Investigation of Mitochondrial Common Deletion and BRCA Mutations for Detection of Familial Breast Cancers in Archival Breast Cancer Materials

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Abstract

Bachground and Aim: Mutation analysis of mitochondrial genome and BRCA genes are helpful in the early diagnosis of familial breast cancers. In this study, we investigated mitochondrial common deletion and BRCA mutations through multiplex PCR and clinical parameters for the detection of familial breast cancers in archival breast cancer samples.

Methods: The multiplex PCR was conducted on DNA from 34 archive breast tissue samples and 13 blood samples.

Results: Five mtDNA4977 deletions and three 5382insC mutations were detected from familial breast cancers. The mtDNA4977 deletion was highly prevalent in peripheral blood but it was absent in the breast tissue of the cancer cases. On the other hand, familial breast cancer tumors exhibited different clinical parameters such as higher mitotic activity, higher polymorphism, lower necrosis, lower tubules, higher ER- and PR-negatives and lower TP53-positives compared to the non-familial cancers.

Conclusion: Our results demonstrated that the testing of mtDNA4977 deletion and 5382insC mutation in combination with clinical parameters can serve as major risk factors in the identification of familial breast cancers.

Keywords: breast cancer, mutation detection, nDNA and mtDNA mutations, multiplex PCR, archival breast cancer tissues, retrospective clinical studies

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Introduction

Breast cancer is the second most common cause of cancer-related death among women in Iran [1]. Breast cancer progression involves the accumulation of various genetic mutations, which are present in both nuclear genomes (nDNA) and mitochondrial genomes (mtDNA). However, mutations of the BRCA genes are one of the most common genetic changes found in nDNA; their presence may be prognosticBreast Cancer Information Core (BIC) database indicates that the mutations with the highest number of registrations associated with breast cancer are 185delAG and 5382insC in the *BRCA1* gene; and 6174delT in *BRCA2* gene [5]. Thus, ability to efficiently, rapidly and unambiguously analyze *BRCA* mutations play an important role in the development of molecular diagnostic assays, the applications of which include genetic testing and carrier screening. Furthermore, the important roles of mitochondria in cellular energy production suggest that mitochondria may serve as a key switch in the breast cancer process [6]. However, the frequency of mtDNA mutations is higher compared to nDNA in a variety of human cancers including breast, bladder, head and neck, and lung cancers. and most mutations occur in the D loop region, where the origin of replication and promoter are located [7]. Of these, the most common somatic mutation is deletion (mtDNA4977) which occurs between nucleotides 8,470 to 13,477 and has been reported in a wide range of tumors, stressed tissues, and even in normal appearing tissues [8]. In addition to obtaining DNA from blood samples, sections from archival breast samples are valuable sources for studying molecular diagnosis methods in breast cancers and are the most widely available material for retrospective clinical studies [9]. Interestingly, it has been clearly established that morphologic and immunohistochemical parameters of breast cancers may help to identify patients who are likely to carry germline mutations in familial breast cancers [10-13]. This article focuses on the researchers' experiences concerning the extraction of nucleic acids from archival materials using conventional approaches ,

and it also focuses on the subsequent application of multiplex PCR with morphological and immunohistochemical parameters for the detection of *BRCA* mutation and mtDNA4977 deletion in familial and non-familial breast cancers.

Materials and Methods

Patients

Patient samples were drawn from three medical centers in Iran: Khatam Hospital, Baghiatollah Hospital and Iranian Center for Breast Cancer. We retrieved archive breast tissue samples from women aged 25- 80 years who were diagnosed with breast cancer in 2004 and 2005. All the cases were reviewed using a special questionnaire which allowed taking into account the presence or absence of family history of breast cancer and other pathology information. The family history characteristics were associated with an increased likelihood of carrying a *BRCA1* or *BRCA2* mutation including 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; and 13 blood samples were obtained from 9 familial breast cancer patients and 4 of their relatives. Verification of each cancer reported in a relative was sought through the pathology reports of the hospital records.

DNA Extraction

CINAGEN Inc.'s DNA Extraction Kit was used to isolate blood and DNA tissue. The extraction procedure was performed according to the manufacturer's instruction for blood samples. In the case of paraffin-embedded samples, tumor pathology in archive samples was reviewed and regions of tumor tissue were selected for dissection from the paraffin blocks. Paraffin was removed from the 20-mm sections by agitation in a 200µl solution of Tris-HCL + 0.5% Tween-20 and then it was heated in a 650 W microwave oven for up to 45 s. After that, the tubes were spun whilst heated at 12,000 rims for 15 min and then were placed on ice. Prior to digestion, the solid wax disc was removed using a sterile pipette tip. Then 5 µl of 10mg/ml Proteinase K was added to each tube and was digested for 3-5 h at 65 °C, with 10 s gentle vibration in every hour. Finally, we followed the boiling method [14,15] and a commercial Kit (CinaGene) process for each sample.

Multiplex PCR

A simple and rapid method was used to detect the simultaneous detection of three common mutations: 185delAG , 5382insC, 6174delT and mtDNA4977 deletion. For each *BRCA* mutation, three primers (one common, one specific for the mutant, and one specific for the wild-type allele) were used [16]. The PCR-primers are described in Table 1. For mtDNA4977 deletion, PCR amplification was performed using two primer pairs of ND6A/ND6B and HSAS8542/HSSN8416 [17]. PCR amplification was performed using 100 ng of DNA derived from archive samples with primers and PCR amplification conditions as published by Pak Cheung R and et al and Zullo S.J. [16,17]. 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

Table 1: *Nucleotide sequences of the primer sets*

Mutation	Primer sequence	Size of amplicon
BRCA1 185delAG	Common forward (P1) *5´*-ggttggcagcaatatgtgaa	335 bp
	Wild-type reverse (P2) *5´*-gctgacttaccagatgggactctc	
	Mutant reverse (P3) *5´*-cccaaattaatacactcttgtcgtgacttaccagatgggacagta	354 bp
BRCA1 5382insC	Common reverse (P4) *5´*-gacgggaatccaaattacacag	271 bp
	Wild-type forward (P5) *5´*-aaagcgagcaagagaatcgca	
	Mutant forward (P6) *5´*-Aatcgaagaaaccaccaaagtccttagcgagcaagagaatcacc	295 bp
BRCA2 6174delT	Common reverse (P7) *5´*-agctggtctgaatgttcgttact	151 bp
	Wild-type forward (P8) *5´*-gtgggatttttagcacagctagt	
	Mutant forward (P9) *5´*-cagtctcatctgcaaatacttcagggatttttagcacagcatgg	171 bp
mtDNA4977 deletion	5'-TTC TCC TAG ACC TAA CCT GA-3'	485 bp
	5'-GGA TAT ACT ACA GCG ATG GC- 3'	
	5'-TGT GGT CTTTGG AGT AGA AAC C-3'	127 bp
	5'-CCT TAC ACT ATT CCT CAT CAC C-3'	

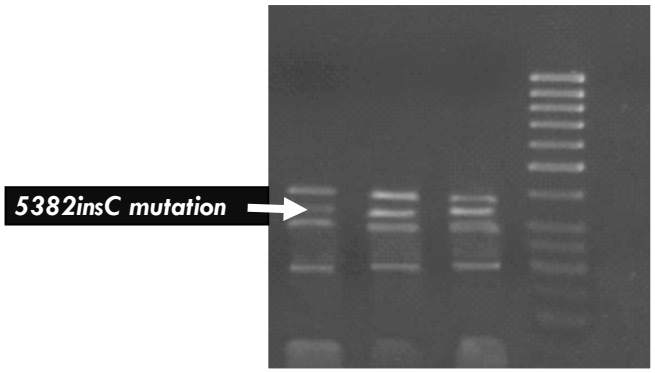

Figure 1: *Electrophoresogram of multiplex PCR products for diagnosis of BRCA mutation Lane 4, 100-bp ladder; lanes 1,2 and 3, samples with 5382insC mutation*

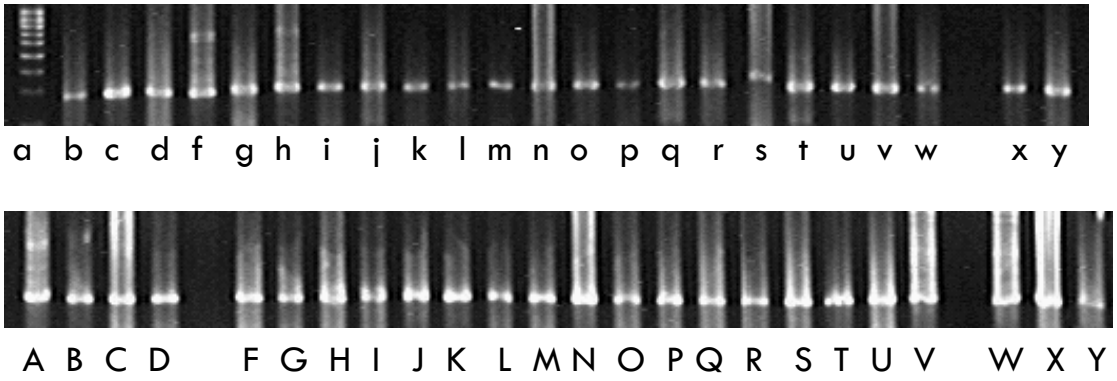

Figure 2: *Electrophoresogram of PCR products*
Lane a, 100-bp ladder; lanes f, h, A, V, and X, samples with mtDNA4977 deletion; lanes b, c, d, g, i, j, k, l, m, n, o, p, q, r, s, t, u, v,w, x, y, B, C, D, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, X and Y samples without mtDNA4977 deletion

extension was performed at 72°C for 10 min in thermocycler (Techne, England). The PCR fragments were run in 2% agarose gel, and were visualized by ethidium bromide staining.

Clinical Parameters

Clinical parameters of 16 familial and 18 non-familial breast cancers were retrieved from their hospital records. The tubules, mitotic activity, necrosis, polymorphism and grade of breast cancer were staged by Nottingham histological grading [21]. Immunohistochemical staining of the sections from the paraffin wax embedded tissues was carried out using avidin biotin complex procedure (DAKO, Glostrup, Denmark) for the expression of ER, PR and p53. 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 was used to compare the familial and non-familial patients. Statistical analyses were performed using the 3.3.2 version of the Epi Info(TM) 2005 software.

Results

Extraction of Nucleic Acids from Archival Breast Cancer Tissues

Successful DNA extraction was assessed by PCR amplification of three fragment of the BRCA gene. For paraffin-embedded tissues, DNA extracted by the simple boiling method yielded higher proportions of successful gene amplifications (70 %) than the DNA extraction Kit (35 %) with microwave. Our study revealed that the highest yield of DNA was obtained with DNA isolation procedure using 5 µl of

Table 2: *Clinical characteristics of Familial Breast Cancer (FBC) and Non-Familial Breast Cancer (NFBC)*

Parameters	FBC (n=16)	NFBC (n=18)	Odds Ratio (OR)
	Number (%)	Number (%)	
Cancer type		16(89%)	1,00
Ductal carcinoma	13(81%)	2(11%)	1,91
Lobular carcinoma	3(19%)		χ^2 =2,50(P<0,2)
Ductal carcinoma in situ		11(61%)	1,00
Present	11(69%)	7(39%)	0,70
Absent	5(31%)		χ^2 =1,40(P<0,3)
Necrosis		1(6%)	1,00
Present	4(25%)	17(94%)	0,19
Absent	12(75%)		χ^2=13,7(P<0,0003)
Tumor size		4(22%)	1,00
< 2 cm	4(25%)	12(67%)	0,91
2–5 cm	11(69%)	2(11%)	0,48
≥ 5 cm	1(6%)		χ^2 =1,07(P<0,3)
Grade		3(17%)	1,00
I	3(19%)	11(61%)	0,65
II	7(44%)	4(22%)	1,50
III	6(37%)		χ^2 =1,82(P<0,2)
Tubules		12(67%)	1,00
3(< 10%)	13(78%)	6(33%)	0,49
2(10-75)	3(22%)	0(0%)	0,0
1(>75%)	0(0%)		χ^2 =4,54(P<0,04)
Mitotic activity		13(72%)	1,00
Low	6(38%)	3(17%)	3,46
Moderate	5(31%)	2(11%)	5,34
High	5(31%)		χ^2=22,5(P<0,0001)
Polymorphisms		6(33%)	1,00
1	12(75%)	11(61%)	0,04
2	1(6%)	1(6%)	1,39
3	3(19%)		χ^2 =8,49(P<0,004)
ER		11(61%)	1,00
+	4 (25%)	7 (39%)	4,69
-	12(75%)		χ^2=26,3(P<0,0001)
PR		12(67%)	1,00
+	5 (31%)	6 (33%)	4,52
-	11(69%)		χ^2=25,8(P<0,0001)
TP53		5 (28%)	1,00
+	10(63%)	13(72%)	0,23
-	6 (37%)		χ^2=24,6(P<0,0001)

χ^2 = Chi-square test for trend

10mg/ml Proteinase K added to each tube and digested for 3-5 h at 65 °C, with gentle agitation in every hour (a modification method from boiling).

Amplification of nDNA from Archival Breast Cancer Tissues

When the quality of isolated DNA was checked using agarose gel electrophoresis prior to PCR amplifications, DNA was then successfully extracted from every block. The success of DNA amplification after microwave treatment and purification using simple boiling occurred in 34 samples. Three 5382insC mutations were detected by multiplex PCR from 34 samples (fig, 1). The following are the reasons for the failure of PCR using DNA isolated from paraffin-embedded tissues: the presence of inhibitory substances, the absence of a detectable amount of target DNA in the small tissue samples or biopsy specimens and the degradation of target DNA (fig, 1). The mentioned factors limit the use of PCR analysis of DNA from the archive samples in mutation detection and molecular epidemiologic studies in breast cancer. Optimization of multiplex PCR was very important in multiplex PCR for mutation detection in nDNA.

Amplification of mtDNA from Archival Breast Cancer Tissues

The success of DNA amplification after microwave treatment and purification using simple boiling for 34 formalin-fixed and paraffin-embedded samples was 100 % but no mtDNA4977 deletion was detected in these samples. In addition to the archive samples, we detected some DNA samples which were isolated from 9 familial blood samples and 4 blood samples of healthy persons for mtDNA4977 deletion. Five-mtDNA4977 deletions were detected by multiplex PCR in 9 familial blood samples (fig, 2). The mtDNA4977 deletion was highly prevalent in peripheral blood (56%) but absent from the archive samples. The results demonstrate that the frequency of mtDNA4977 deletions was higher in blood samples ($P < 0.001$) compared to the archive samples. Wild type of mtDNA4977 was the smallest gene fragment that we amplified (127 bp), while the mutation type of mtDNA4977 (485 bp) was longer.

Comparison of Results of Multiplex PCR with Clinical Parameters

In total, DNA and breast tissues of 16 familial breast cancer patients and 18 non-familial breasts cancer patients were investigated. The ovarian cancer was observed in 2 familial breast cancer patients. Table 2 demonstrates the distributions of clinical parameters of tumors in the familial and non-familial cases. Tumors in familial breast cancer 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 tubules (OR= 0,49, P<0,04), compared with the non-familial cancers. Estrogen receptor ER (OR= 4,69, P <0.0001) and progesterone receptor PR (OR= 4,52, P <0.0001) were observed less frequently in the familial cancer cases than the non-familial ones. A significantly higher level of TP53 (OR=0,23, P<0,0001) expression was observed in tumor tissues in the familial breast cancer patients compared with the non-familial cases. The frequency of 5382insC and mtDNA4977 deletions was higher in the familial breast cancer cases (P<0.0001).

Discussion

Mutation analysis of mtDNA and nDNA are helpful in the determination of early diagnosis and gene therapy for breast cancer. Several studies have shown that high degradation of target DNA after electrophoresis may occur due to long time lapses between surgical tissue removal and fixation, the type of fixative used, and the duration of the fixation [9,15,18,19]. In this study, we focused on paraffin removal procedures using microwave and a commercial kit. We found that DNA extracted by a modified boiling method with microwave ,yielded higher proportions of successful gene amplifications (70 %) than the DNA extraction Kit (35 %) with microwave. Coombs et al. [22] found that successful DNA amplification after microwave treatment and simple boiling method was 19.2% but we were able to amplify DNA up to 70% with modified methods through multiplex PCR. The DNA extraction Kit yielded good results, but DNA yields were lower than that of the boiling method; the fraction of DNA suitable for amplification of BRCA mutation was also relatively low.

The most common gene changes in breast cancer are those of the *BRCA1* and *BRCA2* genes. However, methods widely used in research laboratories miss nearly a third of the *BRCA* mutations that are detected by DNA sequencing [20]. The analysis of the BIC data base files indicates that the 5382insC mutation is the second most frequent (800 records) of the total of the mutations associated with breast cancer. Three 5382insC mutations were identified from 16 archival familial patients after amplification (19%) and five-mtDNA4977 deletions were detected by multiplex PCR from 9 blood familial breast cancers. This deletion was highly prevalent in peripheral blood (56%), but it was absent in the breast tissue of the cancer cases. Although this study focused on detecting *BRCA* mutations in nDNA, our findings revealed that success of DNA amplification for mtDNA4977 deletions was 100 % in mtDNA. The DNA extracted may not be long enough; thus, the amplification of smaller gene fragments may be successful. However, the amplification of longer gene fragments may be slightly limited. Multiplex PCR is a powerful tool to examine several point mutations in a high volume of patient samples. Optimization of primers and PCR reactions is a limitation of current method to handle this complexity in a multiplexed format of analysis.

A positive family history of the disease is one of the strongest risk factors for developing breast cancer, especially among young women. However, ascertaining the patient's family history is a difficult task and significantly restricts the use of this parameter in clinical practice. Alternative individual criteria that can be used to identity *BRCA1* gene carriers would, therefore, be of great value. It was recently predicted that individual morphological and immunohistochemical parameters might prove to be useful tools of establishing the *BRCA1* status [10-13, 23]. In this study, we established that familial breast cancer tumors exhibited higher mitotic activity, higher polymorphism, lower necrosis, lower tubules, higher

ER- and PR-negatives and lower TP53-positives than the non-familial cancers (tab,3).

Conclusion

In summary, the primary goal of this study was to compare our own experiences concerning the extraction of nucleic acids from archive using conventional approaches. In addition, we focused on the subsequent application of multiplex PCR with morphological and immunohistochemical methods for the detection of *BRCA* mutation and mtDNA4977 deletion in familial and non-familial breast cancers. We observed that DNA derived from archival breast cancer tissues that archived for less than 1 year could be used successfully for detecting *BRCA* mutation and mtDNA4977 deletion by multiplex PCR. These results demonstrate that DNA extracted by the simple boiling method yielded higher proportions of successful gene amplifications than the DNA extraction Kit with microwave. Furthermore, the difference in successful amplification may be related to the size and number of the gene fragment amplified. Finally, our analysis indicates that testing of mtDNA4977 deletion and 5382insC mutation may be extremely effective .In addition, it is an inexpensive tool for testing high-risk familial breast cancer patients. Combination of above mutations provides a more accurate marker of familial breast cancer by using morphological and immunohistochemical parameters.

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