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APPLICATION OF MULTIPLEX PCR WITH HISTOPATHOLOGIC FEATURES FOR DETECTION OF FAMILIAL BREAST CANCER IN FORMALIN-FIXED, PARAFFIN-EMBEDDED HISTOLOGIC SPECIMENS

Breast cancer is the most common malignancy among females in the world. Age and familial history are the major risk factors for the development of this disease in Iran. Mutations of BRCA1 and BRCA2 genes are associated with a greatly increased risk for development of familial breast cancer. Frequency of BRCA mutations was identified in familial breast cancers (FBC) and non-familial breast cancers (NFBC) by molecular genetics, morphological and Immunohistochemical methods. Thirty forth formalin-fixed, paraffin-embedded breast tissue tumors were analyzed from 16 patients with FBC and 18 patients with NFBC. Three 5382insC mutations detected by multiplex PCR in 16 familial breast cancers. Immunohistochemical method was used to detect estrogen receptor (ER) and progesterona receptor (PR) and TP53. Comparison of ER, PR and TP53 exhibited high difference ($P < 0.0001$) in familial breast cancers and non-familial breast cancers. Our results demonstrated that 5382insC mutation, ER, PR, TP53, mitotic activity, polymorphism, necrosis and tubules can serve as the major risk factors for the development of FBC.

Introduction. Breast cancer with age-adjusted incidence rate of 13.5 per 100 000 people per year and age-adjusted mortality rates of 5.5 per 100,000 people per year is the first most common cancer in Iranians women. Incidence rates are higher in the most of the developed areas (in Europe and America) than in Asia [1]. A family history of breast cancer is one of the major risk factors for the development of this disease in Iran [2]. Familial breast cancer is characterized by early age at onset, bilaterality, vertical transmission through both maternal and paternal lines, and familial association with tumors of other organs, particularly the ovary and prostate gland. It has been shown that, even after adjusting for age, Iranian breast cancer patients relatively younger than their Western counterparts [3]. Although the majority of breast cancer cases are to be regarded as sporadic forms (90–95 %), approximately 5–10 % breast cancer cases in the general population have been suggested to be attributed to an inherited cancer susceptibility gene such as BRCA1 and BRCA2, p53, PTEN, and STK11/LKB1 [4]. The most common gene changes in breast cancer are those of the BRCA1 and BRCA2 genes. BRCA mutations are associated with a greatly increased risk for breast cancer development, with risk estimates ranging from 59–87 % for BRCA1 and from 38–80 % for BRCA2 mutations [4–8]. Known BRCA mutations have been collected in the Breast Cancer Information Core (BIC) database [9]. Nearly 2,000 distinct mutations have been identified in both BRCA1 and BRCA2, and several founder mutations have been identified in each of these two genes in various populations. There is not a perfect method to screen for unknown mutations; combinations of several methods may be necessary for accurate genetic diagnosis. Although genetic testing for BRCA mutations within high-risk families is available but it is expensive and time consuming procedures because of the large size of both genes, the absence of hot spots for mutations throughout their entire coding region, and the low percentage of mutated cases. Most reported germline mutations are located in the coding sequence of BRCA and represent single nucleotide substitutions and small sequence deletions or insertions causing truncations of the BRCA proteins. Three founder mutations, 185delAG, 5382insC and 6174delT are relatively common in Ashkenazi Jewish individuals with a combined prevalence of 2.3 % in this population, compared with a general

population prevalence of approximately 0.1 % for BRCA mutations [10]. Women who know that are the carrier of BRCA mutation, may use this information to make more informed decisions about their health care, including whether to use tamoxifen and/or prophylactic surgery to delay or prevent the onset of cancer. The morphologic and immunohistochemical profiles of breast cancers may help identify patients who are likely to carry germline mutations in BRCA1 and BRCA2. Breast cancers arising in carriers of mutations in the breast cancer susceptibility genes, BRCA1/2, differ histologically from each other and from breast cancers unselected for a family history [11–14]. Archival materials are a valuable source for the study of molecular diagnosis methods in breast cancer and they are the most widely available material for retrospective clinical studies. Comparison mutation detection from archival tissues and blood for BRCA genes was shown that mutation detection was the most accurate for newer archival breast cancer tissues, high fidelity Taq with shorter PCR amplicon length yielded the highest mutation detection success rate and lowest artifact rate; and base substitutions were more often correctly identified than frameshift mutations or wild-type sequences [15]. Although the yield of DNA from archival tissues is less (four times less than that from fresh tissue and 30 % of the amount that can be extracted from frozen tissue) or can be fragmented, many of these problems can be circumvented by amplification [16]. Breast cancers in patients with familial history are more often negative for Estrogen receptor (ER), Progesterone receptor (PR), and Human Epidermal Receptor 2 (HER-2), and are more likely to be positive for p53 protein compared with controls. The use of morphologic and immunohistochemical features provides a helpful and cost-effective tool for those making decisions about genetic screening for carrier of BRCA mutation. In this article, we will analyse the three founder mutations, 185delAG and 5382insC (in BRCA1) and 6174delT (in BRCA2) by multiplex PCR in formalin-fixed, paraffin-embedded tissue blocks. This study evaluates the pathological and molecular diagnostic profiles of familial and non-familial breast cancers.

Materials and Methods. *Case Selection.* We reviewed archival formalin-fixed, paraffin-embedded tissue blocks from women with breast cancer diagnosed the age of 25–80 years, selected from the histopathology archives of the Department of Pathology, Khatam and Baghiatollah Hospital in Iran for the years 2004 and 2005. Verification of every cancer reported in a relative was sought through pathology reports, hospital records. All cases were reviewed using a special questionnaire, which allowed taking into account the presence or absence family history of breast cancer and also other pathology information. Family history characteristics associated with an increased likelihood of carrying a BRCA1 or BRCA2 mutation include multiple cases of breast cancer in the family. We analyzed 34 formalin-fixed, paraffin-embedded tissue blocks from 16 familial breast cancer patients and 18 non-familial breast cancer patients.

DNA Extraction. In the case of paraffin-embedded samples, tumor pathology was reviewed and tumor tissues were selected for dissection from paraffin blocks. Paraffin was removed from the 20-mm sections by being agitated first in 200µl solution Tris-HCL + 0.5 % Tween-20 and then heating in a 650 W microwave oven for up to 45 s. The tubes were spun whilst warm at 12,000 rims for 15 min and placed on ice. Using a sterile pipette tip the solid wax disc was removed prior to digestion. So 5 µl of 10 mg/ml Proteinase K was added to each tube and digested for 3–5 h at 65 °C, with gentle agitation every hour. 200 µl Tris-HCL was added to each digest solution, heated to 99 °C for 10 min, spun at 12,000 rims for 15 min and placed on ice. Any hardened wax was removed, the sample re-spun at 12,000 rims for 15 min, and top layer removed for later analysis.

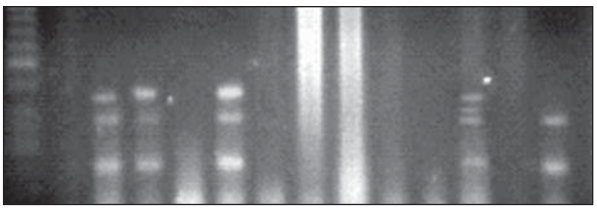
Multiplex PCR. Multiplex PCR was used to detect the simultaneous detection of three common mutations: 185delAG and 5382insC in BRCA1, and 6174delT in BRCA2. PCR amplification was performed using 100 ng of DNA derived from formalin-fixed and paraffin-embedded with primers and PCR amplification conditions as published by Pak Cheung R and et al. [17]. The reaction mixture underwent initial denaturation process at 94 °C for 5 min, followed by 35 cycles at 94 °C for 60 sec, 55 °C for 60 sec, and 72 °C for 35 sec. The final extension was performed at 72 °C for 10 min in Techne Thermocycler. The PCR fragments were run in 2 % agarose gel and visualized by ethidium bromide staining.

Immunohistochemistry. Morphological and immunohistochemical diagnoses of 16 familial breast cancer and 18 non familial breast cancer were retrieved from their hospital records. The Nottingham histological grading system was used to assess the grade of breast cancer samples. Immunohistochemical staining of sections from paraffin wax embedded tissues from these cases for the expression of ER, PR and p53 was carried out using a standard method, the avidin biotin complex (ABC) procedure. Sections from the tissue were immersed in boiling 10 mM sodium citrate at pH 6.5 for 2 min in a pressure cooker. The percentage of stained nuclei, independent of the intensity, was scored for ER, PR and p53. For categorical analysis, a case was considered positive when >10, 10, and 25 % of the cells were stained with ER, PR, and p53, respectively.

Statistical Methods. Chi-square test for Trend or others were used to make comparisons. Statistical analyses were performed using the 3.3.2 version of the Epi Info (TM) 2005 software.

Results. *Results of DNA extraction and multiplex PCR from formalin-fixed and paraffin-embedded samples.* Eighty-two blocks were used for multiplex PCR. For each mutation, three primers (one common, one specific for the mutant, and one specific for the wild-type allele) were used. The PCR-primers are described in Table 1. Successful DNA extraction was assessed by PCR amplification of three fragment of the BRCA gene. DNA was successfully extracted from every block but success of DNA amplification after microwave treatment and purification using simple boiling was 34 of 84 samples (40 %) and 60 % of DNA samples were degraded DNA (Figure). Three 5382insC mutations were detected by multiplex PCR in BRCA1 gene.

Distributions of morphological and immunohistochemical features in familial and non-familial breast cancers. Among the 34 cases with breast cancer selected in our study (20–85 age), 16 (47 %) were positive for family history and 18 (53 %) without any family history of breast cancer. 2 out of 16 familial patients were positive for ovarian cancer. Table 2 lists the distributions of morphological features in familial and non-familial tumors. Tumors associated with family history exhibited higher mitotic activity (OR = 5,34, P < 0,0001), higher polymorphism (OR = 1,39, P < 0,004), lower necrosis (OR = 0,19, P < 0,0003) and lower



1 2 3 4 5 6 7 8 9 10 11 12 13 14

Electrophoregram of PCR products Lane *1*, 100-bp ladder; *2, 5, 7, 10,11* and *13* – samples without amplification; *12* – sample with mutation; *3, 4, 14* – wild type samples; *8, 9* – samples with degraded DNA

tubules (OR = 0,49, P < 0,04), than non-family history cancers. The immunohistochemical diagnoses of 16 familial breast cancers and 18 non familial breast cancers were retrieved from their hospital records. Immunohistochemical staining of sections from paraffin wax embedded tissues from these cases for the expression of ER, PR and TP53 was carried out using the avidin biotin complex (ABC) procedure. Overall, Estrogen Receptor ER (OR = 4,69, P < 0.0001), Progesterone Receptor PR (OR = 4,52, P < 0.0001), expression was observed less frequently in familial tumors cases than non-familial cases. Breast cancers occurring in familial tumors cases had significantly higher levels of TP53 (OR = 0,23, P < 0,0001) expression compared with cancers occurring in non-familial cases.

Comparison of results of Multiplex PCR with pathological and immunohistochemical features in familial and non familial breast cancer. Comparison

Table 1

Nucleotide sequences of the primer sets

Mutation	Primer sequence	Size of amplicon
BRCA1	Common 5'-ggttggcagcaatatgtgaa	
185delAG	Wild-type 5'-gctgacttaccagatgggactctc	335 bp
	Mutant 5'-cccaaattaatacactcttgtcgtga-cttaccagatgggacagta	354 bp
BRCA1	Common 5'-gacgggaatccaaattacacag	
5382insC	Wild-type 5'-aaagcgagcaagagaatcgca	271 bp
	Mutant 5'-aatcgaagaaaccaccaaagtcc-ttagcgagcaagagaatcacc	295 bp
BRCA2	Common 5'-agctggtctgaatgttcgttact	
6174delT	Wild-type 5'-gtgggatttttagcacagctagt	151 bp
	Mutant 5'-cagtctcatctgcaaatacttcagg-gatttttagcacagcatgg	171 bp

Table 2

Distribution of molecular and immunohistochemical features in Familial Breast Cancer (FBC) and Non-Familial Breast Cancers (NFBC)

Characteristics	Groups	FBC (n = 16)	NFBC (n = 18)	Odds Ratio (OR)
		Number (%)		
Cancer type	Ductal carcinoma	13 (81 %)	16 (89 %)	1,00
	Lobular carcinoma	3 (19 %)	2 (11 %)	1,91
				χ^2 = 2,50(P<0,2)
Other cancers	Yes	2 (13 %)	3 (17 %)	1,00
	No	14 (87 %)	15 (83 %)	1,37
				χ^2 = 0,62(P<0, 5)
DCIS	Present	11 (69 %)	11 (61 %)	1,00
	Absent	5 (31 %)	7 (39 %)	0,70
				χ^2 = 1,40(P<0,3)
Necrosis	Present	4 (25 %)	1 (6 %)	1,00
	Absent	12 (75 %)	17 (94 %)	0,19
				χ^2 = 13,7(P<0,0003)
Tumor size	< 2 cm	4 (25 %)	4 (22 %)	1,00
	2–5 cm	11 (69 %)	12 (67 %)	0,91
	⩾ 5 cm	1 (6 %)	2 (11 %)	0,48
				χ^2 = 1,07(P<0,3)
Age groups (years)	<30	2 (13 %)	1 (6 %)	1,00
	31–40	2 (13 %)	3 (17 %)	0,35
	41–50	7 (44 %)	7 (39 %)	0,52
	51–60	1 (6 %)	4 (22 %)	0,13
	61–70	3 (18 %)	0 (1 %)	8,31
	>71	1 (6 %)	3 (17 %)	0,16
				χ^2 = 1,49(P<0,3)
Grade	I	3 (19 %)	3 (17 %)	1,00
	II	7 (44 %)	11 (61 %)	0,65
	III	6 (37 %)	4 (22 %)	1,50
				χ^2 = 1,82(P<0,2)
Tubules	3 (< 10 %)	13 (78 %)	12 (67 %)	1,00
	2 (10–75)	3 (19 %)	6 (33 %)	0,49
	1(>75 %)	0 (0 %)	0 (0 %)	0,0
				χ^2 = 4,54(P<0,04)
Mitotic activity	Low	6 (38 %)	13 (72 %)	1,00
	Moderate	5 (31 %)	3 (17 %)	3,46
	High	5 (31 %)	2 (11 %)	5,34
				χ^2 = 22,5(P<0,0001)
Polymorphisms	1	12 (75 %)	6 (33 %)	1,00
	2	1 (6 %)	11 (61 %)	0,04
	3	3 (19 %)	1 (6 %)	1,39
				χ^2 = 8,49(P<0,004)
ER	+	4 (25 %)	11 (61 %)	1,00
	–	12 (75 %)	7 (39 %)	4,69
				χ^2 = 26,3(P<0,0001)
PR	+	5 (31 %)	12 (67 %)	1,00
	–	11 (69 %)	6 (33 %)	4,52
				χ^2= 25,8(P<0,0001)

Finish of Table 2

Characteristics	Groups	FBC (n = 16)	NFBC (n = 18)	Odds Ratio (OR)
		Number (%)		
TP53	+	10 (63 %)	5 (28 %)	1,00
	–	6 (37 %)	13 (72 %)	0,23
				χ^2 = 24,6(P<0,0001)
5382insC	+	3 (19 %)	0 (<1 %)	1,00
	–	13 (81 %)	18 (>99 %)	0,04
				χ^2 = 17,9(P<0,0001)

Note. χ^2 = Chi-square test for Trend.

of incidence of 5382insC mutation in 16 familial and 18 non-familial breast cancer patients has shown that incidence of 5382insC mutation is high and effective in familial patients (P < 0.0001) relatively non-familial patients. Significantly higher, than in non-familial breast cancer, incidence of 5382insC indicates that this may be founder BRCA1 mutation characteristic for familial breast cancers. In combination with this mutation, use of morphological and immunohistochemical staining provides a more accurate predictor of familial breast cancer (Tabl. 2).

Discussion. In our study, we used multiplex PCR and pathology methods to analyze 34 routinely submitted formalin-fixed, paraffin-embedded tissue blocks from breast cancer patients. The analysis of archival formalin-fixed, paraffin-embedded tissue samples becomes increasingly important for molecular genetic studies. A molecular genetic analysis of human tissue often entails the use of PCR with previously extracted DNA as the template. The results of the current study indicate that the pathology familial breast cancers clearly differ on the basis of several measured indices from the pathology of breast cancers did not any family history of breast cancer. Breast cancer results from genetic and environmental factors leading to accumulation of mutations in essential genes. These mutations can be either germline or sporadic events. Environmental risk factors for breast cancer may vary in different geography of the world and they are of greater importance than genetic factors. The BRCA1 and BRCA2 genes are most common gene changes in familial breast cancers. The morphologic and molecular phenotype of breast cancers may help to identify patients who are likely to carry germline mutations in BRCA1 and BRCA2. Breast cancers in patients with BRCA1 germline mutations exhibited higher mitotic counts, higher grade and are more often negative for ER, PR, and HER-2, and are more likely to be positive for p53 protein compared with controls. In contrast, c-MYC amplification was present in 18.2; 62.5 and 12.5 % of BRCA1, BRCA2, and non-BRCA1/2 carrier, respectively, and 31 % in the control group. In combination with ER and morphology, use of cytokeratin staining provides a more accurate predictor of *BRCA1* mutation status.

Numerous founder mutations have been reported in BRCA1 and BRCA2 in different population. The BIC database indicates that three founder mutations have been observed in different population especially in Ashkenazi Jewish patients. These mutations with the highest number of registrations associated with breast cancer are 5382insC, 185delAG (in BRCA1) and 6174delT (in BRCA2). The BRCA2 6174delT mutation has been seen only in Ashkenazi Jews [18], with a frequency of 0.9–1.5 % [19, 20]. The founder BRCA1 185delAG mutation, with a frequency of 0.8–1.1 % in Ashkenazi Jews [19, 21], is also observed in Sephardic Jews, indicating an older origin. The 185delAG mutation has also been observed in individuals of English origin but on a different haplotype, which suggests a different origin. It is concluded that the 185delAG BRCA1 mutation occurs in some non-Ashkenazi populations at rates comparable with that of Ashkenazim especially in Iranian Jews. The 185delAG BRCA1 mutation originated before the dispersion of Jews in the diaspora and is not limited to Ashkenazim [22]. The third founder

mutation, BRCA1 5382insC, has a frequency of 0.13–0.3 % in Ashkenazi Jews. The 5382insC mutation is observed in many populations, and the vast majority of carriers share the same core haplotype. The analysis of the BIC data base files indicates that the 5382insC mutation is the second most frequent (200 records) of the total of the mutations associated with breast cancer. Based on the frequency and geographical distribution in Europe, it has been suggested that the 5382insC mutation originated in the Baltic area during the medieval period approximately 38 generations ago with a decreasing prevalence from the eastern to the western regions in Europe [23]. No molecular genetic study has yet been carried out on the 5382insC mutation in Iran. We have screened cancer patients from 16 familial patients and 18 non-familial patients from Iran for 5382insC, 185delAG (in BRCA1) and 6174delT (in BRCA2) mutations. One of three mutations (5382insC) was found in 3 of the 16 familial patients after amplification (19 %). Significantly higher, than in non familial breast cancer, incidence of 5382insC indicates that this may be founder effect for familial breast cancer. In Russian familial breast/ovarian cancer patients it accounts for 12 % of 25 the mutations identified [24]. It is also present in 10 % of 60 breast and ovarian cancer families in north-eastern Poland [25]. A high frequency of this mutation (13.3 %) has been described in 30 Canadian breast/ovarian cancer families [26]. It also constitutes a frequent mutation in 4 % of 248 Germans high-risk breast cancer population [27]. In contrast, Scandinavian studies show a surprisingly low frequency (1 %) in 100 finnishes breast/ovarian cancer families [28]. Likewise, the 5382insC mutation was not identified in any of the 47 families with familial breast/ovarian cancer studied in Southern Sweden, in 106 families in Sweden, or in 25 families in Norway [29–31]. The results suggest that cancers from non-familial history patients are of lower mitotic activity, lower polymorphism, higher tubules and higher necrosis and more positive for ER, PR, and are more likely to be negative for p53 protein than breast cancers with family history. Our analysis shows that testing of 5382insC BRCA1 mutation in combination with morphological and immunohistochemical staining should be extremely effective and inexpensive tool in testing familial breast cancer aimed to identify individuals with high risk of breast. Currently, population screening is limited to cystic fibrosis mutation analysis for adults of reproductive age, but several diseases such as breast cancer meet minimum criteria for mutation-based screening in adult.

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РЕЗЮМЕ. Рак молочной железы (РМЖ) является наиболее частым видом злокачественных опухолей у женщин в мире. В Иране возраст и наличие заболеваний РМЖ у ближайших родственников являются главными факторами риска развития этого заболевания. Мутации гена BRCA1/2 обусловливают высокий риск развития в течение жизни РМЖ. Исследованы частоты мутаций BRCA у лиц с семейным раком молочной железы (СРМЖ) и несемейным раком молочной железы (НСРМЖ) из Ирана. Для достижения поставленной цели использовали молекулярно-генетические, морфологические и иммуногистохимические методы. Проанализированы 34 ткани опухолей, зафиксированных в парафине, у 16 больных СРМЖ и 18 больных НСРМЖ. При исследовании генов BRCA1 и BRCA2 с мультиплексным ПЦР идентифицированы три мутации (5382insC) у 16 больных СРМЖ. Иммуногистохимическим методом определяли рецептор эстрогена (ЭР), рецептор прогестерона (РП) и TP53. Сравнение ЭР, РП и TP53 в тканях СРМЖ и НРМЖ показало высокие достоверные различия ($P < 0.0001$). В результате исследований выявлено, что мутация 5382insC, ЭР, РП, TP53, митотическая особенность, полиморфизмы, некроз и тубулы могут быть использованы как главные факторы риска развития СРМЖ.

РЕЗЮМЕ. Рак молочної залози (РМЗ) є найбільш частим видом злоякісних пухлин у жінок в світі. В Ірані вік та наявність захворювань РМЗ в найближчих родичів є головними факторами ризику розвитку цього захворювання. Мутації гена BRCA1/2 зумовлюють високий ризик розвитку протягом життя РМЗ. Досліджено частоти мутацій BRCA в осіб з сімейним раком молочної залози (СРМЗ) та несімейним раком молочної залози (НСРМЗ) з Ірану. Для досягнення поставленої цілі використовували молекулярно-генетичні, морфологічні та імуногістохімічні методи. Проаналізовано 34 тканини пухлин, зафіксованих в парафіні, у 16 хворих СРМЖ і 18 хворих НСРМЖ. При дослідженні генів BRCA1 та BRCA2 з мультиплексним ПЦР ідентифіковано три мутації (538insC) в 16 хворих СРМЖ. Імуногістохімічним методом визначали рецептор естрогена (ЕР), рецептор прогестерона (РП) і TP53. Порівняння ЕР, РП і TP53 в

тканинах СРМЖ та НРМЖ показало високі достовірні відмінності ($P < 0.0001$). В результаті досліджень виявлено, що мутація 5382insC, ЭР, РП, ТР53, мітотична особливість, поліморфізми, некроз і тубули можуть бути використані як головні фактори ризику розвитку СРМЖ.

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