (figure 2), while 5 (10%) of 50 breast cancer sample were BAT 26 positive with a PCR product size 121bp as shown in (figure 3). Two out of 13 samples were positive for both BAT 25 and BAT 26, the remainder 37 (74%) out of 50 samples showed a microsatellite stability. All benign breast tumor samples showed negative result for MSI and give 100% for microsatellite stability.

Statistical analysis showed presence of significant differences ($p < 0.001$) toward the role of MSI in invasive ductal breast carcinoma when compared with negative control. However, the results showed there is no correlation of the presence of MSI in IDC with the age of the patient. This study also trying to find an association between the stage of IDC and the existence of MSI but the statistical analysis showed no correlation between these variants.

Regarding the differentiation of the cell in tissue sample of IDC, it was well in all samples and when compared between positive samples of MSI with negative one in this parameter, the results showed no significant differences between the two groups.
Figure 1: Gel electrophoresis (2% agarose, 7 v/cm², 1. hrs) of PCR positive products for \( b\)-globin 167bp, L1:-25/100bp DNA ladder was used, L2 positive control.

Figure 2: Gel electrophoresis (2% agarose, 7 v/cm², 1. hrs) of PCR positive products for BAT25 was shown in 124bp, L1: - 25/100bp DNA ladder was used, L2 positive control.

Figure 3: Gel electrophoresis (2% agarose, 7 v/cm², 1. hrs) of PCR positive products for BAT26 was shown in 121bp, L1: - 25/100bp DNA ladder was used, L2 positive control.

Discussion

DNA mismatch repair is a system for recognizing and repairing erroneous insertion, deletion, and mis-incorporation of bases that can arise during DNA replication and recombination. Mismatch repair is strand-specific. During DNA synthesis the newly synthesised (daughter) strand will commonly include errors. In order to begin repair, the mismatch repair machinery distinguishes the newly synthesised strand from the template (parental). Any defects in this system cause an error in the replication of simple nucleotide repeat segments. This condition is commonly known as microsatellite instability (MSI) because of the frequent mutations of microsatellite sequences [16]
Microsatellite instability is associated with many tumor such as endometrial cancers, colon, gastric and pancreatic cancer. There are many studies investigating microsatellite instability in breast carcinoma at different loci by using different microsatellite markers but still inconsistent. [17,18]

In this study we have detected microsatellite instability (MSI) at the BAT 25 and BAT 26 in 13 patients out of 50 patients. These data provide firm evidence that the instability seen was specific to the breast cancer. This result was similar to many other investigation working in this field as studies by Walsh., et al [19], Adem, et al [20] and Cindy J., et al [21].

Two tumors showed instability at multiple marker BAT 25 and BAT 26, this result may suggest that the instability seen in breast tumours represents a random background instability, this result comes in accordance with those obtained in others studies of Soo-Chin Lee et al [22]; Thomas et al [23]. who found microsatellite instability may be an early event in the genesis of breast cancers.

In summary, we have detected somatic microsatellite instability in 26% of 50 breast cancer. These data may suggest strong evidence that microsatellite instability (MSI) at the BAT 25 and BAT 26 are involved in the pathogenesis of the great majority of breast cancers. But these markers have been seen just as a part of this process.

**Conflict of Interest**

There was no financial support and no conflict of interest in this study.

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**References**


