Early Diagnosis of Breast Cancer using Molecular, Biochemical and Pathological Markers

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Abstract: Problem statement: Laboratory diagnosis of breast cancer in most of the hospitals has traditionally been performed using cell culture and the direct hormone receptor assay, which are money and time consuming. **Approach:** This study was performed in order to direct the attention toward increasing the efficiency of early diagnosis in clinical laboratories at the western region of KSA and Egypt using recent PCR-dependent protocols i.e., Randomly Amplified Polymorphic DNA (RAPD's) and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Expression of HER4 oncogene using RT-PCR and immunohistochemical (IHC) staining have been assessed as early diagnostic for breast cancer. RT-PCR has detected HER4 expression in 52% of Invasive Duct breast Cancer (IDC) and this expression was significantly correlated with HER4 gene expression by IHC. Also we have selected sixteen 10-mer RAPD primers that have shown high percentage of identity to exons of different human oncogenes, such as V-myc, HER2, HER4, BRCA1 and BRCA2. **Results:** Only 13 out of the selected RAPD primers have revealed 19 distinguishable polymorphic markers between patient and normal females. Moreover, analysis of total protein profile identified extra markers and some differences at the level of Low Molecular Weight (LMW) protein among the two classes of females. **Conclusion:** These data will provide molecular, biochemical and pathological markers that can be used in KSA and Egypt clinical laboratories as additional efficient tools for early diagnosis of breast cancer.

Key words: Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Randomly Amplified Polymorphic DNA (RAPD's), Immunohistochemical (IHC), Transcriptase-Polymerase, Invasive Duct breast Cancer (IDC), Tyrosine Kinase Receptors (RTK), breast cancer

INTRODUCTION

Breast cancer is ranking the first one after urinary bladder tumors and malignant lymphomas (Kumar, 2009). In spite of improvements in health care and disease prevention, breast cancer remains an important cause of mortality in different regions of the world and is a major public health issue. According to a recent analysis, 5-10% of the cases of breast cancer in women can be attributed to inheritance, whereas 90-95% are sporadic, i.e., cases that appear randomly and are not predetermined genetically. Intensive research efforts culminated in the identification of two breast cancer susceptibility genes, BRCA1 and BRCA2, which account for the majority of cases of hereditary breast cancer. BRCA 1 and BRCA2 are the most important breast cancer susceptibility genes identified. A recent meta-analysis of 22 population-based studies of breast cancer estimated the risk by age 70 years in

BRCA1 and BRCA2 carriers to be 65 and 45%, respectively (Antoniou *et al*., 2003). BRCA1 and BRCA2 mutations occur in approximately 20% of families with evidence of inherited susceptibility to breast cancer (Antoniou *et al*., 2003).

BRCA1 and BRCA2 are two high-penetrance breast cancer susceptibility genes, (Lu *et al*., 2007). BRCA1 gene is a human gene located on the long arm of the seventeenth chromosome,(17q21).This gene, called the breast cancer susceptibility gene 1 (BRCA1), encodes a protein involved in many nuclear processes related to transcription, chromatin remodeling, gene silencing and DNA repair (Mahfouz *et al*., 2010 and Starita and Parvin, 2003). BRCA1 gene composed of 22 exons and 21 introns covering approximately 100KB. BRCA1 gene encodes a 1,863 amino acid. BRCA2 gene located on the long arm of the thirteenth chromosome at position 12.3, (13q 12.3). The BRCA2 is also a tumor suppressor gene involved in the repair of

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chromosomal damage. BRCA2 gene composed of 27 exons encoding a protein of 3418 amino acids. The structures of both genes are very different, but their functions appear to be similar (Cabarcas *et al*., 2010 and Lee-Hoeflich *et al*., 2008). Most of cancers due to hereditary mutations involve BRCA1 and the close homologous BRCA2 gene. Women carrying mutations in the BRCA1 or BRCA2 genes are subjected to high risk of breast cancer development, being 7% in the cases due to alterations of these two genes (Butcher *et al*., 2004).

The type 1 Tyrosine Kinase Receptors (RTK) are a group of four growth factor receptors HER1(c-erbB1/epidermal growth factor receptor), HER2(c-erbB2/neu), HER3(c-erbB3) and HER4(c-erbB4) characterized by their homology to the avian erythroblastosis virus transforming protein (Yarden *et al*., 2001), which have a significant influence on the prognosis of invasive breast cancer.

However, although breast cancer is one of the most intensely studied human tumors, the genetic analyses of chromosomal alterations and gene mutations have not established the critical sequence of events that lead to the development of sporadic breast cancer (Wijdan, 2009; Pickl and Ries, 2009 and Diermeier-Daucher *et al*., 2008). It is possible that genetic heterogeneity may have obscured the basis for sporadic breast cancer and may explain, at least in part, why the genetic alterations are still not understood completely.

Whether genetic or sporadic in origin, the conversion of normal cells to malignant cancer cells requires multiple alterations at both the gene and chromosome levels (Hollmen *et al*., 2009 and Tovey *et al*., 2006). Evidence in favor of this concept comes from the observation of multiple genetic alterations such as deletions, insertions, amplifications, rearrangement, recombination and point mutations in the tumor genome. Identification and characterization of all possible genetic alterations in the genome that predispose to the development of breast cancer are of at most importance in gaining a complete understanding of the exact molecular events involved in the development of tumor-genesis. Knowledge of all the genetic alterations in the genome will allow for a better interpretation of the interplay between genes that is involved in the induction of breast cancer. Several PCR-based methods have been developed to detect mutations, including random amplified polymorphic DNA (RAPD), also called arbitrarily primed PCR (AP-PCR); multiplex PCR; Single Strand Confirmation Polymorphism (SSCP); and Short Tandem Repeats (STR). All of these methods, except for AP-PCR/RAPD, are based on the use of known gene sequences as primers for amplification (Mufti *et al*., 2009; Jones, 2008 and Naresh *et al*., 2006).

In this study, we have selected 16 different random primers that have showed high percentage of identity to different exons of human oncogenes such as Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (myc); ; H. sapiens v-erb-b2 erythroblastic leukemia viral oncogene homologe 2 (avian) (ERBB2 or HER2); H. sapiens v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) (ERBB4 or HER4); H. sapiens breast cancer 1 (BRCA1) and H. sapiens breast cancer 2 (BRCA2)), to be used for detection any molecular polymorphism between patient and normal females. Also RT-PCR, immunohistochemistry and total protein profile analysis techniques have been applied in order to prepare extra efficient tools for breast cancer early diagnosis.

MATERIALS AND METHODS

Patients: The present study was performed on 17 diagnosed female breast cancer patients (12 from KSA and the rest from Egypt) and 15 normal females with family history with breast cancer (11 of them from KSA and the rest from Egypt). Their age ranged from 37-65 years. The tumor tissues were fixed in 10% neutral buffered formalin (18-24 h) and processed for histological, immunohistological, DNA and RNA extraction.

Histological diagnosis: For histological diagnosis, tissues were fixed in 4% phosphate-buffer formalin and routinely processed to wax. Paraffin sections (5 μm) were stained with Heamatoxilene and eosin and examined with the light microscope. For electron microscopy examination, paraffin embedded tissues (small pieces of tissue about 1 mm) were fixed in 2.5% phosphate-buffered glutaraldehyde for 4 h at 4°C. The tissues were subsequently osmicated, dehydrated and embedded in resin. Ultra thin sections having a thickness of 70-80 nm were counterstained with lead citrate and uranyl acetate before being examined by transmission electron microscope (Bancroft and Gamble, 2007).

Immunostaining: HER4 protein expression was detected immunohistochemically using autostainer machines. HER4 was categorized into negative showing membrane staining (<10% of the tumor cells) and positive showing staining of the entire membrane (>10% of the tumor cells) (Boenish *et al*., 2001).

DNA extraction and PCR analysis: DNA extraction from paraffin sections was carried out according to Coombs *et al*. (1999). Gene copy determination using Polymerase Chain Reaction (PCR) was followed according to (Lőnn *et al*., 1995). A master mix of PCR reagents was prepared containing 100 μL 10 mL^{-1} of 10x buffer, 200 mM of dNTPase, 1 mm of each

primer, 6 μL 25 mm MgCℓ2, 5 μL Taq DNA polymerase (2.5μ). Then the correct volume of 100 μL master mix is added to each sample (1 μg). Finally, the samples were loaded in the thermal cycler blocks. PCR was performed in the thermal cycler, Roobycycler gradient 96 Strata gene. Initially, samples were heated for 5 min at 94°C for denaturation and then cycled 35 times at 94°C for 1 min, 36°C for 1 min and 72°C for 1 min, followed by an extension cycle at 72°C for 5 min.

RNA isolation: Total RNA was isolated from breast sections using RNeasy total RNA isolation kit (Qiagen, West Stussex, UK).

RT-PCR: The access RT-PCR system (Promega Corporation, USA), was used for one-step RT-PCR, β-actin primers were used in each RT-PCR reaction as RNA loading control. The primers used for amplification of β-actin and HER4 are shown in Table 1. To ensure exponential phase amplification, the number of PCR cycles was determined and optimized at 30 cycles. RT-PCR was performed in a final volume of 25 μL, in the presence of 10 pm of each primer, 0.2 mm dNTP mixture, 1 mm MgSO4, 2.5 units of AMV reverse transcriptase and 2.5 units of Taq-DNA polymerase. cDNA synthesis was carried out at 48° for 45 min, followed by 94°C for 2 min (to denature the cDNA and RNA duplex and inactivate the reverse transcriptase. Cycles conditions were denaturing at 94°C for 45s, with annealing and extension at 52°C nd 68°C for 1 min, respectively.

RAPD's: RAPD's (Randomly Amplified Polymorphic DNA) technique was done according to protocol of (Williams *et al.*, 1990).

RESULTS

Histology, Imunohistochemistry and RT-PCR analysis: A total of 17 patients and 15 normal females with family history with breast cancer were involved in these examinations. Light microscope was used to study the cases of malignant breast lesions, from Invasive Duct Carcinoma (IDC) of the breast by hematoxelin and eosin stain. Normal shaped cells and normal level of mitotic division were recorded in normal female sections (Fig. 1a), while malignant cells and high level of mitotic division were observed in IDC female cases (Fig. 1b).

The RT-PCR analysis, provided a specific molecular marker that can be used in order to detect the differences between breast cancer patients and normal females. We have used a pair of primers specific to HER4 gene (232 bp) and a control gene thymidine kinase (160 bp) (Table 1).
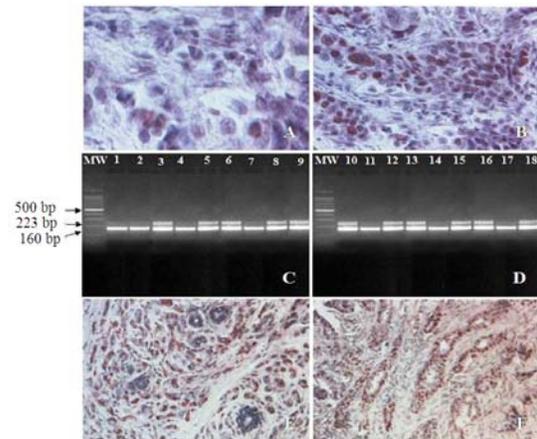

Fig. 1: Histology, Imunohistochemistry and molecular analysis (A) normal female section by Hematoxelin and eosin stain (×400) showing mitosis: (B) A case of IDC by Hematoxelin and eosin stain (×400) showing mitosis; (C-D) Electrophoresis separation of HER4 gene (232 bp) and control gene thymidine kinase (160 bp) PCR amplified fragments on 2% agarose gel. Lanes 3, 5, 6, 8, 9, 10, 12, 13, 15, 16 and 18: IDC positive. Lanes 1, 2, 4, 7, 11, 14 and 17 negativ for HER-4/neu gene amplification. Lane MW: 100 bp molecular weight Marker; (E) IDC showing expression of HER4+ and (F) IDC showing expression of HER4++

An amplified 232bp fragment specific to HER4 gene was detected in all 17 patient females, while the 15 females with family cancer history did not show the 232bp fragment, but only the 160 bp fragment has recorded (Fig. 1C and D, have showed 11 out of the 17 patients and only 7 out of 15 normal females).

Moreover, HER4 gene expression was detected by immunohistochemical and RT-PCR techniques. Both techniques showed almost the same sensitivity. The immunohistochemical analysis has showed positive correlation between number of cells and HER4 gene expression (product: receptor tyrosine-protein kinase (c-erbB-4) isoform). The different breast cancer sections have showed different levels of expressions, some of them were less in cell number and less HER4 expression, HER4+ (Fig. 1E), while we have recorded high level of cell number and gene expression, HER4++ (Fig. 1F).

Both of RT-PCR and immunohistochemical analyses have confirmed each other. Ninety 4% of the patient females who showed 232 bp HER4 specific PCR fragment, were positive to HER4 (IHC) analysis (Table 2A and B), while 100% of the normal females were negative to IHC analysis.

Table 1: Primers used to amplify HER4 gene

Primer name	Primer sequence	PCR product Length (bp)
HER4 (Sense)	5'-CTC TGG TGG TCT TCC TTC TAC C-'3	232 bp
HER4 (Antisense)	5'-TGA TAG TAG GCA GCA TTG CC-'3	
thymidine kinase (Sense)	5'-CTT TGA TTG CAC ATT GTT GT-'3	160 bp
thymidine kinase (Antisense)	5'-GAA AGC AAT GCT ATC ACC TC-'3	

Table 2a: Correlation between HER4 expression by RT-PCR and diagnosed by Immunohistochemistry (IHC): (A) HER4 expression using RT-PCR

	HER4 expression by (PCR)			
HER4 expression	No. of patient females (17)	(%)	No. of normal females (15)	(%)
+ve expression	17	100	0	0
-ve expression	0	0	15	100

Table 2b: HER4 Immunohistochemistry (IHC)

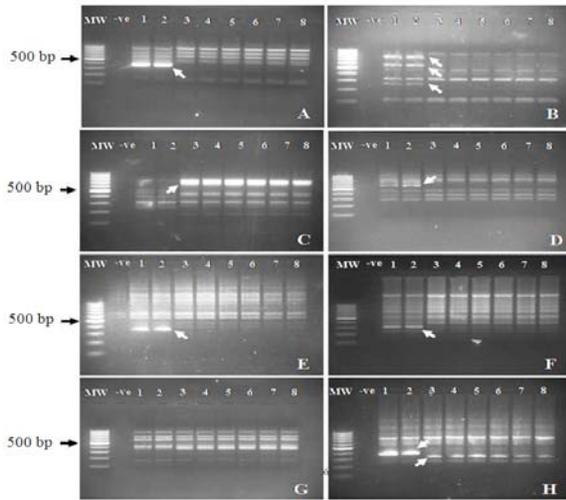|	HER4 expression by (IHC)			
HER4 expression	No. of patient females (17)	(%)	No. of normal females (15)	(%)
+ve expression	16	94	0	0
-ve expression	1	6	15	100

Fig. 2: Developing of RAPD markers specific to breast cancer. MW lane, is a 100 bp ladder, -ve lane, is a negative PCR (no DNA) reaction, Lanes 1 and 2 are normal females, Lanes 3, 4, 5, 6, 7 and 8 are breast cancer patients. The polymorphic bands are highlighted with white arrows. (A) OPC-5 primer ; (B) OPF-18 primer (C) OPD-2 primer ; (D) OPB-7 primer ; (E) OPC-14 primer ; (F) OPC-16 primer (G) OPA-13 primer and (H) OPE-20 primer

RAPD's analysis: Looking for point mutations within oncogenes sequence or involved in the development of tumor-genesis or interplay between genes that are involved in the induction of breast cancer, we have selected sixteen different 10 mer RAPD primers, that showed high identity percentage with some exons of five different human oncogenes. Only 13 out of the selected primers have showed a clear, reproducible and specific polymorphism to either normal or patient females.

For V-myc (Homo. sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (myc)) gene, two primers were used and have showed reproducible data. The 3' end of these two primers, OPC-5 and OPF-18, was identical with regions 450 and 1750 of V-myc cDNA respectively. Among the RAPD's reaction using primer OPC-5, a polymorphic band between the normal and patient females has recorded. This fragment (350 bp) was present among the normal females and absent in all patients (Fig. 2A). While primer OPF-18 has showed three polymorphic bands among the two classes of females. These polymorphic band sizes are 800, 550 and 280 bp respectively, were present in normal females, while absent in the patient females (Fig. 2B).

Two RAPD 10 mer oligonucleotides, OPD-2 and OPB-7, identical to regions 1600 and 2850 bp. of HER2 or ERBB2-cDNA (H. sapiens v-erb-b2 erythroblastic leukemia viral oncogene homologe 2 (avian), have been selected and showed reproducible data. Among the RAPD's reaction using primer OPD-2, we could record a polymorphic band between the normal and patient females. This fragment (750 bp) was absent among the

normal females and present in all patients (Fig. 2C). Also, primer OPB-7 has showed another polymorphic band among the two classes of females.
This polymorphic band (600 bp) was present in normal females, while absent in the patient females (Fig. 2D).

Concerning HER4 (H. sapiens v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) (ERBB4 or HER4)), four 10 mer oligonucleotides, OPC-14, OPC-16, OPA-13 and OPE-20, identical to different exons of HER4 gene, have been selected. They showed reproducible data. From OPC-14 primer products, we could detect a polymorphic band between the normal and patient females. This fragment (350 bp) was present among the normal females and absent in all patients (Fig. 2E). Also, primer OPC-16 has showed another polymorphic band among the two classes of females. This polymorphic band (300 bp) was present in normal females, while absent in the patient females (Fig. 2F). The primer OPA-13 did not show any differences among samples (Fig. 2G), while OPE-20 primer showed interesting results. This primer has produced two polymorphic fragments, with molecular weights 350 bp and 300 bp respectively (Fig. 2H). The fragment with size 350 bp was present in normal females but absent in patients and the second fragment was found only in the background of the diagnosed females with breast cancer.

We have selected four primers that were identical from their 3' with different regions of H. sapiens breast cancer 2 (BRCA2)-cDNA. These cDNA regions are 1850, 4700, 5150 and 5550 bp. respectively. The RAPD's product of four primers has been screened. Primer OPG-1 products have revealed two constant polymorphic bands with size 450 bp and 350 bp respectively. These two fragments were absent from normal females genetic backgroung, while present in all patients (Fig. 3a). Another polymorphic band (350 bp) between the two classes of females has been found among the primer OPF-20 product. This DNA fragment only present among normal females, but not the patients (Fig. 3b). RAPD's product of the other two primers OPC-10 and OPI-1 did not reveal any polymorphism among the normal and patient female samples (Fig. 3c and D).

Six primers identical to different exons of H. sapiens breast cancer 1 (BRCA1) oncogene were designed. Only four of them have produced several polymorphic fragments specific to either the normal or patient females. The PCR product of primer OPA-8 has showed three different polymorphic fragments, the first product (750 bp) present in the normal females, while the other two bands (500 bp and 270 bp respectively), appear only among the patient females (Fig. 3E). While the RAPD's of primers OPD-8 and OPD-20 have revealed one polymorphic band, one for each with molecular weight 250 and 800 bp respectively.
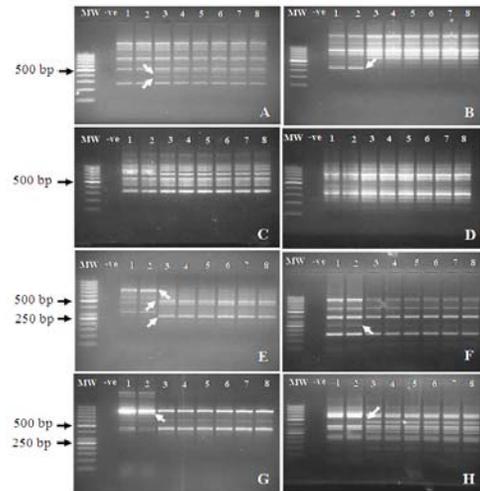

Fig. 3: Developing of RAPD markers specific to breast cancer. MW lane, is a 100 bp ladder for panels (A-D) , MW lane, is a 50 bp ladder for panels (E-H) , -ve lane, is a negative PCR (no DNA) reaction, Lanes 1 and 2 are normal persons, Lanes 3, 4, 5, 6, 7 and 8 are breast cancer patients. The polymorphic bands are highlighted with white arrows. (A) OPG-1 primer ; (B) OPF-20 primer; (C) OPC-10 primer ; (D) OPI-1 primer; (E) OPA-8 primer ; (F) OPD-8 primer (G) OPD-20 primer and (H) OPB-16 primer
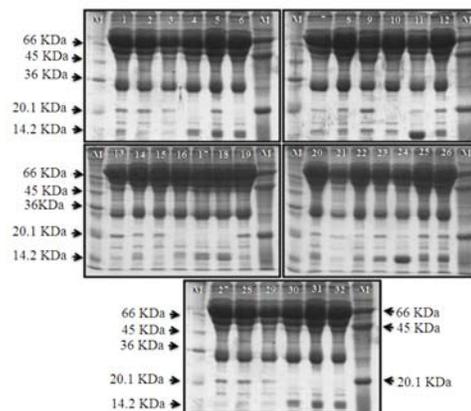

Fig. 4: Total protein analysis of breast cancer patients and normal females with breast cancer history. M, is protein molecular weight marker; 1, 2, 3, 7, 8, 9, 13, 14, 15, 19, 20, 21, 22, 23, 27, 28 and 29 are diagnosed preast cancer females; 4, 5, 6, 10, 11, 12, 16, 17, 18, 24, 25, 26, 30, 31 and 32 are normal females with breast cancer family history

Both fragments appeared only in normal females (Fig. 3F and G). Finally, primer OPB-16 has produced another

important fragment of size 450 bp and this product recorded only among the patient females (Fig. 3H).

Moreover, a preliminary experiment of Cleaved Amplified Polymorphic Sequence technique "CAPS" using different human oncogenes specific primers has been applied on the blood DNA of patients and normal females, but there was no detected polymorphism among the two classes of females (data not shown).

Total protein profile analysis: Thirty two females are divided into 17 diagnosed breast cancer females (females number, 1, 2, 3, 7, 8, 9, 13, 14, 15, 19, 20, 21, 22, 23, 27, 28 and 29) and 15 normal females (not diagnosed) with history of breast cancer (females number, 4, 5, 6, 10, 11, 12, 16, 17, 18,, 24, 25, 26, 30, 31 and 32), have been involved in this study. Total protein has been isolated and separated using SDS-PAGE system. 100% of the diagnosed patients have showed expression of Low Molecular Weight (LMW) isoforms at molecular weight 20.1 kDa, While 40 % of females with breast cancer history have showed same LMW protein forms (females number 5, 11, 12, 24, 25, 26) (Fig. 4). It was shown in Porter *et al.*, (2001) that cyclin E protein is cleaved into its LMW forms by the elastase class of enzymes through proteolytic processing of the full-length cyclin E in tumor but not normal cells.

DISCUSSION

Laboratory diagnosis of breast cancer has traditionally been performed using cell culture and the direct hormone receptor assay. However, these assays are increasingly being replaced in clinical laboratories using PCR dependent protocols i.e., Randomly Amplified Polymorphic DNA (RAPD's) and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

The main objective of this study, is to find more markers to be used for breast cancer early diagnosis and consequently direct the direction to use more efficient markers in clinical laboratories at the western region of KSA and Egypt.

RT-PCR technology using specific primers to HER4 or *C-erbB-4* (H. sapiens v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian) gene has provided a specific and trustable molecular marker. Also RAPD's analysis of different female samples recorded extra interesting markers. Because of the RAPD's amplification nature, we understand that the reproducible polymorphic product of the thirteen primers might be due to some point mutations inside the five oncogene sequences or belong to point

mutations distributes all over the genomic DNA of the normal or patient females. But due to the fixed polymorphism among the two classes of females, these primers and their polymorphic product bands can be used as a tool for breast cancer early diagnosis.

Morimotor *et al*. (2003) have recorded that the super oxide radicals and the metabolic product H_2O_2 lead to fat oxidation in cell membrane may have caused mutagenicity and carcinogenicity, which induce damage to nucleic acids. This may explain the increase in chromosomal breaks and chromosomal rearrangements in breast cancer patients.

Moreover total protein analysis among females provided a biochemical marker at the level of low molecular weight (LMW) protein forms. In tumor cells, the LMW forms of cyclin E are hyperactive compared to the full-length cyclin E in terms of cell cycle regulation, including: decreased length of G1 phase, more rapid transition of G1 to S phase, also increasing cyclin E kinase activity and increasing genomic instability (Akli and Keyomarsi, 2004). Since the LMW isoforms of cyclin E are generated through proteolytic cleavage by elastase, inhibition of elastase specific neutrophli inhibitor, CE-2072, should halt the appearance of the LMW forms (Akli and Keyomarsi, 2004).

CONCLUSION

In this study, we could apply the new PCR-depended techniques, such as RT-PCR on RNA extracts from patients and normal samples, RAPD's "Randomly Amplified Polymorphic DNA" and CAPS "Cleaved Amplified Polymorphic DNA" markers on DNA extracts for the same samples. Also Imunohistochemistry and biochemical analysis techniques have been used. The Imunohistochemistry and molecular analysis have confirmed each other, when both techniques showed almost the same sensitivity, and most of the immunohistochemical positive samples for *C-erbB-4* gene gave a heavy band by PCR. The results have provided several specific molecular, biochemical and pathological markers that can be easily applied in KSA and Egypt hospitals laboratories, in order to avoid money or time consuming.

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