Detection of genetic mutations in patients with breast cancer from Saudi Arabia using Ion AmpliSeq™ Cancer Hotspot Panel v.2.0

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Abstract. Next-Generation Sequencing allows for quick and precise sequencing of multiple genes concurrently. Recently, this technology has been employed for the identification of novel gene mutations responsible for disease manifestation among breast cancer (BC) patients, the most common type of cancer amongst Arabian women, and the major cause of disease-associated death in women worldwide. Genomic DNA was extracted from the peripheral blood of 32 Saudi Arabian BC patients with histologically confirmed invasive BC stages I-III and IV, as well from 32 healthy Saudi Arabian women using a QIAamp® DNA Mini Kit. The isolated DNA was quantified using a Qubit™ dsDNA BR Assay Kit with a Qubit 2.0 Fluorometer. Ion semiconductor sequencing technology with an Ion S5 System and AmpliSeq™ Cancer Hotspot Panel v2 were utilized to analyze ~2,800 mutations described in the Catalogue of Somatic Mutations in Cancer from 50 oncogenes and tumor suppressor genes. Ion Reporter Software v.5.6 was used to evaluate the genomic alterations in all the samples after alignment to the hg19 human reference genome. The results showed that out of the 50 genes, 26 mutations, including 17 (65%) missense point mutations (single nucleotide variants), and 9 (35%) frameshift (insertion/deletion) mutations, were identified in 11 genes across the cohort in 61 samples (95%). Mutations were predominantly focused on two genes, PIK3CA and TP53, in the BC genomes of the sample set. *PIK3CA* mutation, c.1173A>G located in exon 9, was identified in 15 patients (46.9%). The *TP53* mutations detected were a missense mutation (c.215C>G) in 26 patients (86.70%) and 1 frameshift mutation (c.215_216insG) in 1 patient (3.33%), located within exon 3 and 5, respectively. This study revealed specific mutation profiles for every BC patient, Thus, the results showed that Ion Torrent DNA Sequencing technology may be a possible diagnostic and prognostic method for developing personalized therapy based on the patient's individual BC genome.

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Introduction

Breast cancer (BC) is the most common malignancy in females and the second leading cause of cancer-related death after lung cancer worldwide (1). BC has a significant impact on a women's health (2), and its incidence rates have been steadily increasing in recent years in Arab-speaking communities, with a significant number of cases being diagnosed in the first instance at advanced stages of the disease (3). BC incidence varies widely globally (4), and its incidence amongst Saudi Arabian women has progressively increased (3). BC is a complex and multifactorial disease, and genetic, hormonal and environmental factors contribute to its pathogenesis (5). The interplay between the genetic background and the environment in BC development has been proposed, but with mixed results on the importance of each (6-8). Female sex, age and ethnicity are the strongest risk factors associated with increased incidence (9). In addition, obesity (10), age at first delivery of a child (11), early menarche/late menopause (12), ionizing radiation exposure (13), breastfeeding (14), past/current estrogen treatment (15), breast tissue density (16), smoking and alcohol consumption (17) and steroid hormone receptors (18) are all known risk factors of BC.

BC aggregates in families, and an estimated 5-10% of BC cases are hereditary in origin caused by a myriad of susceptibility genes, transmitted from parent to child (19). These include rare variants in *BRCA1*, *BRCA2*, *PALB2*, *ATM* and *CHEK2* genes, which reportedly confer a moderate-high lifetime risk of the disease. Other variants of >70 loci, which were identified through genome-wide association study, and large-scale replication studies (20), were also reported to confer heightened risk of disease, though to varying extents.

While hereditary BC is linked to a well-established set of susceptibility genes, the exact contribution of these genes to

disease pathogenesis remains largely unknown (21). *BRCA1* (chromosome 17) and *BRCA2* (chromosome 13), described as regulators of DNA repair, transcription and cell cycle progression in response to DNA damage, were confirmed genetic loci associated with genetic susceptibility to BC (22,23). In this regard, it was shown that pathogenic *BRCA1*/*BRCA2* mutations account for almost 30% of BC cases in high-risk families (24). Polymorphisms in other genes are also involved in BC, but to variable extents (25), suggesting that genetic variations may explain the heterogeneous nature of BC, and thus inter-individual differences with regard to tumor behavior (20).

AKT1, *PIK3CA*, *PTEN* and *TP53* have been identified as recurrently mutated genes, and somatic mutations in these genes are found at a high frequency in BC patients. According to the Catalogue of Somatic Mutations in Cancer (COSMIC) database (26), high prevalence rates of *PIK3CA* (26.4%), *TP53* (24.7%), *PTEN* (3.8%), and *AKT1* (2.8%) were reported for BC.

In addition to the aforementioned mutated genes, two kinds of genomic instability have been often reported in BC and seldom in proliferative breast disease: Microsatellite instability (MSI) and loss of heterozygosity (27). Clinical testing for MSI involves immunohistochemistry and PCR testing for four proteins of the mismatch repair pathway: MSH2, MSH6, MLH1 and PMS2. MSI has been documented in BC, but at a lower frequency compared with other types of cancer. Previous studies reported that 0.9% of primary Triple Negative BC (28) and 1.53% of BC of all subtypes (29) have MSI.

In view of its heterogeneous etiology, the manifestations of BC vary widely among individual patients, with each patient having a unique profile, hence highlighting the potential value of precision medicine and individualized therapies for effective management (30-32). Next-Generation sequencing (NGS) was recently employed to improve identification of novel gene mutations responsible for disease manifestation amongst BC patients (33-36).

Ion Torrent™ technology allows the parallel sequencing of several genes, thus overcoming the problems inherent with conventional sequencing. In this study, Ion semiconductor sequencing technology with the Ion S5 System and AmpliSeq™ Cancer Hotspot Panel v2 was used to analyze ~2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes in a cohort of 32 BC cases from Saudi Arabia. Therefore, the present study aims to investigate the efficiency of AmpliSeq™ Cancer Hotspot Panel v2 on the detection of mutations in the genomic DNA extracted from the whole blood of the Saudi BC patients.

Patients and methods

Study subjects. Ethical approval for the present study was obtained from the Ethics Committee of King Fahad Medical City (KFMC; Riyadh, Saudi Arabia; IRB approval no. FWA00018774), and the study was performed in accordance with the guidelines described in the Helsinki Declaration (37). A total of 32 Saudi Arabian patients with BC (mean age 48.5±8.2 years; median age 45 years [age range, 31-85 years; interquartile range (IQR) 42.5-55.5)], and with histologically confirmed invasive BC, were recruited from Medical Oncology Department, KFMC. None of the patients had a history of other cancer types and were not subjected to chemo-, radio or hormone therapy. In addition, 32 healthy Saudi women with no familial history of any cancer types served as the controls (mean age 49.1±11.0 years; median age, 47.5 years (age range, 35-71 years; IQR, 42.5-55), were recruited into this retrospective case-controlled study from the blood bank (Table I). Demographic and clinical data of BC patients and control women were collected from the hospital records. All participants provided signed informed consent prior to inclusion in this study.

DNA extraction and quantification. Peripheral blood (2 ml) was collected in EDTA tubes from each participant. Genomic DNA was extracted using a QIAamp® DNA Mini Kit according to manufacturer's instructions (Qiagen GmbH) then quantified using a Qubit™ dsDNA BR Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Inc.), following the manufacturer's instructions.

Library preparation. Manual library preparations were performed using an Ion AmpliSeq™ Cancer Hotspot Panel v.2, Ion Xpress barcoded adapters, and an Ion AmpliSeq Library Kit 2.0 (Thermo Fisher Scientific, Inc.). The panel consisted of 207 amplicons, covering ~20,000 bases surveying hotspot regions, including up to 2,855 COSMIC mutations in 50 oncogenes and tumor suppressor genes, all with known cancer associations. The genes included in this panel were: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNA11*, *GNAS*, *GNAQ*, *HNF1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RB1*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL*.

Multiplex PCR was performed using 10 ng genomic DNA with a premixed primer pool and Ion AmpliSeq HiFi master mix (Ion AmpliSeq Library Kit 2.0). The amplicons were treated with 2 μl FuPa reagent to partially digest the primer sequences and phosphorylate the amplicons. Amplicons were ligated to adapters with the diluted barcodes of Ion Xpress Barcode Adapters kit (Thermo Fisher Scientific, Inc.). The adapter-ligated amplicons (library) were purified using the Agencourt AMPure XP reagent (Beckman Coulter, Inc.). Quantification of the final libraries was performed using an Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific, Inc.) on the Applied Biosystems® 7500 Real-Time PCR System, following the manufacturer's protocols.

Template preparation and chip loading. After library dilution to ~100 pM, the clonal amplification of barcoded DNA library (AmpliSeq libraries) onto ion spheres was performed on an Ion Chef™ Instrument using Ion 520™ & Ion 530™ Kit-Chef, according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Template-positive spheres from barcoded libraries were multiplexed and loaded onto Ion 530™ Chips following the manufacturer's protocol, and sequencing was run on the Ion Gene Studio S5 system (Thermo Fisher Scientific, Inc.).

Statistical analysis. Samples were evaluated for genomic alterations, including single nucleotide variants (SNVs), and insertions and deletions, using Ion Reporter Software v.5.6

Table I. Demographics and clinical characteristics of the cohorts.

Characteristics	Healthy controls, n=32	Patients BC, n=32	P-value
Mean age, years[c]	49.09±11.02	48.80±8.28	0.904[d]
BMI, kg/m^2[b]	27.60±5.66	32.89±7.96	0.004[b,d]
Oral contraceptives use			0.5[e]
Yes	19	20	
No	13	12	
Breastfeeding			0.011[a,e]
Yes	19	9	
No	13	23	
Tumor size			-
<2 cm	-	4	
≥2 cm	-	28	
Tumor stage			-
I	-	3	
II	-	11	
III	-	13	
IV	-	5	
Histological classification			-
IDC	-	29	
ILC	-	2	
DCIS	-	1	
Tumor location			
Left	-	28	
Right	-	4	
ER status			-
ER$^+$	-	23	
ER$^-$	-	9	
PR status			-
PR$^+$	-	21	
PR$^-$	-	11	
HER2 status			-
HER2$^+$	-	13	
HER2$^-$	-	19	

[a]P<0.05, [b]P<0.01. [c]Mean ± standard deviation. [d]Unpaired Student's t-test. [e]χ^2 test. HER2, Human epidermal growth factor receptor; ER, Estrogen receptor; PR, Progesterone receptor; DCIS, Ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma.

(Thermo Fisher Scientific, Inc.), after alignment to the hg19 human reference genome. Of note, higher quality requirements for variant analysis and selection, high-quality SNVs and insertion/deletion variants were strictly followed in this study and were defined as: i) FILTER=PASS, ii) QUAL≥100, iii) depth coverage≥20X, and iv) variant fraction≥20%. The sequencing data analysis using such approach yielded high-quality variants that did not necessary require additional confirmatory testing (such as through Sanger sequencing validation) as recommended by Arteche-López group (38). Furthermore, the 'bam' files of each clinically actionable variant were carefully reviewed in order to provide additional confidence to the accuracy and reliability of the NGS calls.

Qualitative and quantitative data were analyzed using SPSS v.21 (IBM Corp.). Qualitative data are presented as the frequency and percentage of total, and these data were compared using a χ^2 goodness-of-fit test, while continuous variables are presented as the mean ± SD, and were compared using an unpaired two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Study subjects. The clinical and demographic characteristics of the study subjects are summarized in Table I. Patients were clinically characterized in terms of tumor size, location, stage, histological classification and presence/absence of tumor markers, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). In addition, age, body mass index (BMI), use of oral

contraception and breastfeeding were compared between the two groups. No statistically significant differences were noted between patients and controls regarding mean age (P=0.90), and oral contraceptive use (P=0.50). However, a significant difference was noted between BC patients and healthy subjects for mean BMI, which was higher in patients compared with the control group (P=0.004), and breastfeeding (P=0.011), which was higher in the healthy control group.

Invasive ductal carcinoma of no specific type (n=29, 90.6%) was the predominant histological type of primary tumor, followed by invasive lobular carcinoma (n=2, 6.3%), and ductal carcinoma *in situ* (n=1, 3.1%). The majority of cases were stage III (n=13, 40.6%) and stage II (n=11, 34.4%), whereas stage I (n=3, 9.3%) and stage IV (n=5, 15.6%) were less common. In addition, 4 patients (12.5%) had ER^+, PR^+, $HER2^-$ tumors; 21 patients (65.6%) had ER^+, and/or PR^+, $HER2^+$ tumors, 4 patients (12.5%) had ER^-, PR^-, $HER2^+$ tumors, and 3 patients (9.4%) had triple-negative (ER^-, PR^-, $HER2^-$) tumors.

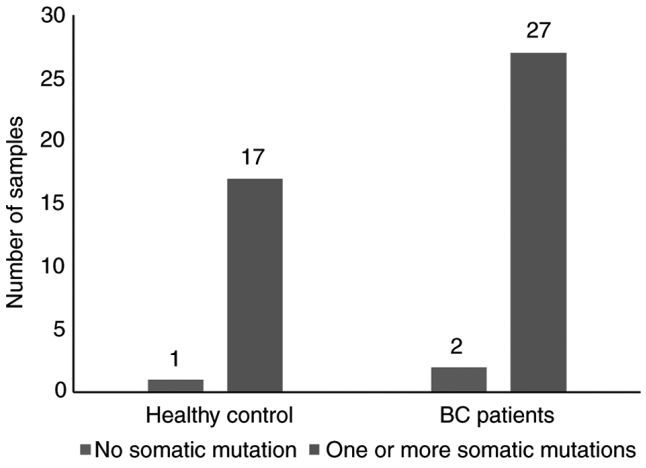

Figure 1. Bar graph showing the distribution of somatic mutations in BC patients and healthy controls. BC, breast cancer.

Somatic mutations. There were only three unclassified samples, two from patients (sample #2 and sample #13) and 1 from a control individual (sample #4) that did not possess any identified mutations (Fig. 1). The observed mutations were detected with varied frequencies across BC patients (Table II) and healthy controls (Table III); 26 mutations, including 17 (65%) missense point mutations (SNV), and 9 (35%) frameshift (insertion/deletion) mutations in 11 genes (out of 50); *TP53*, *PIK3CA*, *KDR*, *KIT*, *ATM*, *HRAS*, *ERBB2*, *FGFR3*, *GNAQ*, *APC* and *JAK3* (Fig. 2) across the cohort in the 61 samples (95%).

Amongst BC patients, 27 patients (84.8%) were positive for >1 somatic mutation, compared with 17 control subjects (53.1%) (Fig. 1). The most frequently observed concurrent mutations were some combination of c.215C>G (*TP53*), c.1173A>G (*PIK3CA*), c.1416A>T (*KDR*) and c.1621A>C (*KIT*) in patients (Fig. 3A), and *c.215C>G* (*TP53*), *c.1173A>G* (*PIK3CA*) and *c.1416A>T* (*KDR*) in healthy controls (Fig. 3B).

The site of the most frequent mutations within *TP53* and *PIK3CA* differed between samples. The most common *TP53* mutation detected was a missense mutation (c.215C>G) in 26 patients (86.70%), with only 1 frameshift mutation (c.215_216insG) identified in 1 patient (3.33%). The most common *PIK3CA* mutation detected was c.1173A>G, located in exon 9, and was identified in 15 (50%) patients.

Table IV summarizes the distribution of the most frequent missense mutations in *TP53* and *PIK3CA* (c.215C>G and c.1173A>G, respectively), between patients and healthy control samples. A significant difference (P=0.020) was observed in the frequency of c.215C>G (Pro72Arg), which was higher in patients (0.87) than in controls (0.61). Similarly, a significant difference was observed in the frequency of c.1173A>G (Pro72Arg) (P=0.041) that was higher in patients (0.53) compared with the healthy control participants (0.25).

A unique mutational status was identified for every BC patient except for patients #8, #10, #18, and #20 (Table II).

Discussion

BC is the most common type of cancer amongst Arabian women, and the major cause of disease-associated mortality in women worldwide (39). In the Middle East, Arabian women face a significantly higher risk of mortality, as the cancer is often diagnosed at a later stage in disease progression (40-45).

Current efforts to manage BC include on improving prevention, diagnosis and an increased armamentarium of effective treatment choices for patients with BC (46). Due to the heterogeneity of BC and the interactions between genetic and environmental factors, each patient's tumor possibly exhibits an unique gene mutation profile (47). By profiling an individual's cancer genome, it becomes possible to differentiate the oncogenic mechanisms that regulate cancer and, therefore, the genetic biomarkers that may be specifically associated with the disease state (5).

In this study, massively parallel sequencing was performed to identify frequent mutations in 32 BC Saudi Arabian patients and 32 healthy controls using Ion Torrent sequencing technology.

In this study, 32 BC patients were clinically characterized in terms of tumor size, location, stage, histological classification, and the presence or absence of tumor markers such as ER, PR, and HER2. In addition, four common risk factors, including age, BMI, oral contraceptives use and breastfeeding, were also evaluated and compared between healthy controls and BC patients. The statistical analysis showed no significant difference in the mean age between BC patients and healthy controls as the two study groups were already age matched.

BMI was significantly higher in BC patients compared with the healthy control group. This finding is in agreement with several reports, highlighting BMI as one of the most important risk factors for the BC (48,49). Chronic low-levels of inflammation that are usually observed in obese people can lead to BC through the increased likelihood of DNA damage (50). Additionally, fat cells can produce an excess amount of estrogen and adipokines, which can stimulate cell growth, as observed in BC (49,51). Similarly, a significant difference was observed for breastfeeding, which was higher in control group compared to the BC patients group, supporting the previously published literature linking breastfeeding to reduced BC risk (52).

PIK3CA and *TP53* are the most frequently mutated genes, and harbor most of the mutations in this cohort. The p53 tumor suppressor gene, located on chromosome 17p13,

Table II. Mutational status of breast cancer patient samples analyzed using the Ion AmpliSeq™ Cancer Hotspot Panel v2.

No.	Tumor histologic type	Stage	Hormone receptor status			Genes	Mutations detected	Effect	Amino acid change
1	IDC	III	$HER2^{+}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KDR	c.1416A>T	Missense	p.Gln472His
						GNAQ	c.625C>A	Missense	p.Gln209Lys
						TP53	c.215C>G	Missense	p.Pro72Arg
3	IDC	IV	$HER2^{-}$	ER^{-}	PR^{-}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						HRAS	c.84_85insT,	Frameshift	p.Val29fs,
						HRAS	c.80_81insC	Frameshift	p.Val29fs
						TP53	c.215C>G	Missense	p.Pro72Arg
						JAK3	c.394C>A	Missense	p.Pro132Thr
4	IDC	III	$HER2^{-}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KDR	c.1416A>T	Missense	p.Gln472His
						TP53	c.215C>G	Missense	p.Pro72Arg
5	IDC	II	$HER2^{+}$	ER^{+}	PR^{+}	KIT	c.1621A>C	Missense	p.Met541Leu
						TP53	c.21+I9:K95C>G	Missense	p.Pro72Arg
6	IDC	III	$HER2^{+}$	ER^{+}	PR^{+}	KDR	c.1416A>T	Missense	p.Gln472His
						TP53	c.215C>G	Missense	p.Pro72Arg
7	IDC	III	$HER2^{+}$	ER^{+}	PR^{-}	APC	c.3949G>C	Missense	p.Glu1317Gln
						ATM	c.2572T>C	Missense	p.Phe858Leu
						TP53	c.215C>G	Missense	p.Pro72Arg
8	IDC	II	$HER2^{+}$	ER^{+}	PR^{+}	KDR	c.1416A>T	Missense	p.Gln472His
						TP53	c.21G	Missense	p.Pro72Arg
9	IDC	II	$HER2^{-}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						TP53	c.215C>G	Missense	p.Pro72Arg
10	IDC	II	$HER2^{+}$	ER^{+}	PR^{+}	TP53	c.215C>G	Missense	p.Pro72Arg
11	IDC	IV	$HER2^{-}$	ER^{-}	PR^{-}	TP53	c.215C>G	Missense	p.Pro72Arg
12	DCIS	III	$HER2^{+}$	ER-	PR^{-}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KDR	c.1416A>T	Missense	p.Gln472His
						TP53	c.215C>G	Missense	p.Pro72Arg
14	IDC	I	$HER2^{+}$	ER^{-}	PR^{-}	TP53	c.215C>G	Missense	p.Pro72Arg
15	IDC	III	$HER2^{+}$	ER^{+}	PR^{+}	ATM	c.2525C>G	Missense	p.Thr842Ser
						TP53	c.215C>G	Missense	p.Pro72Arg
16	IDC	IV	$HER2^{+}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
					PR^{+}	APC	c.3920T>A	Missense	p.Ile1307Lys
						APC	c.3920_3921delTA	Frameshift	p.Ile1307fs
						APC	c.3920delT	Frameshift	p.Ile1307fs
						TP53	c.215C>G	Missense	p.Pro72Arg
17	IDC	IV	$HER2^{+}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KIT	c.1621A>C	Missense	p.Met541Leu
						TP53	c.215_216insG	Frameshift	p.Val73fs
						TP53	c.215C>G	Missense	p.Pro72Arg
18	IDC	II	$HER2^{+}$	ER^{+}	PR^{+}	KDR	c.1416A>T	Missense	p.Gln472His
						TP53	c.215C>G	Missense	p.Pro72Arg
19	IDC	III	$HER2^{-}$	ER^{-}	PR^{-}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						TP53	c.215C>G	Missense	p.Pro72Arg
20	IDC	II	$HER2^{+}$	ER^{+}	PR^{+}	TP53	c.215C>G	Missense	p.Pro72Arg
21	ILC	I	$HER2^{+}$	ER^{+}	PR^{+}	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						FGFR3	c.2389G>C	Missense	p.Ala797Pro
						ATM	c.7313C>T	Missense	p.Thr2438Ile
						TP53	c.215C>G	Missense	p.Pro72Arg

Table II. Continued.

No.	Tumor histologic type	Stage	Hormone receptor status			Genes	Mutations detected	Effect	Amino acid change
22	IDC	III	$HER2^+$	ER^+	PR^+	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						TP53	c.215C>G	Missense	p.Pro72Arg
23	IDC	III	$HER2^+$	ER^+	PR^+	TP53	c.215C>G	Missense	p.Pro72Arg
24	IDC	III	$HER2^+$	ER^+	PR^+	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						HRAS	c.84_85insT	Frameshift	p.Val29fs,
						HRAS	c.80_81insC	Frameshift	p.Val29fs
						TP53	c.215C>G	Missense	p.Pro72Arg
25	IDC	III	$HER2^-$	ER^-	PR^-	KIT	c.1621A>C	Missense	p.Met541Leu
						TP53	c.215C>G	Missense	p.Pro72Arg
26	ILC	III	$HER2^+$	ER^+	PR^+	KIT	c.1621A>C	Missense	p.Met541Leu
						TP53	c.215C>G	Missense	p.Pro72Arg
27	IDC	II	$HER2^+$	ER^+	PR^+	PIK3CA	c.233A>G	Missense	p.Glu78Gly
						PIK3CA	c.1173A>G	Missense	p.Ile391Met
28	IDC	IV	$HER2^+$	ER^+	PR^+	PIK3CA	c.233A>G	Missense	p.Glu78Gly
						ATM	c.1810C>T	Missense	p.Pro604Ser
						TP53	c.215C>G	Missense	p.Pro72Arg
29	IDC	II	$HER2^-$	ER^+	PR^+	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KDR	c.1416A>T	Missense	p.Gln472His
30	ILC	II	$HER2^-$	ER^+	PR^+	KDR	c.1416A>T	Missense	p.Gln472His
						ATM	c.3905G>T	Missense	p.Gly1302Val
31	IDC	II	$HER2^+$	ER^-	PR^-	TP53	c.215C>G	Missense	p.Pro72Arg
32	IDC	II	$HER2^+$	ER^-	PR^-	PIK3CA	c.1173A>G	Missense	p.Ile391Met
						KDR	c.1416A>T	Missense	p.Gln472His

Del, deletion; ins, insertion; fs, frameshift; HER2, Human epidermal growth factor receptor; ER, Estrogen receptor; PR, Progesterone receptor; DCIS, Ductal carcinoma *in situ*; ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma.

is 20 kb long, encompassing 11 exons encoding a 53 kDa phosphoprotein (53). In the present study the detected mutations in this gene were 1 missense mutations (c.215C>G) in 26 patients (86.70%) and 1 frameshift mutation (c.215_216insG) in 1 patient (3.33%). These results are in agreement with the current release of the International Agency for Research on Cancer *TP53* database (http://www-p53.iarc.fr/), which also shows that all *TP53* mutations were missense mutations in the coding region (54). The detected mutations were located within exons 3 and 5, encoding the Proline-rich domain, which plays a role in *p53*-mediated apoptosis and in the DNA-binding and oligomerization domain. These regions are required for interactions with FBX042, HIPK1 and AXIN1, the DNA major groove, and a domain containing a nuclear export signal (46,55,56). Dysfunction of p53 can cause defective DNA replication and malignant transformation, common in dysplasia's of BC (53). The p53 gene exhibits several genetic alterations in patients with BC (57). This highlights the need to administer effective treatments such as cell-cycle inhibitors in the form of target therapies and combinatorial target therapies against the wide range of *TP53* mutations.

In the current study, the high *TP53* mutation rate in the cohort could be explained by the high number of ER^+ cases, given that 67% of the observed *TP53* mutations occurred in the ER^+ tumors. ER status is tightly associated with the molecular subtypes, and a significantly higher *TP53* mutation rate was demonstrated in the basal-like subtype, mainly ER-, and HER2-enriched (both ER^- and ER^+) tumors compared with the primarily ER^+ luminal type (56,58). A recent study by Bai *et al* (59) conducted in 2021 using NGS to detect *TP53* mutations in the cell free DNA in Chinese metastatic BC (MBC) patients indicated that TP53 mutations could be used as a prognostic marker for worse outcomes in MBC and for the response of adjuvant endocrine therapy. *TP53*-mutated MBC patients had a significantly worse outcome than *TP53* wild-type patients, especially those in the $HR^+/HER2^-$ and triple-negative BC (TNBC) cohorts. *TP53* mutations were also associated with endocrine resistance (59).

In the present study TP53 mutations were not associated with $HER2^-$ tumors, which is comparable to the previously published research. *TP53* mutation status was an independent predictive factor of survival especially in $HR^+/HER2^-$ and TNBC cohorts, but not in the $HER2^+$ cohort (59,60).

In the present study, the somatic TP53 mutation c.215C>G p.(Pro72Arg) was the most frequently detected mutation in all BC patients, particularly those with Stage III BC. A previous

Table III. Mutational status of healthy control samples analyzed using the Ion AmpliSeq™ Cancer Hotspot Panel v2.

No.	Genes	Mutations detected	Effect	Amino acid change
1	KDR	c.1416A>T	Missense	p.Gln472His
	HRAS	c.80_81insC	Frameshift	p.Val29fs
	HRAS	c.80_81insC	Frameshift	p.Val29fs
	ERBB2	c.2380G>T	Missense	p.Val794Leu
2	KDR	c.1416A>T	Missense	p.Gln472His
	TP53	c.215C>G	Missense	p.Pro72Arg
3	TP53	c.215C>G	Missense	p.Pro72Arg
		c.209_215delCTCCCCCinsTCCCCCG	Frameshift	p.Ala70_Pro72delinsValProArg
5	TP53	c.215C>G	Missense	p.Pro72Arg
6	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	APC	c.3920T>A	Missense	p.Ile1307Lys
	TP53	c.215C>G	Missense	p.Pro72Arg
7	TP53	c.215C>G	Missense	p.Pro72Arg
8	PIK3CA	c.233A>G	Missense	p.Glu78Gly
	FGFR3	c.2389G>C	Missense	p.Ala797Pro
	KDR	c.1416A>T	Missense	p.Gln472His
9	TP53	c.215C>G	Missense	p.Pro72Arg
10	TP53	c.215C>G	Missense	p.Pro72Arg
11	APC	c.3920T>A	Missense	p.Ile1307Lys
	APC	c.3920delT	Frameshift	p.Ile1307fs
12	ATM	c.1811delC	Frameshift	p.Pro604fs
	ATM	c.1810C>T	Missense	p.Pro604Ser
	TP53	c.215C>G	Missense	p.Pro72Arg
	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	KIT	c.1621A>C	Missense	p.Met541Leu
13	KDR	c.1416A>T	Missense	p.Gln472His
	APC	c.3920T>A	Missense	p.Ile1307Lys
	APC	c.3920_3921delTA	Frameshift	p.Ile1307fs
14	PIK3CA	c.233A>G	Missense	p.Glu78Gly
	TP53	c.215C>G	Missense	p.Pro72Arg
15	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	GNAQ	c.625C>A	Missense	p.Gln209Lys
	TP53	c.215C>G	Missense	p.Pro72Arg
	TP53	c.209_215delCTCCCCCinsTCCCCCG	Frameshift	p.Ala70_Pro72delinsValProArg
16	KDR	c.1416A>T	Missense	p.Gln472His
	TP53	c.215C>G	Missense	p.Pro72Arg
17	PIK3CA	c.1173A>G	Missense	p.Ile391Met
18	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	KDR	c.1416A>T	Missense	p.Gln472His
	ATM	c.2572T>C	Missense	p.Phe858Leu
	TP53	c.215C>G	Missense	p.Pro72Arg
19	KDR	c.1416A>T	Missense	p.Gln472His
	JAK3	c.394C>A	Missense	p.Pro132Thr
20	TP53	c.215C>G	Missense	p.Pro72Arg
21	ATM	c.2572T>C	Missense	p.Phe858Leu
	TP53	c.215C>G	Missense	p.Pro72Arg
22	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	KDR	c.1416A>T	Missense	p.Gln472His
23	KIT	c.1621A>C	Missense	p.Met541Leu
	KDR	c.1416A>T	Missense	p.Gln472His
	TP53	c.215C>G	Missense	p.Pro72Arg

Table III. Continued.

No.	Genes	Mutations detected	Effect	Amino acid change
24	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	APC	c.3920T>A	Missense	p.Ile1307Lys
	TP53	c.215C>G	Missense	p.Pro72Arg
25	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	TP53	c.215C>G	Missense	p.Pro72Arg
26	KIT	c.1621A>C	Missense	p.Met541Leu
	TP53	c.215C>G	Missense	p.Pro72Arg
27	TP53	c.215C>G	Missense	p.Pro72Arg
28	TP53	c.215C>G	Missense	p.Pro72Arg
29	PIK3CA	c.1173A>G	Missense	p.Ile391Met
	APC	c.3920delT	Frameshift	p.Ile1307fs
	TP53	c.215C>G	Missense	p.Pro72Arg
30	TP53	c.215C>G	Missense	p.Pro72Arg
31	TP53	c.215C>G	Missense	p.Pro72Arg
32	TP53	c.215C>G	Missense	p.Pro72Arg

Del, deletion; ins, insertion; fs, frameshift.

Table IV. Frequency distributions of the most frequent mutations TP53 and PIK3CA between the breast cancer patients and the healthy controls.

Gene	Mutation	Amino acid change	Chromosome	Exon	NCBI 1000 Genomes Browser ID	Variant frequency in the patients	Variant frequency in the healthy controls	P-value[b]
TP53	c.215C>G	p.Pro72Arg	17	3	rs1042522	0.87	0.61	0.020[a]
PIK3CA	c.1173A>G	p.Ile391Met	3	9	rs2230461	0.53	0.25	0.041[a]

[a]P<0.05. [b]χ^2 test.

study showed that TP53 mutation NM_000546.5:c.824G>A p.(Cys275Tyr) was the most common mutation detected in 82 patients with Stage I-III BC who underwent NGS using tissue and blood samples, and they showed that TP53 pathogenic somatic mutations were associated with an 8-fold risk of recurrence in the univariate Cox regression analysis (61). The same study showed that the coexistence of TP53 and PIK3CA mutations was a common finding in BC patients (61). *PIK3CA* mutation (c.1173A>G) and p.(Ile391Met) located in exon 9, was identified in 15 (50%) patients. This exon encodes the helical domain, and mutations, represented by single amino acid substitutions, in this domain are associated with increased lipid kinase activity, and thus induce oncogenic transformation (62,63).

The PI3K pathway has been identified as a major player in cancer development and progression (62-66). PI3K is a heterodimeric enzyme composed of a p110α catalytic subunit encoded by the PIK3CA gene and a p85 regulatory subunit encoded by the PIK3R1 gene (67). In the present study, 47% of the BC patients were carriers of *PIK3CA* mutations. This result corroborates the findings of the previous work, reporting that *PIK3CA* mutations occur in 20-40% of BC and ≈30% of tumors of the prostate, cervix and endometrium (68,69). Several studies have suggested that *PIK3CA* mutations are more frequent in ER^+ and $HER2^+$ BC cases (68,69). Accordingly, the low mutation rate can be explained by a bias in the subtype distribution of the cohort. In the present study, 2 out of 5 (40%) of the *PIK3CA* mutations occurred in ER^+ primary tumors. The small size of the cohort may have influenced this distribution. Martínez-Sáez *et al* (70) showed that 28% of *PIK3CA* mutations identified in circulating tumor DNA (ctDNA) in 48 patients with advanced $HR^+/HER2^-$ BC were not part of the therascreen® *PIK3CA* test (QIAGEN GmbH), a FDA approved kit used to select patients who possessed *PIK3CA* mutations in tumor tissue specimens and/or in circulating tumor DNA (ctDNA) isolated from plasma specimens (71). Therascreen PIK3CA detects 11 *PIK3CA* hotspot mutations, mostly found in exons 9 and 20 (71).

It is important to remember that only a subset of genes was examined in the present study, and that for a deeper understanding of the mutational profiles of BC patients, considerably more extensive sequencing is required. The hotspot panel was not specifically developed for BC, and it includes areas

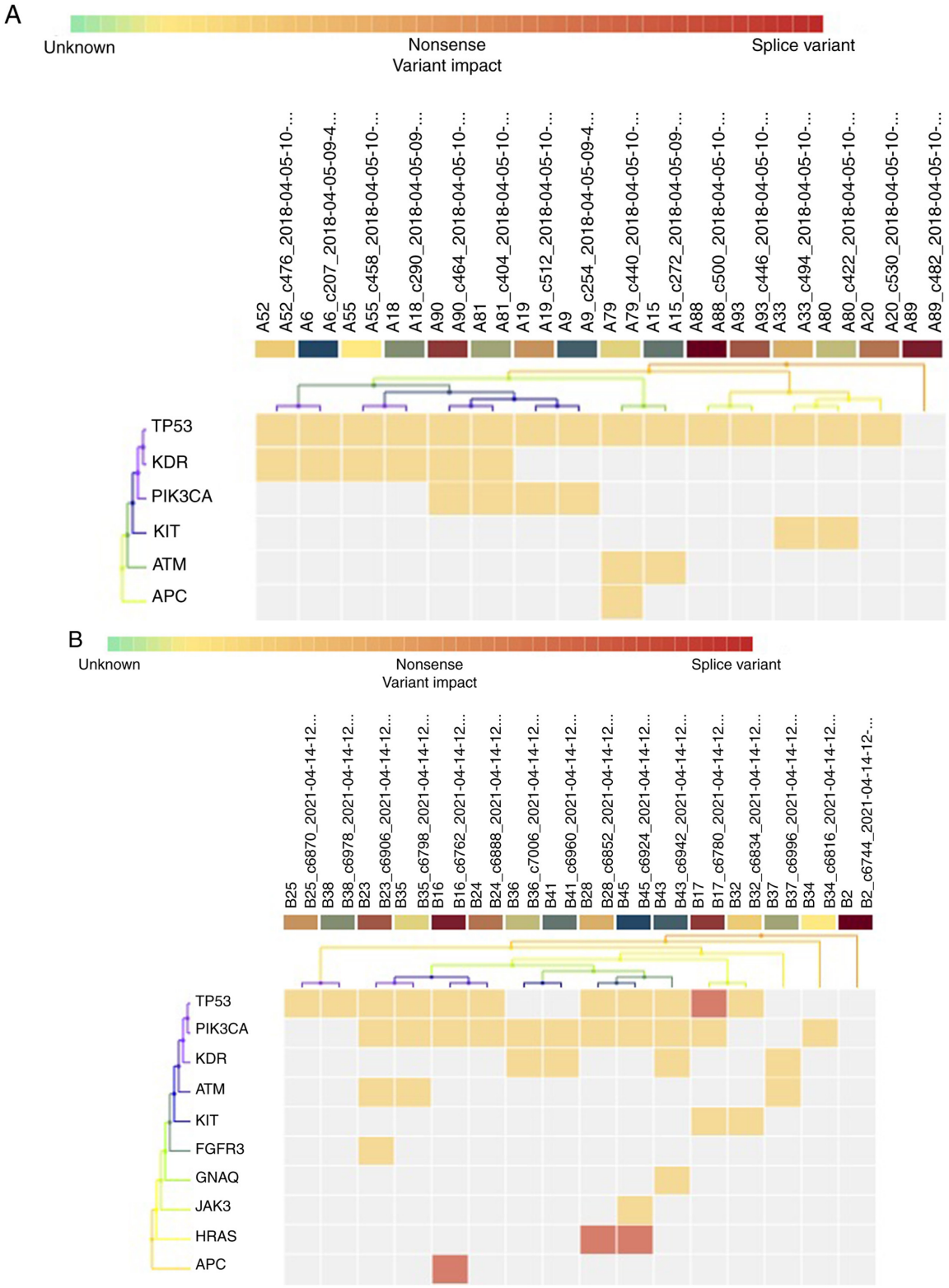

Figure 2. Continued.

and genes that are more commonly mutated in other cancer types (72).

Recently, Hempel *et al* (73) utilized a wide NGS panel to investigate 41 MBC samples and found that *PIK3CA* mutations appear in 34% of the patients. A recent study reported that 22% of the total population and 28% of patients with HR^+ BC have a *PIK3CA* mutation (74). Further research using a large NGS panel targeting 1,021 genes in 193 MBC samples by Tang *et al* (75) detected 36 (18.7%) mutations in the kinase domain and 26 (13.5%) substitutions in the helical domain, with 10 (5.2%) additional alterations distributed in the remaining *PIK3CA* sequence.

PIK3CA mutations in the ctDNA of patients with BC have also been reported (76). Board *et al* (77) was able to detect

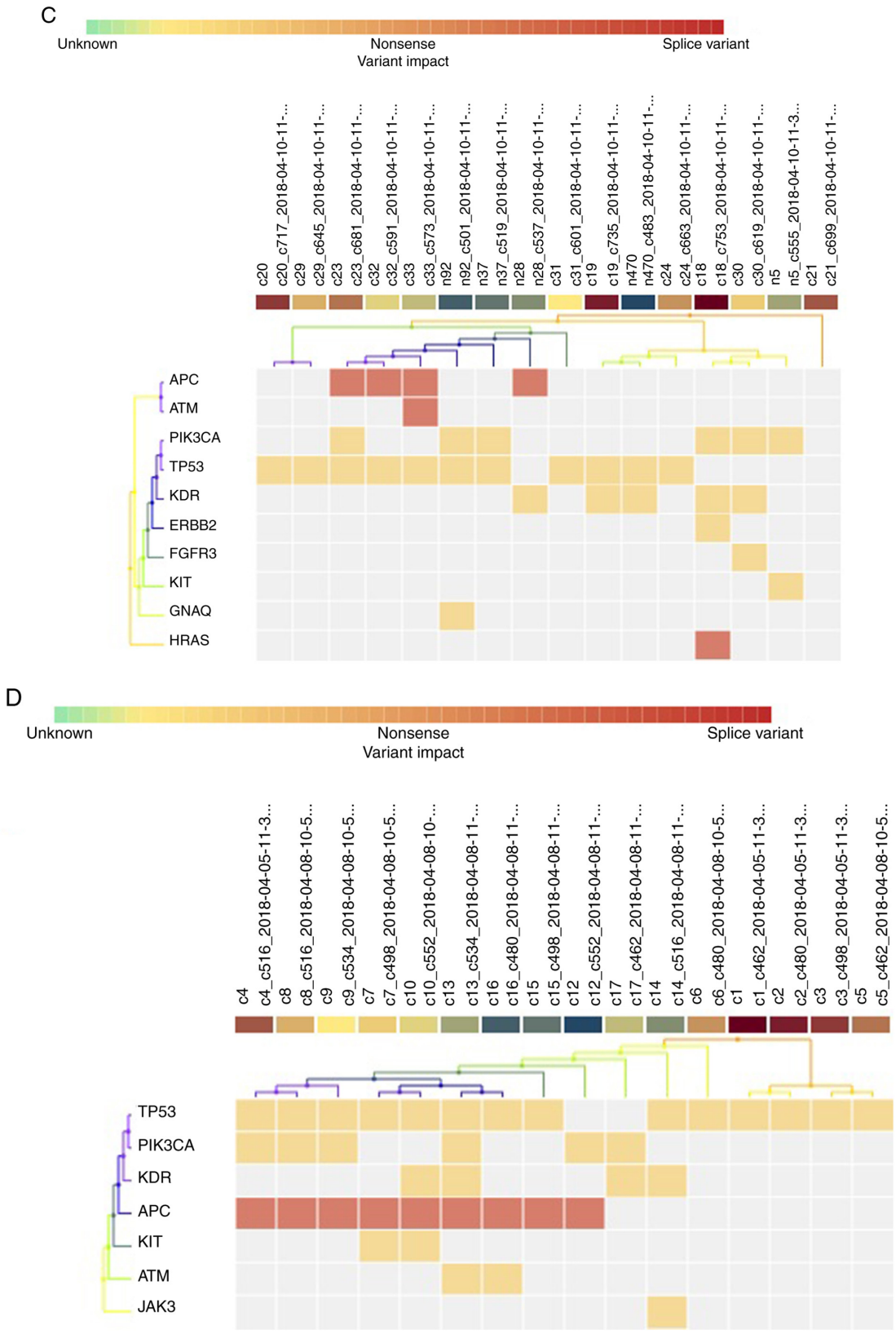

Figure 2. Heat maps displaying the combination of the different mutations in the 32 BC patients (A and B) and the 32 healthy controls (C and D).

PIK3CA mutations in the vast majority (80%) of ctDNA samples from *PIK3CA*-mutated MBC, but not in early BC (78).

In conclusion, the results of this investigation showed that Ion Torrent DNA Sequencing technology using AmpliSeq Cancer Hotspot Panel v2 was found to be a suitable method to perform molecular characterization of the genotype of BC patients and healthy controls using peripheral blood samples. The present study revealed specific mutational profiles for every BC patient; for this reason, it may be a possible to improve diagnosis and prognosis, and recommend

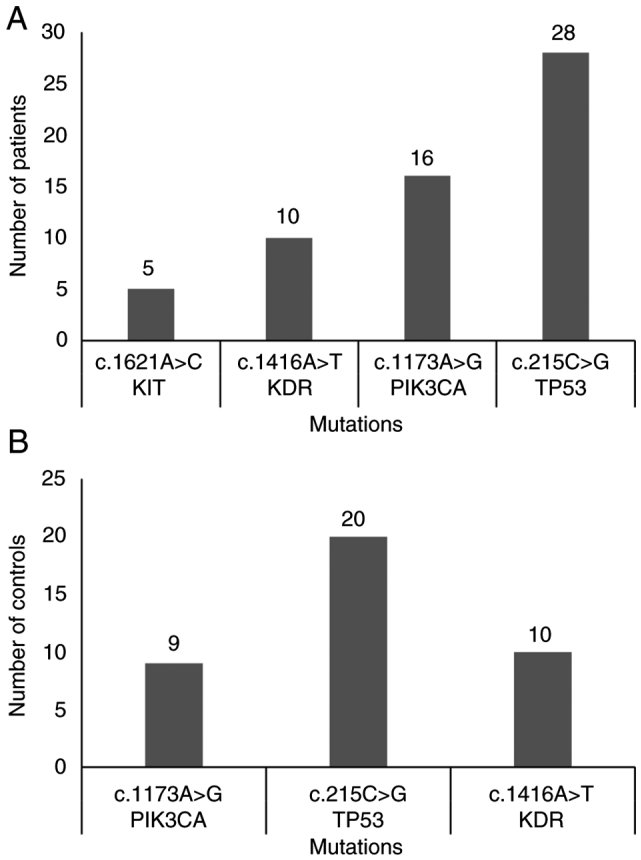

Figure 3. Bar graphs showing the distribution of the different mutations. Distribution of mutations in the (A) 32 BC patients and (B) 32 healthy controls. The most frequent mutations were found in the genes *PIK3CA*, *TP53* and *KDR*.

personalized treatments for each BC patient based on the mutational profile. The primary benefits of NGS are that it allows for the identification of multiple mutations at the same time, eliminating the need for sequential individual tests. Therefore, this technique could be routinely implemented in cancer diagnosis, as it can precisely identify fusions, SNPs, copy number variants, and insertion/deletions. Additionally, confirmation of NGS variants must be carefully investigated and validated through Sanger sequencing to avoid false positive outcomes. The results of the present study add to the existing body of knowledge and practice in the diagnosis and treatment of BC patients.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SAM conceived the study, curated the data, analyzed the data, performed the experiments and wrote and reviewed the manuscript. NAAS, BA and SRB contributed to the design of the study, performed the experiments and drafted the manuscript. ABA and AMA performed the experiments and contributed to data analysis and interpretation. MA, AB and WYA conceived the study, performed data interpretation, and wrote and critically reviewed the manuscript. SAM, MA and AB confirm the authenticity of all the raw data. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for this study was obtained from the Ethics Committee of King Fahad Medical City (KFMC; Riyadh, Saudi Arabia; IRB approval no. FWA00018774), and the study was performed in accordance with the guidelines described in the Helsinki Declaration. All participants provided signed informed consent prior to inclusion in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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