

## The Using of BAT25 Marker for Detection of Microsatellite Instability in Malignant and Benign Breast Tumors

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**Abstract:** Microsatellites are small DNA sequences repeat (1 to 6 base pairs in length) spread throughout the genome. Microsatellite instability (MSI) is the spontaneous loss or gain of nucleotides from repetitive deoxyribonucleic acid (DNA) segments. This study was aimed to detect MSI in benign breast tumor (fibroadenoma) and breast cancer (ductal carcinoma) by using BAT25 marker. The formalin fixed paraffin embedded (FFPE) samples were taken from 21 cases with benign tumor and 23 cases with malignant breast tumor. Patients' age range was 16-72 year with mean of (43 ± 2.1 SEM. years). Tumor and corresponding normal DNA of the same patient was extracted from about 25mg of FFPE samples. Conventional PCR was used for amplification of BAT25 locus the detection of MSI was done by using high resolution (HR) agarose. The results revealed that the incidence of at BAT25 locus in benign cases was (14%), while the incidence of MSI at BAT25 locus in malignant cases was (13%). Moreover, no significant ( $P > 0.05$ ) correlation was found between MSI and age. As a conclusion, MSI represented by BAT25 marker can be detected in benign and malignant breast tumor.

**Keywords:** Breast cancer, Microsatellite instability, BAT25, PCR, high resolution agarose.

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### I. Introduction

Microsatellite are genetic loci that consist of sequential repeats of a short motif of one to six nucleotide bases. The number of tandem repeats varies from lower than ten to hundreds or even thousands of successive copies. Microsatellites are characterized as greatly polymorphic and unstable motifs, in humans most common repeats are dinucleotide repeats (CA)<sub>n</sub>/(GT)<sub>n</sub> and mononucleotide repeats (A)<sub>n</sub>/(T)<sub>n</sub> [1].

The loss or gain of nucleotides from repetitive DNA tracts is defined as microsatellite instability (MSI). It is a diagnostic phenotype for endometrial, gastrointestinal and colorectal tumors, yet the instability events across a various types of cancer remains poorly understood. MSI can arise from abnormal function in the mismatch repair (MMR) system, which limits correction of spontaneous mutations in repetitive DNA sequences [2][3].

Breast cancer is a multifactorial disease that compose of different tumor subtypes that different in prognosis and response to drug [4]. World Health Organization (WHO) 2013 announced that breast cancer is the most commonly diagnosed cancer among women in the great majority (140 of 184) of countries worldwide, making it the merely cancer that is common among women in all regions of the world and is the most commonly diagnosed cancer in women worldwide with approximately 1.7 million new cases diagnosed in 2012, accounting for 25% of all new cancer cases in women [5].

Current classification methods of breast cancer group tumors into genetically- and molecularly-defined intrinsic sub-types including, but not limited to: luminal A, luminal B, HER2-overexpressing, and triple negative cancers [6]. Benign breast disorders encompass a heterogeneous group of conditions. These conditions include masses, cysts, abnormalities detected by imaging, nipple discharge, breast pain (mastalgia), inflammatory breast disease, and skin disorders of the breast [7].

Fibroadenomas are the most common cause of breast masses in adolescent girls and young women. The median age at which patients present with fibroadenomas is 25 years. Fibroadenomas also can be present in older women, accounting for 12% of all masses in menopausal women [8].

c-kit, also known as KIT or CD117, is a proto-oncogene that encodes a transmembrane tyrosine kinase receptor, which is a type 3 transmembrane receptor for mast cell growth. C-kit was first identified as the cellular homologue of the feline sarcoma viral oncogene v-kit. Mutations in this gene are associated with various types of malignant tumors. C-kit expression was shown to be reduced in breast carcinomas whereas normal epithelium showed an almost 100% expression [9].

## II. Material and Methods

FFPE samples of 44 females with breast tumor were collected from the archive of Al-Yarmook hospital and Baghdad medical city in Baghdad through the period from November 2016 to May 2017. These samples belong to newly diagnosed (untreated) patients with an age range of 16-72 year. The tumor and normal tissue have been chosen from the same patient. Thirty two malignant cases and 21 benign case were diagnosed and enrolled in this study. Ethical permission to conduct the research was obtained from Al-Nahrain Biotechnology College and from these institutions.

The patients data information for each patient were obtained, which included: name, age, address, and laboratory data, from medical records. Pathological data including: histologic tumor type and tumor grade were revised and confirmed by a specialist histopathologist.

Amplification of microsatellite region was achieved by using simple sequence repeats (SSRs) specific primer of BAT25 locus. This marker have been chosen from five markers from a panel of markers that were selected by a National Cancer Institute in this study and supplied by MacroGen Company (USA) in a lyophilized form. Lyophilized primers have been dissolved in a free DNase/RNase water to give a final concentration of 100 pmol/μl as a stock solution.

**Table (1): Oligonucleotide primer used for the amplification of BAT25 locus**

Primer		Sequence (5'- 3')	tm	Product Size(bp)	Location
BAT25	Forward (20b)	TCGCCTCCAAGAATGTAAG T	56.4	110-130	c-kit gene 4q12
	Reverse (21b)	TCTGCATTTTAACTATGGCT C	55.5		

Total genomic DNA was extracted from 88 FFPE samples collected from the archive of females' breast tumors. DNA extraction was carried out by using gSYNCT<sup>TM</sup>100 DNA extraction kit supplied by Geneaid/Taiwan. PCR was carried out in a total volume of 25 μl using Techne (tc-5000) (UK). The reaction components are indicated in Table (2). Extracted DNA from FFPE samples and BAT25 marker were used in PCR.

**Table (2): Components of reaction mixture for BAT25 marker.**

Component	Volume (μl)
Master Mix: <i>Taq</i> DNA Polymerase, dNTPs, MgCl <sub>2</sub> , and reaction buffer.	12.5
Forward Primer	1
Reverse Primer	1
DNA Template	4
D.W.	6.5

Polymerase chain reaction was done according to the amplification program shown in Table (3), and then amplification products were analyzed on agarose (Promega, USA) gel (2%) in presence of 100pb DNA ladder marker.

**Table (3): PCR amplification program for BAT25 locus.**

Steps	Temperature (°C)	Time	No. of Cycles
Initial Denaturation	94	5min.	1
Denaturation	94	30sec.	35
Annealing	60	30sec.	
Extension	72	30 sec.	
Final Extension	72	10min.	1

Aliquot of 3 μl of pure PCR product solution was loaded into the wells and the electrophoresis was carried out in HR agarose (Benchmark Scientific, USA) for 120 minutes at 70v and 400 mA 5v/cm<sup>2</sup>. DNA bands were visualized by gel documentation system (BIO-RAD, USA).

### Statistical Analysis

The statistical significance of the correlation among various groups was determined by Chi-square test and Fisher exact test by IBM SPSS (statistical package for social sciences) version 23. Differences were considered statistically significant for P-value less than 0.05.

## III. Results

Patient's had been distributed into two groups malignant (ductal carcinoma) and benign

(fibroadenoma) breast tumor according to histopathological report. Malignant group comprised 23 patients (52.3%) with age range from 38 to 72 with mean of (52.34 ± 1.7 SEM. years). Benign group comprised 21 patients (47.7%) with age range from 16-50 with mean of (32.76 ± 2.48 SEM years) as shown in Table (4).

**Table (4) Descriptive Statistics of studied group's ages**

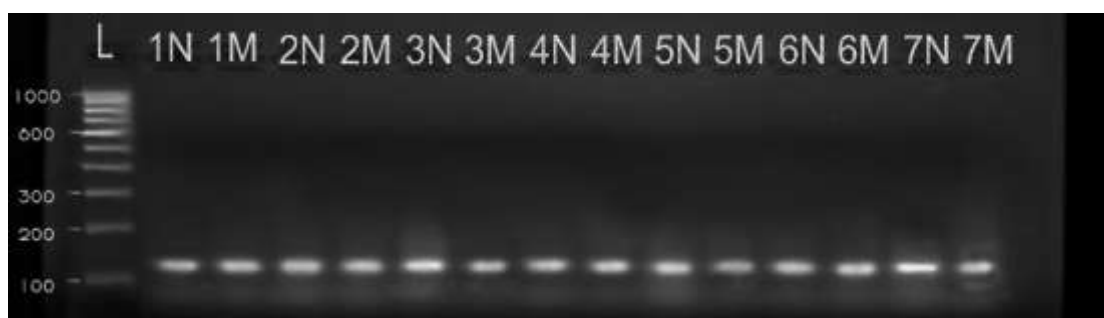
Age	N	Minimum Age	Maximum Age	Mean	SEM
Malignant	23	38	72	52.34	1.7
Benign	21	16	50	32.76	2.48

According to the immunohistochemistry (IHC) test of the hormones receptors status the malignant patients distributed into four groups, luminal A, luminal B, HER2 positive and Basal like. Luminal A group comprised 11 patients (48%), luminal B comprised 3 patients (13%), HER2 positive comprised 4 patients (17%) and basal like comprised 5 patients (22%).

According to histological examination malignant patients distributed to three groups, Grade 1, Grade 2 and Grade 3. Grade 1 group comprised 3 patients (13%), Grade 2 group comprised 13 patients (57%) and Grade 3 group comprised 7 patients (30%).

In this study, BAT25 locus was amplified in the DNA solutions which were extracted from each FFPE samples of patients with breast tumor. PCR was achieved under optimum amplification conditions using the specific primers.

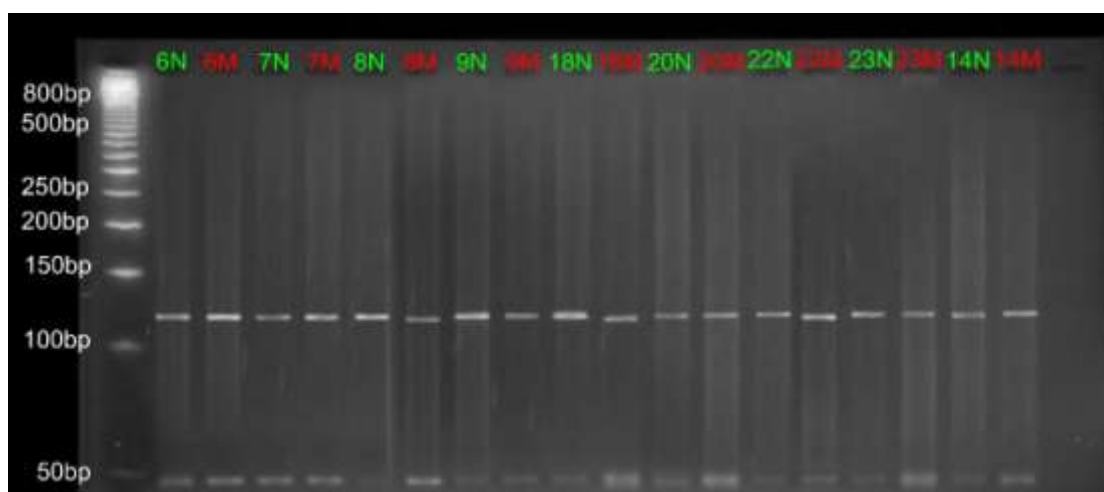
Results illustrated in (Fig.1) showed that the PCR products after electrophoresis on agarose gel (2%) were appeared as clear bands with molecular size of 110 - 130 base pair for each FFPE sample. These fragments represent the region of BAT25 locus [10].



**Figure (1): PCR products of BAT25 locus electrophoresis on agarose gel (2%) at 5v/cm2 volt for 45 minutes in the presence of 100bp Ladder (N): PCR products from DNA of normal tissue of patient. (M): PCR products from DNA of malignant tissue of the same patient.**

Using of HR agarose to detect MSI at BAT25 locus

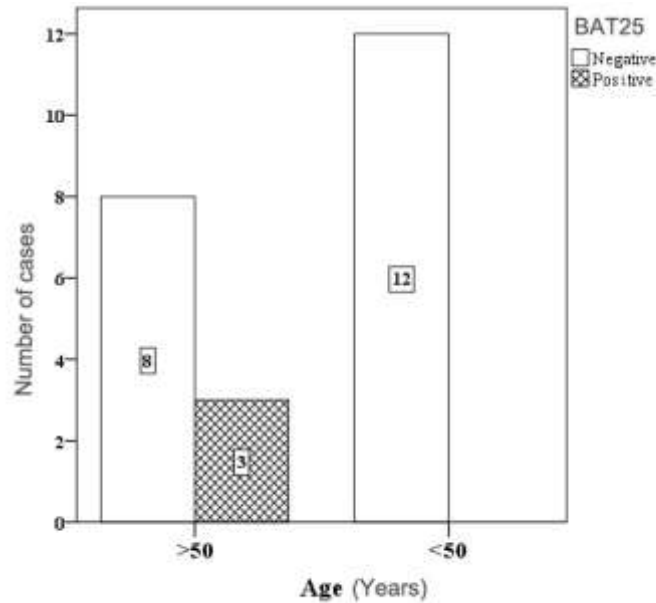
In the current study, the cases No 8, 18 and 22 which represent 3 of 23 (13%) of malignant cases showed MSI at BAT25 locus as shown in Fig. 2.



**Figure (2): PCR products of BAT25 locus electrophoresis on HR agarose gel (5%) at 5v/cm2 volt for 120 minutes in the presence of 50bp Ladder (N): PCR products from DNA of normal tissue of patient. (M):**

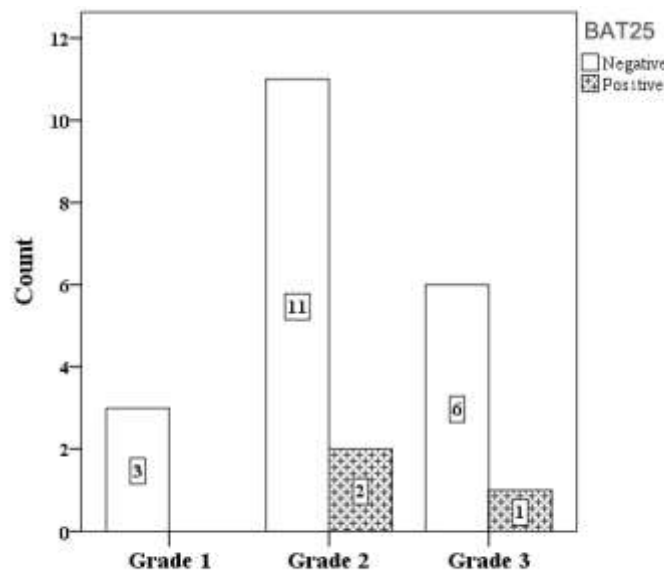
**PCR products from DNA of malignant tissue of the same patient.**

All the MSI cases was above 50 year but statically there is no significant ( $P < 0.05$ ) correlation between MSI at BAT25 locus and age in malignant cases in this study as shown in Fig. 3.



**Figure (3): Bar chart of correlation between age and MSI at BAT25 locus in malignant cases.**

Two of three MSI malignant cases at BAT25 were grade II and one case was grade I. Statically there is no significant ( $P > 0.05$ ) correlation between MSI at BAT25 and grade as shown in Fig. 4.



**Figure (4): Bar chart of correlation between grade and MSI at BAT25 locus in malignant cases.**

Two of three MSI cases at BAT25 were Luminal A and the other one was Basal like. Statically there is no significant ( $P > 0.05$ ) correlation between MSI at BAT25 and hormone receptor state as shown in Fig. 5.

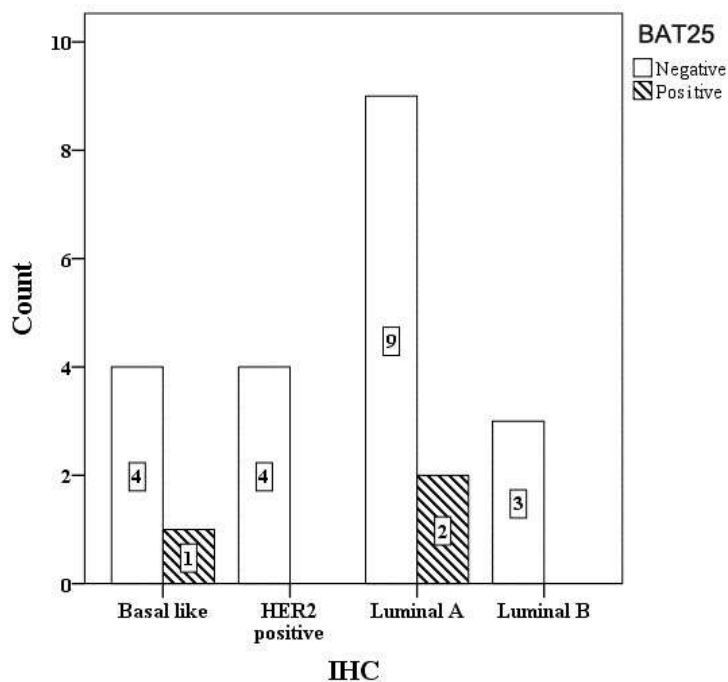


Figure (5): Bar chart of correlation between IHC and MSI at BAT25 locus in malignant cases

The cases No 9, 11 and 14 which represent 3 of 21 (14%) of benign cases showed MSI at BAT25 locus as shown in Fig. 6.

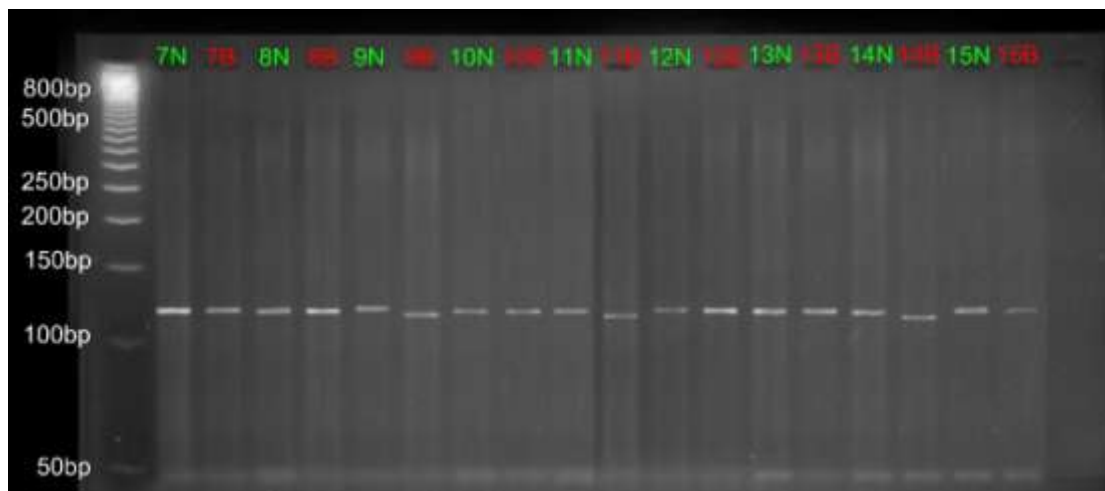


Figure (6): PCR products of BAT25 locus electrophoresis on HR agarose gel (5%) at 5v/cm2 volt for 120 minutes in the presence of 50bp Ladder (N): PCR products from DNA of normal tissue of patient. (B): PCR products from DNA of benign tissue of the same patient

Two of three of the MSI cases was above 30 year. Statically there is no significant ( $P>0.05$ ) correlation between MSI at BAT25 locus and age in benign cases in this study as shown in Fig. 7.

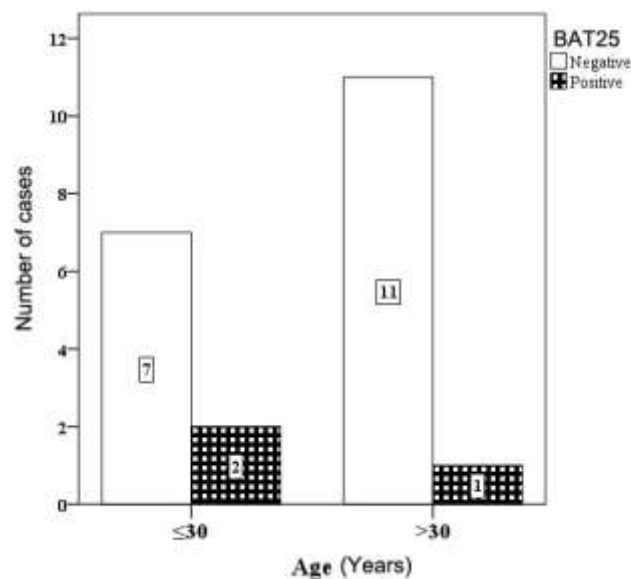


Figure (7): Bar chart of correlation between age and MSI at BAT25 locus in benign cases.

#### IV. Discussion

The genomic instability step is important in the molecular process of tumorigenesis [11]. The MMR system inactivation is responsible for "deficient MMR" phenotype which is characterized by MSI and abnormality in the expression pattern of the MMR proteins in tumor tissue [12].

Microsatellite alterations analysis is a promising and new method to inspect genetic and somatic changes that arise during tumorigenesis [13].

In this study 3 of 23 (13%) of malignant cases showed MSI at BAT25 locus while 3 of 21 (14%) of benign cases showed MSI, so the percentage is similar in both tumors which means that the MSI at BAT25 locus is not specific to malignant cases. Fatimata *et al.* (2015) [14] reported that this locus reveals the sensitivity of MSI which proves itself via decrease of repeated mononucleotide number leading to a reduced size in the majority analysis of cancer tissues and c-kit oncogene BAT25 marker was 89.47% in breast cancer among Senegalese, while Siah *et al.* (2000) [15] found that there was no MSI at BAT25 marker in 66 breast cancer patients with age under 45 years at the time of diagnosis. Demokan *et al.* (2006) [16] observed in head and neck cancer, BAT-25 instability in 15% of cases and Caliman *et al.* (2012) [17] found that MSI at BAT25 locus (37.8%) in ovarian cancer. As shown in Fig.2, the malignant cases No 8, 18, 22 showed deletion on BAT25 so the deletion pattern is obvious in this locus which is agreed with Fatimata *et al.* (2015) [14] as they reported large deletions ranging from 4 to 6 bp and agreed with Pyatt *et al.* (1999) [18] whom demonstrated that BAT25 has sensitivity to MSI, which manifests as decrease of mononucleotide repeats which lead to new alleles with shortened size in common MSI analysis of tumors.

Some studies like Zheng *et al.* (2012) [19] and Buhard *et al.* (2006) [20] reported that BAT25 is quasimonomorphic, it means that it could be used for tumor MSI determination without the requirement for matching normal DNA. But in this study normal and tumor tissue of the same patient were used because there is no study of polymorphism of BAT25 in Iraqi population. Also some recent studies still use normal and tumor tissue of the same patient in MSI studies at BAT25 locus to obtain a correct MSI identification like Watson *et al.* (2016) [21] and Chen *et al.* (2017) [22]. Also the benign cases No (9, 11 and 14) that have MSI as shown in Fig.6 showed decrease in size in BAT25 locus.

The MSI in BAT25 locus was found in malignant cases with older than 50 years while in benign cases the MSI on all cases was below 45 years. Also this result indicates that there is no significant ( $P > 0.05$ ) correlation between MSI on BAT26 with age in breast tumors as shown in Fig.3 and 7), which is agreed with Siah *et al.* (2000) [15] whom found that the age does not correlate with MSI incidence in breast cancer, and it is infrequent in early-onset breast cancer. Ndiaye *et al.* (2017) [23] reported that there is no correlation between age and MSI at BAT25 locus in colorectal cancer. As shown in Figure (4) there is no significant ( $P > 0.05$ ) correlation between MSI at BAT25 and grade in malignant cases. Also there is no significant ( $P > 0.05$ ) correlation between hormone receptor status and MSI at BAT25 as shown in Fig. 5.

As the BAT25 locus includes a 25 repeat of thymine base found in intron 16 of c-kit oncogene which is located on chromosome 4 in the position 12 (4q12), so the MSI in this locus may be correlated to expression of c-kit. Eroglu & Sari (2007) [24] showed that c-kit proto-oncogene product expression in breast cancer was significantly increased compared to those of fibroadenoma.

## V. Conclusion

From the results of the current study, it can be concluded that the MSI at BAT25 can be found in malignant and benign breast tumor. There is no significant ( $P>0.05$ ) correlation between MSI at BAT25 and age in malignant and benign cases. No significant ( $P>0.05$ ) correlation between grade and MSI at BAT25 in malignant case. Also there is no significant ( $P>0.05$ ) correlation between MSI at BAT25 and hormone receptor status in malignant cases.

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