Identification of Genomic Markers By RAPD-PCR Primers in Iraq Breast Cancer Patients

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Abstract

Random Amplification of Polymorphic DNA (RAPD) analysis was used in this study to direct the attention toward increasing the efficiency of early diagnosis of breast cancer in clinical laboratories at Iraq using recent PCR-dependent protocols and investigate DNA polymorphisms in addition to the detection of genomic markers. Blood samples were collected from 12 diagnosed females with breast cancer (malignant) patients, 12 females with breast benign tumor and 12 controls (normal females). DNA was extracted and RAPD-PCR was performed. The results showed unique profiles of amplified DNA fragments produced in genomic DNA of breast tumors by an arbitrary primers of A8, A11, A12, A13, A15 and A18. Out of the 6 primers used, 1 primer produced monomorphic bands namely primer A13. While other 5 primers produced polymorphic bands. Efficiency and discriminatory power of the polymorphic primers ranged from 0.173-0.057 and 9.5-28.5% respectively. Primer A18 produced the highest number of bands (21 bands) and primer A15 produced the largest molecular band (3.204 Kb) while primer A8 and A15 produced the lowest number of bands (11 bands), primer A11 produced the smallest molecular weight band of (0.166 Kb). Primer A11 produced a band of (0.559 Kb) which showed significant frequency of 100% with Breast benign tumor patients and 83.33% with Breast malignant tumor patients in comparison to control individuals which is completely absent. So, the detected DNA polymorphisms by the arbitrary primers might find application in developing efficient RAPD primer for breast cancer early diagnosis in clinical laboratories at Iraq.

Keywords: Breast cancer, RAPD, PCR, Primer, Polymorphic

التحري الجزيئي عن التغايرات الوراثية في مرضى سرطان الثدي في العراق باستخدام تضاعف الدنا متعدد الاشكال العشوائي لانزيم بلمرة الحامض النووي

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الخلاصة

تهدف هذه الدراسة إلى إيجاد مؤشرات وراثية من الحامض النووي ذات علاقة بمرض سرطان الثدي وذلك باستخدام تضاعف الحامض النووي متعدد الأشكال العشوائي لإنزيم بلمرة الحامض النووي وذلك لزيادة كفاءة الفحص المبكر لهذا المرض في العراق. جمعت نماذج الدم من 12 أنثى مصابة بسرطان الثدي (ورم خبيث) ومن 12 أنثى مصابة بورم الثدي الحميد ومن 12 أنثى غير مصابة كنماذج سيطرة. بعد ذلك تم استخلاص الحامض النووي وطبقت عليه تقنية تفاعل البلمرة حيث تم استخدام ست بادئات عشوائية مختلفة. أظهرت

النتائج انه من بين الـ 6 بادئات التي استخدمت في هذه الدراسة، أعطى باديء واحد نمط تضاعفي غير متغاير في حين أعطت الخمس بادئات الأخرى نمط تضاعفي متعدد الأشكال وهي A8, A11, A12, A15 و A18، حيث أعطت هذه البرايمرات قيمة كفاءة تتراوح بين 0.057−0.173 أما القوة التمييزية كانت تتراوح بين 9.5− 28.5% وأعطى الباديء A18 اكبر عدد من الحزم (21 حزمة) وأعطى برايمر A15 اكبر حجم بين الحزم المتضاعفة (3.204 كيلو قاعده) بينما أعطى البادئان A8 و A15 اقل عدد من الحزم وسجلت للباديء A11 اصغر حزمة متضاعفة بحجم (0.166 كيلو قاعده). نتج من استخدام الباديء A11 حزمة بحجم (0.559 كيلو قاعده) وبتكرار 100% في الإناث المصابات بورم الثدي الخبيث وبتكرار 83.33% في الإناث المصابات بورم الثدي الخبيث في حين غابت هذه الحزمة تماما في نماذج السيطرة. نستنتج بأنه يمكن التحري عن التغايرات الوراثية في مرضى أورام الثدي باستخدام طريقة تضاعف الحامض النووي متعدد الأشكال العشوائي لإنزيم بلمرة الحامض النووي وكذلك بالإمكان إيجاد مؤشرات وراثية من الحامض النووي تساعد في التشخيص المبكر لمرضى أورام الثدي في العراق.

Introduction

Breast cancer is considered the most commonly diagnosed cancer of women and the second leading cause of cancer deaths among women after urinary bladder tumors and malignant lymphomas in developed countries [1,2]. Though there are many developments in health care and disease prevention, breast cancer is considered the major public health issue because of increasing the death toll with this disease in the world. Recently, an analysis shows that the inheritance is the cause of 5-10% of the cases of breast cancer in women, while 90-95% are cases that appear randomly and are not predetermined genetically [3].

In Iraq, breast cancer it the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers according to the Iraqi cancer registry in 2004 [4].

Several years ago, there were many studies in molecular genetics have been extensively achieved to clarify the molecular events related with initiation, development and progression of human cancers [5]. There were many PCR-based methods have been developed to detect mutations, including Random Amplified Polymorphic DNA (RAPD), multiplex PCR, Single Strand Confirmation Polymorphism (SSCP), and Short Tandem Repeats (STR). All the above methods are based on the use of known gene sequences as primers for amplification except RAPD [6,7,8].

RAPD can be defined as a DNA fingerprinting technique based on Polymerase Chain Reaction (PCR) amplification of random fragments of genomic DNA with single short primers of arbitrary nucleotide sequences and It was first developed in 1990, using PCR to randomly amplify anonymous segments of nuclear DNA with an identical pair of primers 10 (G,C rich) in length [9,10], depending on this technique, a single piece of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template [11]. The RAPD method can simply and rapidly detect genetic alterations in the entire genome without knowledge of specific DNA sequence information unlike the microsatellite instability analysis which can only detect specific microsatellite loci [12].

RAPD-PCR analysis has been used as a mean for identifying and investigating genetic instabilities associated with human tumors development and revealed frequent occurrence of genetic polymorphisms in various types of tumors, for example, lung cancer [13], squamous cell carcinoma of the head and neck [14], brain tumor [15], ovarian cancer [16], and breast cancer [17, 18].

In this study, six different random primers were selected, trying to investigate breast cancer genetic and breast benign tumors alterations by using RAPD technique in order to determine genetic markers associated with their genes which may prepare extra efficient tools for breast cancer early diagnosis and help in providing the adequate health management offering such patients and their families valuable prognostic, diagnostic and therapeutic benefits.

Materials and Methods

Blood Samples Collection & DNA Extraction

The present study was performed on 12 diagnosed females with breast cancer (malignant) patients, 12 females with benign breast tumor and 12 control (healthy females).

Their age ranged from 18- 70 years. Samples were collected from donors attending National Center for tumors pre-diagnosis/Medical City, from different regions of Iraq. Three millimeters of blood were obtained from each patient and control, placed in tubes containing anti-coagulant (EDTA) transferred immediately to the laboratory. Genomic DNA was extracted from whole blood within 24 hrs. following the instructions of the DNA purification kit, obtained from Alpha DNA Company. This research was performed in the central laboratory in Biotechnology Department/ College of Science / University of Baghdad.

DNA Amplifications

DNA amplifications were carried out in volume of 25 µL reaction mixture containing 10.3 µL mili Q water, 12.5 µL of Go Taq®Green Master Mix (Promega-USA), with concentration (1X) containing (10mM Tris-HCl with (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP) and 1 unit DNA polymerase), 10 pmol of the primer, and 1.2 µl of template DNA. The mixture was incubated in (MultigeneTM Gradient Thermal Cycler, Labnet International, Korea) programmed for initial denaturation (1 cycle) on 94°C for 5 min followed by 45 cycles, each one consisting of a denaturation step (1 min at 94°C), one annealing step (1 min at 36°C) and an extension step (2 min at 72°C), and an extra extension step was performed for 10 min at 72°C. The amplification was carried out with 6 random primers synthesized by Alpha DNA which were selected for the RAPD amplifications. Primer sequences matched corresponding primers from Operon Technologies Incorporation [19, 20].

Gel Electrophoresis

RAPD-PCR products were analyzed by electrophoresis in a 1.2% agarose gel at 5Volt/cm for 2 hours in 0.5X TBE buffer in the presence of 1Kb DNA ladder (Promega-USA) as a molecular size marker, agarose gel were visulised by staining with Ethedium Bromide 0.5 µg/ml for 20-30 minutes. Gel images were captured using a gel documentation System.

Molecular Weight Analysis

Molecular weight estimations were carried out through Photo-CaptMwt version 10.01 computer soft ware, based on comparing the RAPD-PCR products with the known size of DNA fragments of a 1 Kb DNA ladder (consist of 14 bands from 0.250 to 10.000 Kb).

RAPD Analysis

RAPD analysis was prepared for each PCR products of the primers producing amplification patterns. At first, DNA bands were scored in tables for their presence 1 or absence 0 in the RAPD profiles. Primer efficiency and the discriminatory power percent were calculated as the formulas:

- **Primer efficiency=** number of uncommon (polymorphic) bands for each primer/total number of amplified fragments [21].
- **Primer discriminatory power percent=** the number of uncommon bands for each primer/the number of uncommon bands of all primers [22].

Band frequency was estimated for amplification profiles produced by polymorphic primers only and calculated by counting the no. of samples sharing a specific band within each group. The size of the genome screened was calculated by the addition of the sizes of all the individual loci amplified by the random primers [23].

Results

Thirty six samples (malignants, benigns and controls) were screened by RAPD-PCR in the search for diagnostic markers for breast cancer (malignant) patients and if there is any marker that can give a signal for the possibility of inverting of benign to malignant tumor. Of the 6 primers used, 1 primer produce monomorphic bands namely primer A13 Figure 1.

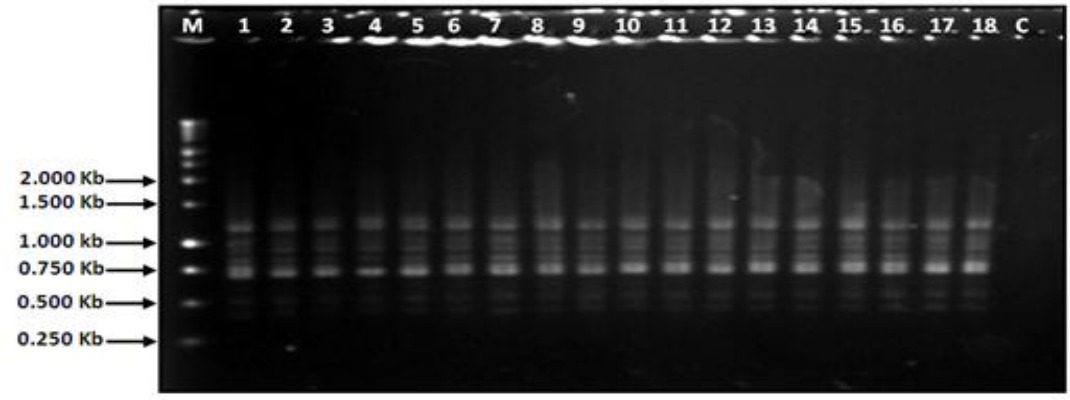

Figure 1- RAPD amplification products generated by primer A13 resolved by electrophoresis in 1.2% agarose gel. M: is 1Kb ladder, lanes 1-6: DNA samples from Breast benign tumor patients, lanes 7-12: DNA samples from Breast malignant tumor patients, lanes 13-18: DNA samples from control subjects and C: negative control.

Other 5primers give polymorphic bands,Table1 lists the primers used in this research, their sequences, and the amplification product. Efficiency and discriminatory power of the polymorphic primers ranged from 0.173-0.057 and 9.5-28.5% respectively Table 2. Primer A18 produced the highest number of bands (21 bands) and primer A15 produced the largest molecular band (3.204Kb) while primer A8 and A15 produced the lowest number of bands (11 bands), primer A11 produced the smallest molecular weight band of (0.166Kb).

Table 1-Primers used for the RAPD analysis, their sequences and the amplification product.

Primer	Sequence 5'-3'	Amplification Pattern
A8	GTGACGTAGG	Polymorphic
A11	CAATGGCCGT	Polymorphic
A12	TCGGCCATAG	Polymorphic
A13	CAGCACCCAC	Monomorphic
A15	TTCCGAACCC	Polymorphic
A18	AGGTGACCGT	Polymorphic

Table 2- Polymorphic primers, efficiency and discriminatory power.

Primer	No. of amplified bands	No. of polymorphic bands	Primer Efficiency	Discriminatory power %
A8	11	4	0.057	9.5
A15	11	6	0.086	14.2
A11	14	12	0.173	28.5
A12	12	10	0.144	23.8
A18	21	10	0.144	23.8

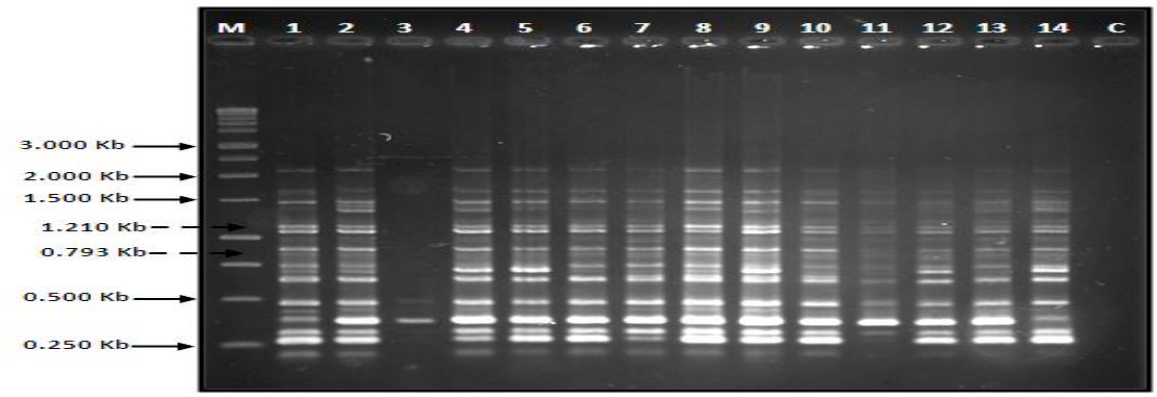

Figure 2- RAPD amplification product generated by primer A18 resolved by electrophoresis in 1.2% agarose gel. M: is 1Kb ladder, lanes 1-6: DNA samples from Breast benign tumor patients, lanes 7-10: DNA samples from Breast malignant tumor patients, lanes 11-14: DNA samples from control subjects and C: negative control.

In general a total of 78 loci were amplified by 6 primers with an average of 13 band per primer, the total length of the genome screened was approximately 70.585Kb. Of the 78 loci, 42 were polymorphic, their size was 38.502 Kb. Polymorphic profile of RAPD amplifications for Breast tumor patients and control subjects with primer A18 are shown in Figure2 in which bands appear with different frequency. Band of 0.793 Kb give a high frequency of 83.33 % in healthy females in comparison with females with tumors Table 3.

Table 3- Frequency of polymorphic bands in patient and control subjects

Primer	Polymorphic band size (Kb)	Frequency of polymorphic bands (100%)		
		Benign	Malignant	Control subjects
A8	**0.636**	50	66.66	41.66
	0.369	41.66	58.33	50
	0.244	41.66	66.66	50
	0.175	91.66	91.66	100
A11	**1.852**	33.33	25	16.66
	1.574	100	83.33	66.66
	1.452	75	83.33	16.66
	1.381	66.66	66.66	16.66
	1.239	0	8.33	0
	0.780	91.66	83.33	83.33
	0.632	100	75	66.66
	0.600	8.33	16.66	8.33
	0.559	100	83.33	0
	0.511	50	41.66	41.66
	0.462	58.33	66.66	33.33
	0.166	8.33	16.66	8.33
A12	**1.047**	8.33	8.33	8.33
	1.020	50	50	50
	0.850	91.66	66.66	100
	0.770	83.33	66.66	91.66
	0.670	91.66	66.66	83.33
	0.630	16.66	16.66	25
	0.517	100	66.66	83.33
	0.439	83.33	66.66	66.66
	0.265	50	58.33	50
	0.220	25	0	25
A15	**3.204**	83.33	91.66	100
	2.734	58.33	75	41.66
	2.529	58.33	66.66	41.66
	1.133	50	58.33	50
	0.764	91.66	91.66	91.66
	0.697	100	91.66	91.66
A18	**1.413**	33.33	25	41.66
	1.382	58.33	50	75
	1.210	75	50	83.33
	1.086	91.66	83.33	91.66
	0.793	25	33.33	83.33
	0.732	66.66	58.33	75
	0.665	66.66	58.33	33.33
	0.428	58.33	25	33.33
	0.392	100	100	91.66
	0.280	83.33	50	66.66

In Figure 3, amplification pattern produced by primer A11 showed significant frequency of 0.559 Kb band in patient (malignant, benign) in comparison to control subjects. This band was present 83.33% in patient with malignant tumors and present 100% with patient with benign tumors while in controls this band was completely absent.

In general polymorphic bands produced by A8, A11, A12, A15, A18 have different frequencies in patients and control subjects Table 3.

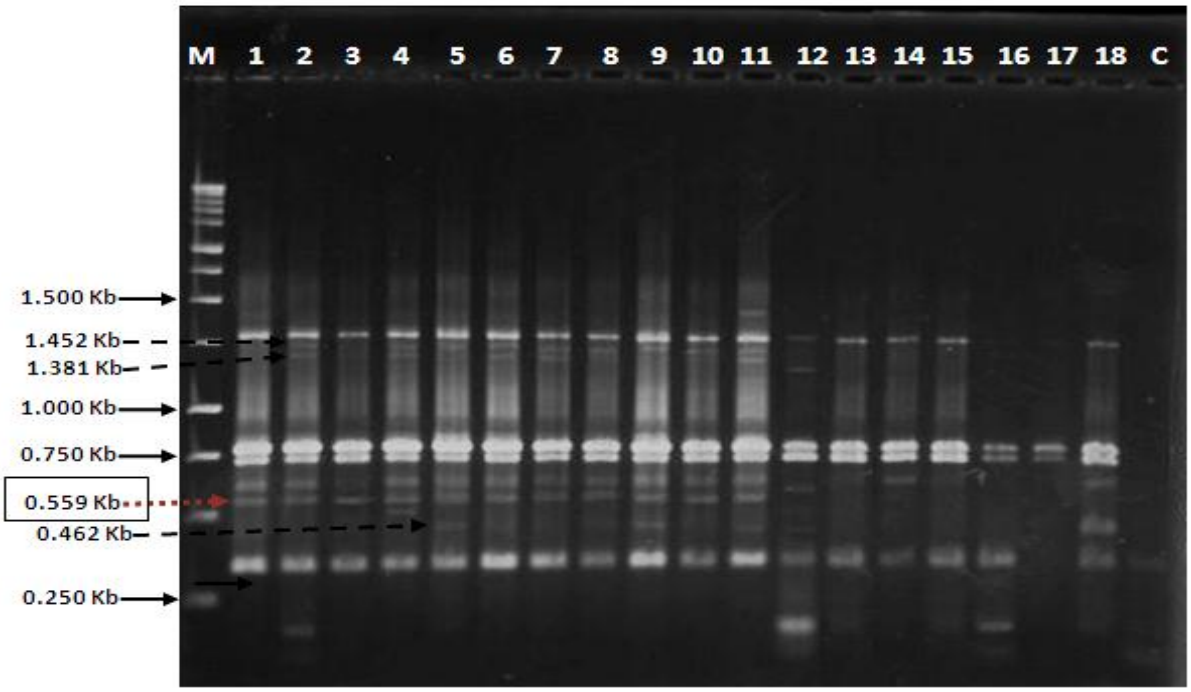

Figure 3- RAPD amplification product generated by primer A11 resolved by electrophoresis in 1.2% agarose gel. M: is 1Kb ladder, lanes 1-6: DNA samples from Breast benign tumor patients, lanes 7-12: DNA samples from Breast malignant tumor patients, lanes 13-18: DNA samples from control subjects and C: negative control.

Discussion

Breast cancer has been traditionally diagnosed in Laboratory by using cell culture and the direct hormone receptor assay, which need a long time to performed. Therefore, clinical laboratories begin to use PCR dependent protocols such as RAPD technique instead of these assays.

The RAPD-PCR analysis has been applied to variety of tumor types to study genomic instability and to identify novel DNA amplifications patterns [12, 13 , 24]. It is worth mentioned the results of our study showed frequent occurrence of DNA polymorphisms in genomic DNA of breast cancer patients in contrast to normal persons.

Polymorphisms between patients with malignant, benign tumors and control subject were detected among the amplification products by 5 out of 6 primers. Highest primer efficiency was produced by A11, primer efficiency values range from 0 to 1 and is defined as the measure of the primer capacity to produce polymorphisms [25].

The primer ability to show polymorphisms in comparison to polymorphisms shown by all primers Is known as primer discriminatory power [22] the primer mentioned above showed high discriminatory power reaching to 28.5% Table 2.

Close frequencies in most of the polymorphic bands between patients and control subjects as shown in Table 3.Is acceptable as most of the genomic DNA is similar and majority of the amplified fragments produced by arbitrary primers are identical between individuals [26] thus monomorphic patterns may reflect amplifications in homogenous genomic regions while slight differences in the percentages of polymorphic bands may be interpreted as heterozygous regions of amplification Figures 1,2 .

Bands such as 1.452 Kb, 1.381 Kb, 0.462 Kb produced by A11 primer and 0.793 Kb band produced by A18 primer are examples of bands

that appear in highly different frequencies in patient in comparison to control subject group and may prove helpful as diagnostic markers in addition to other well identified markers if more studies are conducted to detect their sites and on which gene they are present in the human genome.

Whilst the band 0.559 Kb produced by primer A11 serves best the objective of this study and may be considered as diagnostic DNA marker in patient with breast cancer in view of the fact it appeared in 83.33% of patients with malignant tumor and 100% of patients with benign tumors and was completely absent in control subjects Figure3. However more research is needed to support these results and further identification of DNA fragments need to be conducted to see if they are relevant. Probably a sample with a larger size will offer a better study design, cloning and sequencing of the loci amplified in this study is one way that might reveal mutations of functional importance to the disease and may provide a confirmative conclusion.

The observation of newly added DNA fragment of 0.559 Kb in the genomic DNA of breast cancer might suggest the possibility of using this DNA fragment as a molecular marker for diagnosis and prognosis this disease. So the outcome of present study on genomic DNA of breast cancer showed the possibility of using primer A11 to identify deletion and addition of DNA fragments in genomic DNA of breast cancer patients as compared with normal individuals.

The results of current investigation support the possible application of RAPD-PCR analysis for screening specific molecular markers by using arbitrary primers. These markers might be useful in the diagnosis and to assess prognosis of breast cancer in Iraq.

Conclusion

RAPD decamer primer A11 produced 0.559 Kb DNA fragment in genomic DNA of breast cancer and benign tumor patients, this DNA fragment was not detected in tested normal individuals. The amplified DNA fragment might be proved useful for further development of molecular marker for diagnosis of breast cancer. The study gives new evidence of potential promise of RAPD-PCR analysis in cancer research for detecting and selecting novel genomic markers.

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