TP53 genetic alterations in Arab breast cancer patients: Novel mutations, pattern and distribution

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Abstract. Breast cancer remains a worldwide public health concern. The incidence and mortality of breast cancer varies significantly in ethnically and geographically distinct populations. In the Kingdom of Saudi Arabia (KSA) breast cancer has shown an increase in incidence and is characterized by early onset and aggressiveness. The tumor suppressor *TP53* gene is a crucial genetic factor that plays a significant role in breast carcinogenesis. Furthermore, studies have shown a correlation between certain p53 mutations and response to therapy in breast cancer. In the present study, *TP53* mutations were identified by direct sequencing of the gene (exons 4-9) from 119 breast cancer tissues. The prevalence of *TP53* mutations in Arab breast cancer patients living in the KSA is among the highest in the world (40%). Notably, 73% of the patients whose tumors harbored p53 mutations were less than 50 years of age. Furthermore, for the first time, we identified 7 novel mutations and 16 mutations in breast cancer tissues. Notably, all the novel point mutations were found in exon 4, wherein 29% of the mutations were localized. Furthermore, an excess of G:C→A:T transitions (49%) at non-CpG sites was noted, suggesting exposure to particular environmental carcinogens such as N-nitroso compounds. The results indicate that the *TP53* gene plays a significant role in breast carcinogenesis and the early onset of the disease among Arab female individuals.

Introduction

Breast cancer has a major impact on the health of women worldwide. It is the most frequently diagnosed cancer and a leading cause of cancer-related death, ranking second in Caucasian (1) and Saudi female patients (Cancer Incidence Report, NCR, 2004). The incidence and mortality rates vary between various ethnically and geographically distinct populations, with the lowest incidence reported among Asians and the highest among North Americans (2). Multiple causes characterize breast carcinomas, which may be either familial or sporadic. Genetic predisposition accounts for only about 5-10% of breast cancer, whereas 90% of breast cancer cases are sporadic and their origin remains to be determined (3). The Saudi population comprises more than 50% of females younger than 20 years old. In this population, the majority of breast cancer cases diagnosed are at advanced stages and at an early age (4). Similar characteristics have been found in African-American female individuals (5,6).

Breast carcinogenesis is associated with various types of somatic genetic alterations, such as mutations in oncogenes and tumor suppressor genes (7). The most frequently mutated gene in human malignancies, including breast cancer, is the *TP53* gene (8). This important tumor suppressor gene is a multifunctional transcription factor involved in the control of cell cycle progression, DNA repair, apoptosis and angiogenesis (9). The proportion of *TP53* mutations in various cancer tissues ranges from 10 to 80% (10), while that of *TP53* mutations reported in breast tumors ranges from 15 to 71%, with significant differences among populations. Over 1,400 *TP53* mutations have been identified in breast cancer (11). Of these mutations, 80% are clustered within exons 5-8 (12). Notably, the proportion of *TP53* mutations is higher in younger patients and those with advanced breast cancer (13); these patients comprise the prevalent breast cancer patient group among the Saudis. Furthermore, variations in patterns and distribution of p53 mutations in breast cancer occur according to ethnicity and geographical location, indicating the effect of genetic and environmental factors (14).

Cells lacking normal p53 function have a selective growth advantage and are more resistant to ionizing radiation and frequently used anticancer drugs compared to cells with wild-type p53 protein (15). *TP53* gene mutations predict the response of breast cancer patients to treatment with various

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Table I. *TP53* primers used in the PCR reactions.

Primer	Length (bp)	Sequence (5' to 3')	Size	Annealing temperature (˚C)	$MgCl_2$ (mM)
Exon 4			370	62	1.5
Forward	20	TGA GGA CCT GGT CCT CTG AC			
Reverse	20	CGG CCA GGC ATT GAA GTC TC			
Exon 5			330	62	3.0
Forward	20	TGT TCC AGT TGC TTT ATC TG			
Reverse	20	AGA GCA ATC AGT GAG GAA TC			
Exon 6			180	56-62	2.0
Forward	20	GGC CTC TGA TTC CTC ACT GA			
Reverse	20	GGT CCC CTA AGC AGC AGG AG			
Exon 7			257	62	2.5
Forward	20	CAG GTC TCC CCA AGG CGC AC			
Reverse	20	TGG AAG AAA TCG GTA AGA GG			
Exon 8,9			391	56-62	2.5
Forward	20	CCT TAC TGC CTC TTG CTT CT			
Reverse	20	TGT TAG ACT GGA AAC TTT CC			

chemotherapeutic agents (16,17). Furthermore, it has been shown that the *TP53* mutation status is a crucial survival marker of breast cancer that may provide prognostic data which complements clinical variables (18).

In the present study, the prevalence of *TP53* mutations in Arab breast cancer patients was among the highest in the world (40%), and occurred more frequently in young patients. Notably, 7 novel mutations, including a 15-bp deletion, were identified in these sporadic breast cancer patients.

Materials and methods

Sample collection. A total of 119 archived breast tumor samples were collected from Arab patients living in Saudi Arabia and suffering invasive ductal carcinoma. All of these patients were diagnosed at King Faisal Specialist Hospital and Research Center in Riyadh. The experimental protocol was approved by the institutional Basic Research and Ethics Protocol Committees (RAC proposal no. 2040037). The age of the patients at the time of diagnosis ranged from 22 to 80 years (median 51). A total of 108 fresh blood samples (5 ml) were collected from volunteer healthy Arab female individuals, and used as controls. The age of the healthy Saudi female individuals (controls) ranged from 17 to 76 years (median 47).

DNA purification. Genomic DNA was purified using the Gentra Puregen kit according to the manufacturer's instructions (Gentra Puregene Blood kit; Qiagen, Valencia, CA, USA; cat. no D-50K1-4).

DNA amplification and sequencing of the TP53 gene. Standard PCR was performed to amplify exons 4-9 and their intron/exon borders of the *TP53* gene, using the HotStar Taq polymerase kit (Qiagen, Chatsworth, CA, USA). The primers used for this amplification are listed in Table I. Each PCR reaction was performed in a total volume of 25 μl containing 4 ng of genomic DNA, 0.5 mM dNTPs, 1 mM primers, 0.04 units Taq DNA polymerase and $MgCl_2$ (1.5-3 mM). $MgCl_2$ concentrations were optimized according to the different primers (Table I). Following a denaturation step of 10 min at 94˚C, the PCR amplification consisted of 35 cycles of 45 sec at 94˚C, 45 sec at 62˚C, 45 sec at 72˚C, followed by a final extension step of 10 min at 72˚C. The PCR products were then directly sequenced using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The unincorporated dye labeled terminators were removed using the DyeEx 96 kit (Qiagen). The reaction product was resuspended in a formamide loading buffer, and then separated and detected in the ABI 3730x1 DNA analyzer (Applied Biosystems). The analysis of the obtained sequence was carried out using the GeneBank database, NT_010718. *TP53* somatic mutations were confirmed by two independent experiments.

Statistical analysis. Statistical analysis was carried out using the SPSS program version 17. The Chi-square test (χ^2) was used to test for an association between categorical data. $P \leq 0.05$ was considered to be statistically significant.

Results

Prevalence of TP53 mutations is high among Arab breast cancer patients. Screening for *TP53* mutations was carried out on exons 4-9.

DNA from 119 breast carcinoma tumor samples was amplified and sequenced. A total of 40 of 119 (33.61%) patients harbored mutations in the *TP53* gene; with 6 patients harboring more than one mutation. Subsequently, 47 substitutions were identified in the samples obtained from these 40 patients. Notably, only 19 exonic mutations of these substitutions were previously identified in breast cancer patients (Table II). Different types of mutations were detected: 28 (59.57%) were missense mutations, 6 (12.77%) were silent, 5 (10.64%) were nonsense

Table II. Summary of *TP53* mutations and their nature/location found in breast cancer tissues.

Base change	Structural change	Mutation type	Exon/ Intron	Coding Description	Mut _ ID
GAC>GGC[a]	D48G	Transition	E4	c.143A>G	449
TGG>TAG[a]	W53X	Transition	E4	c.158G>A	502
ACT>CCT[c]	T55P	Transversion	E4	c.163A>C	–
CCA>CTA[c]	P58S	Transition	E4	c.173C>T	–
CCC>CCT[c]	P64L	Transition	E4	c.192C>T	–
GCA>GGA[a]	A76G	Transversion	E4	c.227C>G	753
GCA>GCG[c]	A78A	Transition	E4	c.234A>G	–
GCC>GCT[a]	A84A	Transition	E4	c.252C>T	843
Del of C[a]	A88TdelfsX33	Deletion	E4	c.263del1	887
CCC>CCT[a]	P89P	Transition	E4	c.267C>T	899
TAC>TCC[c]	Y107S	Transversion	E4	c.320A>C	–
GGG>AGG[a]	G117R	Transition	E4	c.349G>A	1209
C>T	No change	Transition	IVS 4-3	c.376-3C>T	5820
C>T	-	Transition	IVS 4-14	c.376-14C>T	–
TCC>TTC[b]	S127F	Transition	E5	c.380C>T	1341
Del CAA[c]	L130-N131delLfsX15	Deletion	E5	c.390-392del3	–
GTG>GCG[b]	V143A	Transition	E5	c.428T>C	1590
CCC>CCT[b]	P153P	Transition	E5	c.459C>T	1761
ACC>ATC[b]	T155I	Transition	E5	c.464C>T	1794
ACC>ACT[b]	T155T	Transition	E5	c.465C>T	1799
ACC>AAC[a]	T155N	Transversion	E5	c.464C>A	1792
Del 15 bp[c]	V157-A161del	Deletion	E5	c.469-483del15	–
ATC>ATT[b]	I162I	Transition	E5	c.486C>T	1932
CAG>TAG[b,d]	Q165X	Transition	E5	c.493C>T	1972
TGC>TAC[b]	C176Y	Transition	E5	c.527G>A	2166
CAT>CGT[b]	H193R	Transition	E6	c.578A>G	2410
CAT>TAT[b]	H193Y	Transition	E6	c.577C>T	2408
TAT>GAT[a]	Y220D	Transversion	E6	c.658T>G	2819
TAT>TGT[b]	T220C	Transition	E6	c.659A>G	2821
GAG>GCG[a,d]	E221D	Transversion	E6	c.662A>C	2833
TAC>TAG[b,d]	Y234X	Transversion	E7	c.702C>G	3029
TAC>AAC[b]	Y234N	Transversion	E7	c.700T>A	3020
TGT>TTT[b]	C238F	Transversion	E7	c.713G>T	3108
C>T	-	Transition	IVS 7-15	c.783-15C>T	–
GTG>TTG[b]	V272L	Transversion	E8,9	c.814G>T	3713
AGA>GGA[b]	R280G	Transition	E8,9	c.838A>G	3844
AGA>ACA[a]	R280T	Transversion	E8,9	c.839G>C	3850
CCT>CTT[b]	P295L	Transition	E8,9	c.884C>T	4084
CAC>TAC[a]	H297Y	Transition	E8,9	c.889C>T	4106
CCC>CTC[a]	P316L	Transition	E8,9	c.947C>T	4348
CAG>CGG[a]	Q317R	Transition	E8,9	c.950A>G	4363
ACC>ATC[a]	T329I	Transition	E8,9	c.986C>T	4501
G>A	-	Transition	IVS 8+18	c.919+18G>A	–
G>A	-	Transition	IVS 9+28	c.993+28G>A	–

Mut, mutations. [a]Mutations reported in cancers other than breast cancer. [b]Mutations reported in breast cancer. [c]Mutations not previously reported. [d]*TP53* mutations reported more than once. International Agency for Research on Cancer database, *TP53* genetic variation in human cancer, IARC release R13 (2008), used as a reference.

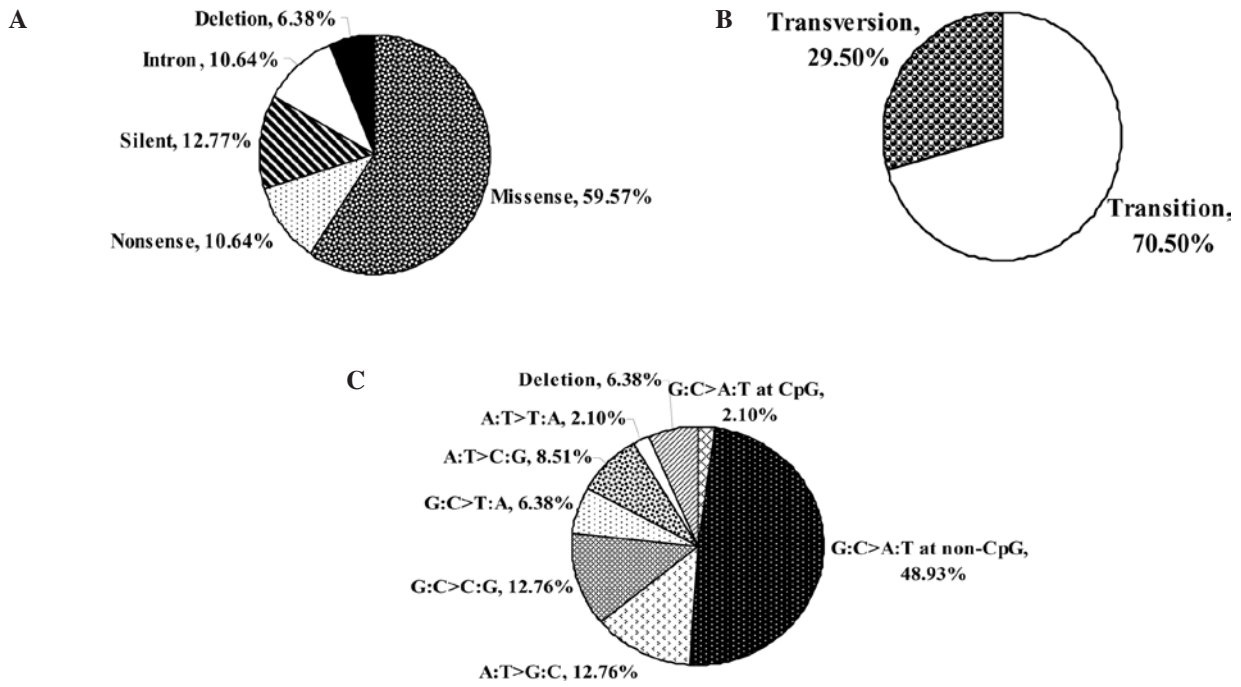

Figure 1. Types and proportions of the different *TP53* mutations identified in the present study. *TP53* mutation patterns are shown as pie charts indicating the proportions of the different types of mutations.

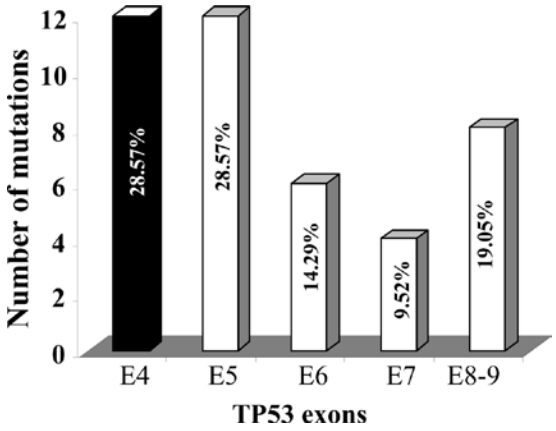

Figure 2. Exon distribution of *TP53* mutations identified in breast cancer patients from Saudi Arabia. The numbers within the columns indicate the proportions of mutations in the different exons (E4 to E8,9) of the gene.

(stop) mutations, and 3 (6.38%) deletions and 5 mutations (10.64%) were found in the intron-exon intersections (Fig. 1A). Two of the 3 deletions led to a premature stop codon (frame shift) (Table II).

The majority of the identified mutations were transitions (Fig. 1B). Only one transition mutation of proline-153 occurred at a CpG site. Furthermore, various base changes were identified in the 47 *TP53* mutations with 24 (51.1%) C:G→T:A transitions (at the CpG and non-CpG sites) representing the most frequent one (Fig. 1C). The frequency of this transition reached 48.93% at the non-CpG sites (Fig. 1C).

Fig. 2 shows the distribution of the *TP53* mutations within exons 4-9 of the gene. The majority of the mutations were identified in exons 4 and 5 (12 mutations, representing 29%, in each). However, only 4 mutations were identified in exon 7, while exons 6 and 8,9 harbored 6 and 8 mutations, respectively. This finding shows that exon 4 is a hot-spot for *TP53* mutations in the Saudi Arabian population. Furthermore, 9 of the 47 mutations were found within the conserved regions (II, III, IV and V) of the *TP53* gene. Of the 9 mutations, 3 were identified in conserved region V at valine 272 and argenine 280 (Table II). Argenine 280 is a significant amino acid involved in direct DNA binding. Another 2 mutations were found at cysteine 176 and 238, in conserved regions III and IV, respectively. These cysteines are also directly involved in the binding of the zinc molecule (Table II). Six (12.8%) mutations were identified within the zinc-binding loop domains L2 and L3 (codons 163-195 and 236-251, respectively) (Table II). No mutation was detected at the 3 hot-spot codons 248, 273 and 175, nor at the highly mutagenic codons 245, 249 and 282 (14). Furthermore, only one mutation was identified at codon 176 and 2 at codon 220.

Identification of novel mutations in the TP53 gene. In the present study, 16 new mutations were identified in the *TP53* gene. These mutations were found in 14 different patients. One of these patients harbored 3 mutations. The majority of these mutations were transitional (10 transitions vs. 6 transversions) (Table II). In addition, 7 novel changes were identified in the *TP53* gene (not previously reported in breast cancer or any other tumor type, IARC database, 2008). These changes (5 base substitutions and 2 deletions) were found in 6 different patients, since the tumor from one patient had 2 of these novel mutations at codons 58 and 64 (Table II). All of the 5 base substitutions were located in exon 4 at codons 58, 64, 55, 78 and 107 (3 transitions

Table III. Association of *TP53* gene mutations with the clinicopathological characteristics of Arab breast cancer patients.

	Total (n)	Positive n (%)	Negative n (%)	P-value
Age				
<50	69	24 (34.8)	45 (65.2)	0.4480
≥50	33	9 (27.3)	24 (72.0)	
Menopausal status				
Premenopausal	68	24 (23.08)	44 (42.31)	0.1690
Postmenopausal	36	8 (7.69)	28 (26.92)	
ER status				
Positive	48	13 (18.31)	35 (49.30)	0.7690
Negative	23	7 (9.86)	16 (22.54)	
PR status				
Positive	1	0 (0.00)	1 (7.69)	0.7640
Negative	12	1 (7.69)	11 (84.62)	
ErbB2 status				
Positive	45	18 (17.48)	27 (26.21)	0.0850
Negative	58	14 (13.59)	44 (42.72)	
Involvement of lymph nodes				
Positive	46	16 (17.20)	30 (32.26)	0.4590
Negative	47	13 (13.98)	34 (36.56)	
Clinical stage of tumors				
I	16	3 (3.19)	13 (13.83)	
II	35	11 (11.70)	24 (25.53)	0.0447
III	22	12 (12.77)	10 (10.64)	
IV	21	4 (4.26)	17 (18/09)	
Histopathological grade of tumors				
I	9	3 (2.88)	6 (5.77)	
II	53	14 (13.46)	39 (37.50)	0.6120
III	42	15 (14.42)	27 (25.96)	

and 2 transversions) (Table II). The 2 novel deletions of 3 and 15 bp were identified in exon 5 (Table II). The second deletion did not lead to a premature stop codon, whereas the first one did following the addition of 15 new amino acids.

The frequency of the 7 novel changes was <1%. Therefore, they were considered as mutations. To verify this, we sequenced exon 4 which encompassed the 5 base substitutions from 108 DNA blood samples from healthy Arab female controls. No substitutions were identified at these sites, confirming that the substitutions identified in the breast cancer tissues were novel mutations. Therefore, the frequency of p53 mutations in the Arab breast cancer patients was 39.49%.

Association between TP53 mutations and the age of Arab breast cancer patients. The potential link between *TP53* mutations and the age of breast cancer patients was investigated. The patients were divided into two subgroups depending on their age; the first group included patients younger than 50 years of age (young patients), and the second included patients of 50 years or older ('old' patients). As expected, most of the Arab breast cancer patients (68%) were under 50 years of age, confirming the early onset of breast cancer in this population. Notably, among 33 patients that harbored *TP53* mutations in their tumors, 24 (73%) were young patients, whereas only 9 (27%) were considered older patients. In each subgroup, patients with tumors harboring *TP53* mutations were compared with those patients with tumors without *TP53* mutations. Table III shows that the *TP53* gene mutations were more frequent in tumors from younger patients with a prevalence of 35%, whereas in the older patients the *TP53* mutations were only 27%. However, the difference was not statistically significant (p=0.45).

Association between TP53 mutations and the clinocopathological characteristics of Arab breast cancer patients. To investigate the potential role of p53 in the development and progression of primary breast tumors, the clinicopathological characteristics of the patients with tumors harboring p53 mutations were compared with those of patients that had tumors without p53 mutations. A statistically significant correlation between the presence of p53 mutations and the clinical stage of the tumors was found (p=0.0447). Patients with locally advanced breast cancer stage III A+B showed the highest proportion of p53 mutations. On the other hand, no statistically significant correlation was found with the other characteristics, such as the menopausal status, the histopathological grade, the presence or absence of lymphatic or vascular invasion, ER/PR status and Her2neu.

Discussion

In the present study, the frequency of *TP53* mutations in Arab breast cancer patients living in Saudi Arabia was found to be 39.49%. This frequency is considered to be relatively high, since it is significantly higher than the previously reported mean proportion of 25% (range 15-71%; examined in 1425 breast tumor samples worldwide) (19). It is also higher than the prevalence of p53 mutations in breast tumors determined in a meta-analysis (18%) (20) and in the IARC mutation prevalence database on all breast cancers, R9 release (28%) (21). Therefore, the frequency of *TP53* mutations in the KSA is one of the highest in the world. It is similar to the frequency found in Kashmir (44%) (22), the USA (45%) (21), Japan (47.5%) (21), the UK (34.5%) (21), and in African-Americans (34.5%) (23). However, it is higher than the prevalence reported in patients from Delhi, India (3%) (24), France (19%) (25), Tokyo (25%) (26) and US midwestern Caucasians (30%) (27). This variation in p53 mutations in breast cancers may be due to factors such as the ethno-geographically diverse populations studied, exposure to various carcinogens, size of the studied population, life-style and dietary habits. Notably, 7 novel mutations (not previously reported in the *TP53* gene) were identified during this study; 5 of the 7 mutations were found in exon 4. Therefore, tumors from Arab breast cancer patients have a high prevalence (28.57%) of *TP53* mutations in exons 4 and 5, whereas the smallest proportion of *TP53* mutations (9.52%) was found in exon 7. However, in the IARC database, exon 5 has the highest proportion of *TP53* mutations in breast cancer (30.6%) followed by exon 7 (23.5%), while exon 4 represents only 4.2% of mutations (IARC *TP53* Database, R14 release, November 2009, http://www.iarc.fr/p53/homepage.htm/). Therefore, even the distribution of *TP53* mutations in the various exons of the gene appears to be population-dependent. Brazilian women of African descent have a higher proportion of mutations in exons 5 and 7, whereas Brazilian women of Caucasian descent have more mutations in exon 8. No mutations were found in Brazilian patients of African descent in exon 4 (29). In the Kashmiri population, no mutation was found in exon 5, and 52.9% of mutations were identified in exon 6 (22). To the best of our knowledge, this study is the first to report a high proportion of mutations in exon 4 of the *TP53* gene.

When we compared the *TP53* mutational pattern in the Arab breast cancer population to the patterns of 15 other populations from low and high breast cancer-risk countries, we found that the Saudi population is characterized by a low frequency (2.1%) of the G:C→A:T transition (at CpG sites) and a high frequency (48.9%) of the mutational type G:C→A:T transition (at non-CpG sites). Thus, the Arab population living in Saudi Arabia possesses the second highest frequency of G:C→A:T transitions at non-CpG sites after a New Orleans population of African or Caucasian descent (57%) (23). On the other hand, the frequency of G:C→A:T transitions at CpG sites in the KSA is the lowest in the world. IARC mutation spectrum data on all breast cancer cases reported frequencies of 17.7% at the non-CpG sites and 21.3% at the CpG sites (21). This variation in the *TP53* mutation pattern among different populations may be due to exposure to various environmental mutagens (23). The association between mutations and specific exogenous mutagens has been observed in the *TP53* gene. The best example is the CC→TT tandem dipyrimidine transition associated with UV light and G→T transversions associated with benzo(a)pyrene (14). In the Saudi breast cancer patients, the most distinguishing feature of the *TP53* mutation pattern was the excess of G:C→A:T transition at the non-CpG sites, which was rarely found at the CpG sites. The transition of cytosine to thymine at the CpG sites may result from spontaneous deamination of methylated cytosine (29). Therefore, the low frequency of this transition in the Saudi breast cancer patients is likely to be due to the low cytosine methylation at the CpG sites. On the other hand, the G:C→A:T transitions at the non-CpG sites is induced by various carcinogens, in particular oxidizing agents and alkylating agents such as N-nitroso compounds (e.g., nitrosoamines and N-nitrosodimethylamine 'NDMA') (14). The carcinogenic effect of the N-nitroso compounds on the mammary gland of laboratory animals is well established, suggesting that human mammary epithelial cells contain DNA adducts due to exposure to these chemicals (30,31). N-nitroso compounds (e.g., N-nitrosdimethylamines) are procarcinogenic agents that are bioactivated by enzymatic metabolism (32,33). These agents lead to guanine alkylation generating O^6-alkylguanine (e.g., O^6-methylguanine), which typically results in G:C→A:T transitions (34). This adduct can be directly repaired by alkylguanine alkyltransferase enzymes (e.g. O^6-methylguanine DNA methyl transferase enzymes) (35). This enzyme has been detected in breast tissue with large inter-individual variations in activity (36). Zaidi *et al* demonstrated that the presence of estrogen increased the amount of O^6-methylguanine in the DNA of breast xenografts (34). Therefore, high exposure to nitrosamines (or NDMA) with insufficient capacity for DNA repair or high levels of estrogen may lead to the accumulation of DNA damage and the formation of mutations that trigger cellular transformation and then breast carcinogenesis. These mutagens and the type of mutations they induce have been shown to play a role in the etiopathogenesis of oesophageal and gastric carcinomas (37-39).

Findings of our study showed that among the 33 patients with tumors harboring *TP53* mutations, 24 (73%) were young patients (<50 years of age), while only 9 (27%) were older patients (≥50 years of age). Furthermore, *TP53* mutations occurred more frequently in tumors from young patients with a prevalence of 34.8% than in the older patients with a prevalence of 27.3%. However, this difference was not statistically significant (p=0.45). Studies have reported the presence of an association between *TP53* mutations and the age of breast cancer onset (13). However, Nagai *et al* who reported on the Brazilian population, found no significant correlation between the age of breast cancer patients and p53 mutations (28).

In the present study, the frequency of p53 mutations in the Arab breast cancer patients was found to be among the highest in the world (40%), with a high proportion of these mutations localized in exon 4 of the gene. Five out of these 12 mutations were identified for the first time. We also identified 2 novel deletions in exon 5. In addition, 16 mutations were identified for the first time in these breast cancer patients. A total of 70% of the patients harboring p53 mutations in their tumors were younger than 50 years of age. Therefore, it can be concluded that the *TP53* gene plays a signficant role in breast carcinogenesis and the early onset of the disease among Arab female individuals.

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