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

Jabbar Salman Hasan[(1)] , Basim Mohammed Hanon[(2)] , Ahmed Hasan Mohammed[(3)]

(1) Department of Microbiology, Al-Nahrain Faculty of Medicine, Baghdad, Iraq

(2) Department of Microbiology,College of Veterinary Medicine, Wasit University, Kut, Iraq

(3) Pathological Analysis Department, College of Science, Thi-Qar University, Nasiriya, Iraq

Abstract:

Background: Breast cancer is the most common cancer among women worldwide, comprising 23% of the 1.1 million female cancers that newly diagnosed each year.

Aims: Investigate the existence of microsatellite instability (MSI) in breast cancer of patients. Settings and Design: Fifty female patients with invasive ductal breast carcinoma (IDC) collected. 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.

Methods and Material: DNA had be 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 bysingleplex 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.

Statistical analysis used: The significance of differences in proportions was analysed by the Fisher's exact testwith SPSS version 20 and P values equal or less than 0.001 considered statistically significant.

Results: 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.

Conclusions: The result suggest 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.

Key-words: Breast Cancer, Microsatellite Instability, PCR

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التحري في عدم استقرار السواتل الجينية الصغرى (Microsatellite Instability) للجينات BAT 25 وBAT 26في مرض سرطان الثدي عن طريق تقنية تفاعل البلمرة الاعتيادي

جبار سلمان حسان[1] ، باسم محمد حنون[2] ، أحمد حسن محمد[3]

ahmedhasan@sci.utq.edu.iq , basimdr28@yahoo.com , jabbarsalman30@yahoo.com

(1) قسم الاحياء المجهرية – كلية الطب – جامعة النهرين – بغداد – العراق

(2) قسم الاحياء المجهرية – كلية الطب البيطري – جامعة واسط – الكوت – العراق

(3) قسم التحليلات المرضية – كلية العلوم – جامعة ذي قار – الناصرية – العراق

الخلاصة:

يعتبر سرطان الثدي من الامراض التي تنشأ عن اسباب مختلفة . واحدة من اهم العوامل التي تؤدي الى ظهور المرض وتطورة هي التغيرات الجينية ويعتبر Microsatellite instability (MSI) والذي هو عدم الاستقرار الجيني المرتبط بخلل في عملية اصلاح الحامض النووي . تهدف هذة الدراسة الى التحري عن وجود Microsatellite instability في كل من BAT 25 and BAT 26 في مجموعة من النساء المصابات ب سرطان الثدي .تماستخراجالحمضالنوويمنعيناتالأنسجةالمجمدة الماخوذة من مرضى سرطانالثدي.باستعمال كت الاستخلاص المجهز من شركة كياجين - الولاياتالمتحدةالأمريكية.

اظهرت الدراسة وجود Microsatellite instabilityفي 13 (26٪) من 50 عينة من المرضى و كانتثمانية (16٪) من 50 عينةسرطانالثدي موجبة BAT 25فيحينأن 5 (10٪) من 50 عينةسرطانالثديكانتBAT 26إيجابية

من هذة الدراسة يمكن الاستنتاج علىأنMicrosatellite instability في كل من BAT 25 and BAT 26 لة دور مهم في تطور وتقدم مرض سرطان الثدي.

كلمات البحث: سرطانالثدي،عدماستقرارميكروساتليت

1- Introduction:

The development of breast cancer 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 alteration 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 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 mutation 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 DNApolymerases, 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 tumours, with sensitivity and specificity approaching 94%–98% for both markers [10]. The quasimonomorphic feature of these markers, defined as little or no polymorphism in these loci across all ethnic populations, allows the testing of tumour tissue without the need for a corresponding normal control[11]. However, some unstable tumours may have stable BAT26 loci due to a large intragenic MSH2 deletion, causing complete absence of the BAT26 loci in the tumour 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 in invasive ductal breast carcinoma (IDC) and its correlation with clinical and pathological parameters.

The objective of this study was detection of MSI in the BAT25 and BAT26 loci of DNA samples extracted from invasive ductal breast carcinoma tissue sample and amplification the product by singleplex PCR.

2- Subjects, Materials and Methods:

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 -20ºC for DNA extraction and PCR.

DNA Extraction

DNA had been extracted from frozen tumor of breast tissues. This protocol done 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, for good PCR-SSP results, DNA is required with an A260/A280 quotient of 1.6 or greater. The sections of tumor tissue should contain more than 50% of neoplastic cells (13) in order to avoid false negatives. Microsatellite marker amplifications are performed as singleplex 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 sample. DNA 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.] Positive control for b-globin: DNA from normal human lymphocytes, 50 ng/ml[15.]

PCR PreMix*AccuPower*® Bioneer, South Korea

The powerful technology for convenient and easy to perform DNA amplification. It contains DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a

pellet. 25/100 bp Mixed DNA Ladder is specially designed for determining the size of double strand DNA from 25 to 2,000 base pairs. The DNA Ladder consists of 17 double strand DNA fragments ranging in size from 25 to 200 bp in 25 bp increments. Single PCR reactions were conducted to amplify the two loci BAT 25 and BAT 26 for screening breast cancer patients

The PCR reaction was performed in a final volume of 50 ml, containing: 0.5 µl b-globin both forward primer, reverse primer (primer designed in Alpha DNA, Canada) (table 1) 30 pmol/ml. 0.3 pmol/µl final, 1 µl of diluted sample DNA, 20 µl mister mix, H2O to volume, Overlay the reaction mixture with 20 ml of mineral oil.

Thermal cycling: 94°C 10 min. + 5 × (94°C 60 sec., 52°C 60sec., 72°C 60sec.) + 35 × (94°C 30 sec. 52°C 30 sec. 72°C 30 sec.) + 72°C 5 min.

Prepare a different master mix for each microsatellite marker (singleplex). PCR was performed in a final volume of 50 µl, containing: 20 µl of master mix, 0.5 µl forward primer and reverse primer, 30 pmol/ml 0.3 pmol/ml final 0.5 µl, (50 ng/ml) 1 µl of diluted sample DNA or 1 µl of undiluted negative control or 1 µl of positive control for amplification and H_2O to volume, Overlay reaction mixture with 20 µl of mineral oil.

Thermal cycling: 94°C 10min. + 5 × (94°C 60 sec., 55°C 60 sec., 72°C 60 sec.) + 35 × (94°C 30 sec. 55°C 30 sec. 72°C 30 sec.) + 72°C 5min'

Table 1: Primers Sequences for Microsatellites instability.

Marker name	Genomic position	Sequences (5′–3′)	*T* °m	Product (bp)
b-globin	11p15.5	F: ACACAACTGTGTTCACTAGC R: GAAAATAGACCAATAGGCAG	58 56	167
BAT25	4q12-4q12	F: TCGCCTCCAAGAATGTAAGT R: TCTGCATTTTAACTATGGCTC	59.7 57.0	124
BAT26	2p16.3-2p16.3	F: TGACTACTTTTGACTTCAGCC R: AACCATTCAACATTTTTAACCC	57. 59.0	121

3- Gel visualization

Mix 10 ml 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. Run at 80 V constant until bromophenol blue reaches 1/2 of the gel. Inspect under a UV source. A single band should be visible in the sample.

4- Statistical analysis:

The significance of differences in proportions was analysed 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.

5- 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 female with benign breast tumor used in this study as negative control.

All samples tested for MSI bysingleplex 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 sample of MSI with negative one in this parameter, the results showed no significant differences between the two groups.

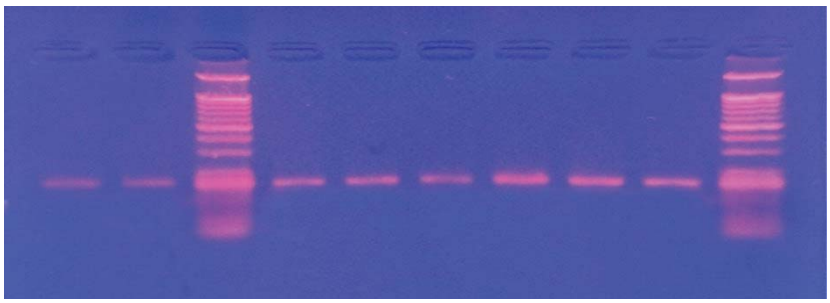

Fig.(1): Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for b-globin 167bp, L1:-25/100bp DNA ladder was used, L2 positive control.

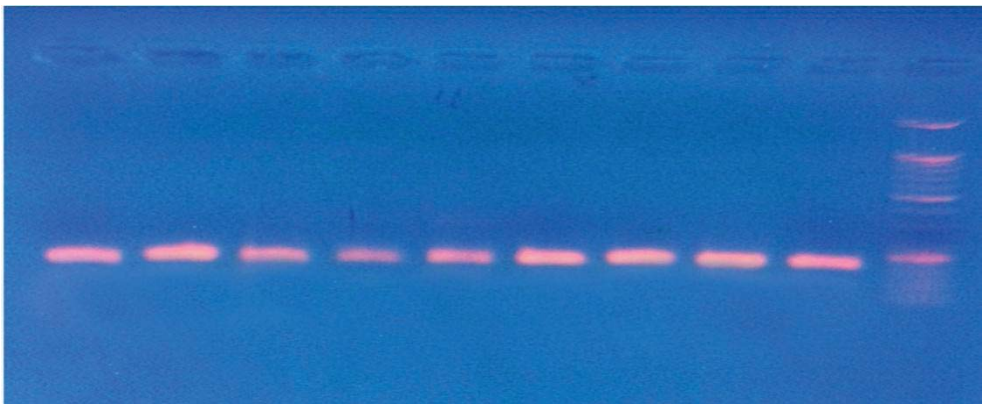

Fig.(2): Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for BAT25 was shown in 124bp, L1:-25/100bp DNA ladder was used, L2 positive control.

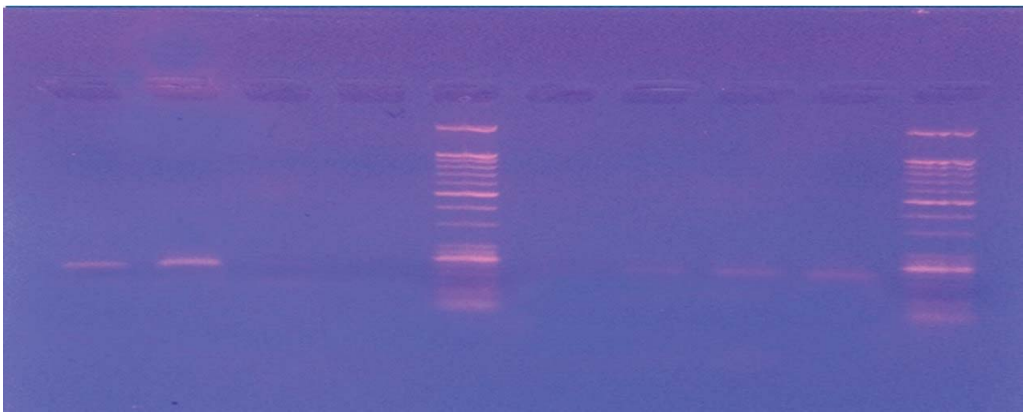

Fig.(3): Gel electrophoresis (2% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for BAT26 was shown in 121bp, L1:-25/100bp DNA ladder was used, L2 positive control.

6- 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 causes an errors 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 and 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].

Study done by Soo-Chin Lee[22], in which 8 breast cancer specimen were examine show all eight cases were microsatellite stable for all markers that amplified. It also is in concordance with the only other systematic study examining the incidence of MSI in medullary breast cancer in which 24 medullary breast cancer specimens were examined, and only two were microsatellite instable.

This discrepancy probably due to the methods of sample homogenization, attributed to inappropriate choice of microsatellite markers, because the mechanisms leading to MSI may differ between different microsatellite markers.

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]. Siah*et al*, [24] in a review of more than 300 microsatellite loci in breast tumors, reported that the size of the microsatellite repeat unit was not associated with the frequency of detecting MSI. Siah*et al*[24]suggested that some loci may be more informative than others in the analysis of breast cancer.

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 marker has be seen just as a part of this process.

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