A Comparative Analysis of Breast and Ovarian Cancer-related Gene Mutations in Canadian and Saudi Arabian Patients with Breast Cancer

YUTAKA AMEMIYA[1,2], STEPHANIE BACOPULOS[2], MOHAMED AL-SHAWARBY[3], DALAL AL-TAMIMI[3], WALID NASER[4], AYESHA AHMED[4], MAHMOUD KHALIFA[5], ELZBIETA SLODKOWSKA[5] and ARUN SETH[1,2,5]

[1]Genomics Core Facility, [2]Sunnybrook Research Institute, Toronto, ON, Canada; [3]University of Dammam, [4]King Fahd Hospital of the University, Dammam, Kingdom of Saudi Arabia; [5]Department of Anatomic Pathology, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

Abstract. *Previous reports have indicated that patients with breast cancer who are from the Eastern Province of Saudi Arabia have a different gene expression profile from that known for their age-matched North American population. In the present study, breast tumor samples from Canadian and Saudi Arabian patients were screened for known and unknown mutations within BRCA1 and BRCA2 as well as 21 additional genes, including, ATM, BARD1, CDH1, P53, EPCAM, MSH6, and RAD50, which have been implicated in breast and ovarian cancer predisposition. A total of 129 non-synonymous mutations were identified by Ion Torrent amplicon sequencing. Forty-one mutations in 18 genes were unique to the Canadian population and 59 mutations in 20 genes were unique to the Saudi Arabian population. A total of 55/129 unique mutations in 22 genes were not previously reported in the database. Twenty-nine mutations in 16 genes were common to both populations; one of these mutations was not previously reported in the database. The most frequently mutated gene in both populations was the BRCA2 gene, followed by BRCA1 and TP53. Unique to this work is the identification of mutations frequently found in the Saudi Arabian population that are rare in the Canadian population. This work will allow direction of genetic analysis resources toward the clinical needs of each particular population.*

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Correspondence to: Professor Arun Seth, Department of Anatomic Pathology, Sunnybrook Health Sciences Centre, 2075 Bayview Ave., S-238, Toronto, ON, M4N 3M5, Canada. Tel: +1 4164806100 ext. 3536, Fax: +1 4164805737, e-mail: arun.seth@utoronto.ca

Key Words: Breast cancer, ovarian cancer, mutations, Canadian patients, Saudi Arabian patients, comparative study, next generation sequencing.

Currently, breast cancer screening includes mammography, and for high-risk families, genetic testing, notably for the breast cancer susceptibility gene 1 (*BRCA1*) and *BRCA2* (1, 2). Distinct ethnic groups, including Icelanders, American Mormons, Newfoundlanders, and Ashkenazi Jews have been found to share similarly higher breast cancer incidence and grade relative to wider populations. It is now clear that each ethnic group has separate and distinct mutations and distributions of mutations, especially across *BRCA1* and *BRCA2*, which underlie clinical similarities. For example, Ashkenazis have a high prevalence of the *BRCA1* mutations185delAG, 5385insC,and6174delT, whereas almost all affected Icelanders share a single deleterious mutation, *BRCA2* 999del5 (2, 3).

A high incidence of breast cancer in the Eastern Province of Saudi Arabia (EPSA) strongly suggests populations bearing unique genetic mutations. Amongst Saudi Arabian women, 21.83% of the malignancies they experience are breast cancer (1). Breast cancer specific to Saudi women tends to be high-grade and locally advanced at diagnosis (1). Moreover, the age at diagnosis for Saudi women is typically between the ages of 46 and 50 years, this is younger than the median age of Western women affected by breast cancer, which is between the ages of 60 and 65 years (1). This is interesting, as the probability that breast cancer is inherited increases with decreased age at presentation (2). Furthermore, the moleculat pattern of tumors found in the Saudi Arabian population differs from that of the Western population. Tumours in patients from industrialized Western countries tend to be of the luminal subtype, with a frequency ranging from 66.9%-78.6% depending on the study (1). In contrast, the luminal subtype is seen in about 19.9% of Saudi patients (1). Additionally, the West experiences fewer *HER2*-positive tumors (4-6.6%) compared to Saudi Arabia (17.31%)(1). Determination of the breast and ovarian cancer-predisposing

Table I. *Clinical characteristics of breast cancer samples.*

	Patient	Age	Histopathological diagnosis	Grading	ER	PR	HER-2
	CA-3	40	Duct, with mucinous differentiation	II/III	+	+	+
	CA-4	48	Invasive ductal carcinoma	III	-	-	-
	CA-6	50	Invasive and In-situ duct carcinoma	II	+	+	-
	CA-10	68	Invasive ductal carcinoma	III	+	+	-
Canadian	CA-13	71	Invasive ductal carcinoma	III	Low+	-	+
	CA-15	65	Invasive lobular carcinoma	II	+	-	-
	CA-16	40	Invasive lobular carcinoma	III	+	-	-
	CA-20	71	Invasive ductal carcinoma	III	-	-	-
	CA-22	61	Invasive ductal carcinoma	III	+	-	+
	CA-23	61	Invasive ductal carcinoma	III	-	-	+
	SA-1	32	Invasive ductal Ca with mucinous component	III	+	+	+
	SA-2	27	Invasive ductal carcinoma	III	+	-	+
	SA-4	34	High grade DCIS with foci of microinvsion	III	+	-	+
	SA-5	45	Invasive ductal carcinoma	II	+	-	-
Saudi Arabian	SA-9	43	Invasive and in situ lobular carcinoma	NA	+	+	-
	SA-11	42	Invasive lobular carcinoma	NA	+	-	-
	SA-17	62	Metastasis to LNs	II	+	-	+
	SA-18	29	Invasive ductal carcinoma	I	-	-	+
	SA-19	49	Invasive ductal carcinoma	III	+	+	-
	SA-21	41	Atypical medullary carcinoma	III	-	-	-

gene mutation status for women from the Eastern Province would be of clinical use within Saudi Arabia. There exists also scientific interest in determining the effects of new mutations, and the discovery of regionally specific mutations.

Herein we used next-generation targeted sequencing technologies to investigate molecular differences between breast cancer mutations in Canadian and Saudi Arabian women. Patient samples were analysed for single nucleotide polymorphisms (SNPs), as well as small insertion and deletion (INDEL) variants, using the Breast and Ovarian Cancer Predisposing panel, which targets 395 coding exons of 23 genes, in 823 amplicons, of DNA repair and other genes linked with breast cancer susceptibility. This pilot study aimed to rapidly screen women for both known and unknown mutations within *BRCA1* and *BRCA2*, as well as 21 additional genes, including *ATM, BARD1, CDH1, P53, EPCAM, MSH6* and *RAD50*, which have been implicated in predisposition to breast and ovarian cancer (4).

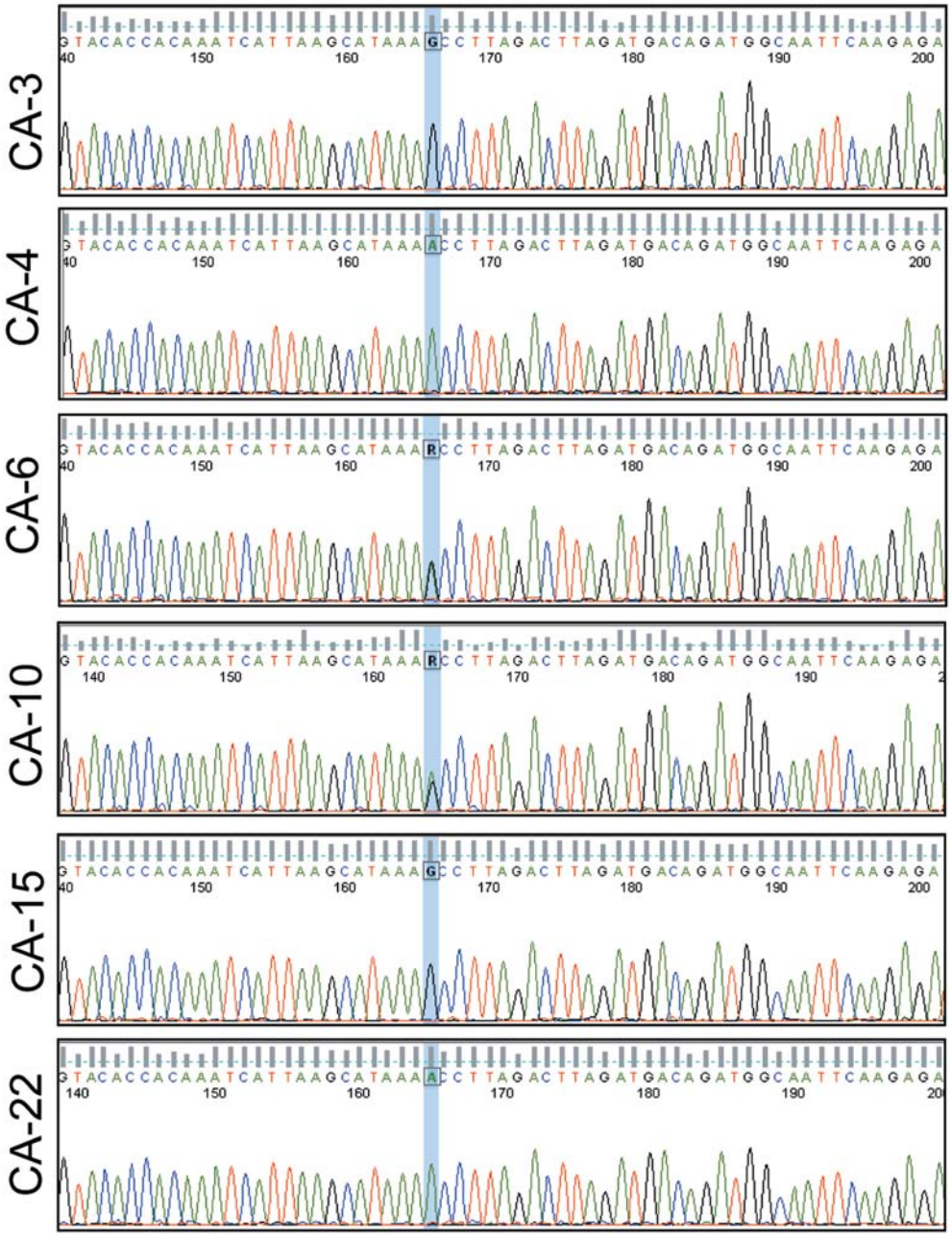
Materials and Methods

Tissue samples. Ten formalin-fixed paraffin-embedded (FFPE) normal breast samples and matched tumor samples were taken from each of the University of Dammam, Saudi Arabia and Sunnybrook Health Sciences Centre, Canada (Table I). Blocks were sectioned and haematoxylin and eosin (H&E)-stained to visualize the sample morphology. H&E-stained slides were sent to the Sunnybrook Health Sciences Centre, Pathology Department to be reviewed and marked for normal and tumor areas by our collaborating breast pathologist. Marked slides were then used as a guide to punch cores from the paraffin blocks.

Isolation of genomic DNA from fresh-frozen and FFPE tissues. Genomic DNA from fresh frozen tumor tissues was extracted with Gentra Puregene Tissue Kit (Qiagen Sciences, Germantown, MD, USA) . Marked H&E slides of FFPE tissue were used as a guide to punch cores from the paraffin blocks. FFPE cores with a diameter of 1 mm were taken and used for extraction. Genomic DNA from FFPE tissue was extracted using the Recoverall Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) . All procedures followed manufacturer's instructions. DNA concentration was determined by Qubit 2.0 fluorometer with dsDNA BR assay kit (Life Technologies).

Primer pool for mutation identification. To assess the genetic differences in breast tumours between the Saudi and Canadian populations, we used a list of genes likely to bear inherited mutations for breast and ovarian cancer provided by Dr. Steven Narod (Breast and Ovarian Cancer Predisposing panel). Primers for each gene mutation were designed using Web-based Ion AmpliSeq Designer software (https://www.ampliseq.com/). This panel targets 395 coding exons of 23 genes, in 823 amplicons, involved in DNA-repair mechanisms or cell-cycle checkpoints along with tumour-suppressor genes linked with breast cancer susceptibility (Table II) (4).

Library preparation. Targeted genomic areas were polymerase chain reaction (PCR) amplified from 1 or 10 ng of genomic DNA from FFPE tissue or frozen tissue. PCR reactions were prepared using the Ion AmpliSeq Library Kit 2.0 and 2× Ion AmpliSeq Breast and Ovarian Cancer Predisposing panel and executed in a 3720 Thermal Cycler (Life Technologies). The PCR conditions for 10 ng of template DNA were 2 min at 99˚C for polymerase activation and 18 cycles of 15 s at 99˚C for denaturation and 4 min at 60˚C for annealing/ extension. For 1 ng of template DNA, the amplification cycles were increased to 18 cycles, and for 10 ng of template DNA

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FAM175A     GTACACCACAAATCATTAAGCATAAAGCCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-3        GTACACCACAAATCATTAAGCATAAAGCCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-4        GTACACCACAAATCATTAAGCATAAAACCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-6        GTACACCACAAATCATTAAGCATAAARCCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-10       GTACACCACAAATCATTAAGCATAAARCCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-15       GTACACCACAAATCATTAAGCATAAAGCCTTAGACTTAGATGACAGATGGCAATTCAAGA
CA-22       GTACACCACAAATCATTAAGCATAAAACCTTAGACTTAGATGACAGATGGCAATTCAAGA
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Figure 1. *Sanger sequencing and nucleotide alignment with reference sequence validating the discovery of a potentially deleterious A>G SNV in the FAM175A gene. Upper panel: Sanger sequencing electropherograms for six representative Canadian samples with a shared single nucleotide polymorphism (SNP) in the FAM175 gene (chr4:84383810, rs12642536) originally found by Personal Genome Machine sequencing in seven of 10 Canadian and five of 10 Saudi Arabian samples, with the location of potentially deleterious A>G SNP highlighted by a blue bar. Lower panel: DNA sequence alignment of the Sanger sequencing results and the FAM175 gene, with potentially deleterious A>G SNVs indicated in red font.*

Table II. *List of DNA repair and tumor suppressor genes linked with breast and ovarian cancer susceptibility. Breast cancer samples from Canadian and Saudi Arabian women were analysed for single nucleotide polymorphisms, as well as small INDEL variants, using the Ion Torrent Personal Genome Machine with the Breast and Ovarian Cancer Predisposing panel, which targets 395 coding exons of 23 genes, in 823 amplicons, of DNA repair and other genes linked with breast and ovarian cancer susceptibility.*

DNA repair genes	
ATM	ATM serine/threonine kinase
BARD1	BRCA1 associated RING domain 1
BRCA1	Breast cancer 1, early onset
BRCA2	Breast cancer 2, early onset
BRIP1	BRCA1 interacting protein C-terminal helicase 1
FAM175A	Family with sequence similarity 175, member A
MLH1	mutL homolog 1
MRE11A	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MUTYH	mutY homolog
NBN	Nibrin
PALB2	Partner and localizer of BRCA2
PMS2	PMS2 postmeiotic segregation increased 2 (S. cerevisiae)
RAD50	RAD50 homolog (S. cerevisiae)
RAD51C	RAD51 paralog C
RAD51D	RAD51 paralog D
STK11	Serine/threonine kinase 11
XRCC2	X-ray repair complementing defective repair in Chinese hamster cells 2
Tumor suppressor genes	
CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
CHEK2	Checkpoint kinase 2
EPCAM	Epithelial cell adhesion molecule
TP53	Tumor protein p53

from frozen tissue, the amplification cycles were decreased to 15 cycles. The resulting amplicons were treated with FuPa Reagent to partially digest primers. Amplicons were ligated to Ion P1 and Ion Xpress Barcode adapters (Life Technologies) and purified using Agencourt AMPure XP Reagent (Beckman Coulter, Brea, CA, USA). Barcoded libraries were then equalized to 100 pM using the Ion Library Equalizer Kit (Life Technologies) following the recommended protocol.

Ion Torrent amplicon sequencing. Template-positive ion sphere particles containing clonally amplified DNA were generated using the Ion Personal Genome Machine (PGM) Template OneTouch 2 200 Kit with the Ion OneTouch 2 Instrument (Life Technologies) and then enriched with the Ion OneTouch ES instrument. Sequencing was performed on an Ion PGM Sequencer using the Ion PGM 200 Sequencing Kit according to the manufacturer's instructions on Ion 316 and 318 Chips.

Sequence data analysis. Ion Torrent platform-specific pipeline software (Torrent Suite version 3.6.2; Life Technologies) was used to separate barcoded reads, generate sequence alignment with the hg19 Human genome reference, perform target region coverage analysis, and filter and remove poor signal reads. Variant calling was performed with Ion Torrent Variant Caller version 3.6.63335 software. A preset parameter of "Germ-Line-High Stringency" was used for normal samples. This setting is optimized for high-frequency variants and minimal false-positive calls. In order to detect somatic mutation events in cancer samples, a preset parameter of "Somatic-High Stringency" that is optimized for low-frequency variant detection (<20%) with minimal false-positive calls was used.

Bioinformatic analysis. Variants found in the Ion Torrent Variant Caller were further analysed to determine the likelihood that the variant was deleterious. Variants at each genomic location were analysed by PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/) with HumDiv model classification (5). The program uses multiple sequence alignment, as well as a probabilistic classifier based on machine-learning method to categorize single nucleotide variants from next-generation sequencing into categories of: benign, potentially deleterious and probably deleterious. Selected potentially and probably deleterious SNP and INDEL were also visually inspected by using the Broad Institute Integrative Genomic Viewer (version 2.1, www.broadinstitute.org/igv) . The frequencies of known mutations with a reference SNP ID number (rsID) of the NCBI single nucleotide polymorphism database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/) in this study were also compared with the 1000 Genome phase 1 population (global minor allele frequency, MAF) using NCBI db SNP database (http://www.ncbi.nlm.nih.gov/snp).

Results

Matched tumor and adjacent normal tissue samples from 10 Saudi and 10 Canadian breast cancer patients were analyzed for genetic mutations (Table I). The average age for patients was 57.5±3.8 and 40.4±3.3 years for Canadian and Eastern Province Saudi Arabian (EPSA) groups, respectively. The majority of breast tumors in both groups were grade III (Canada n=8, EPSA n=5) and invasive ductal carcinomas (Canada n=8, EPSA n=5) (Table I).

DNA from Saudi and Canadian patients was analyzed for genetic differences in 23 genes by population and individual. The target genes are associated with breast and ovarian cancer susceptibility in many populations (http://web.labmed.washington.edu/tests/genetics/BROCA) (Table II) (4, 6, 7). PCR amplification and next-generation sequencing of amplicon libraries generated barcoded DNA sequence data. Ion Torrent platform-specific pipeline software was used to separate barcoded reads, generate sequence alignment with the hg19 Human genome reference, perform target region coverage analysis, and filter and remove poor signal reads. For variant detection, the mean read depth coverage of 892X for tumor tissue (n=21) and 207X for adjacent normal tissue (n=20) over the targeted genomic regions (Breast and Ovarian Cancer Predisposing panel) was achieved. The data of Saudi Arabian sample U5 was not included for further analysis due to its poor quality.

Table III. *Continued*

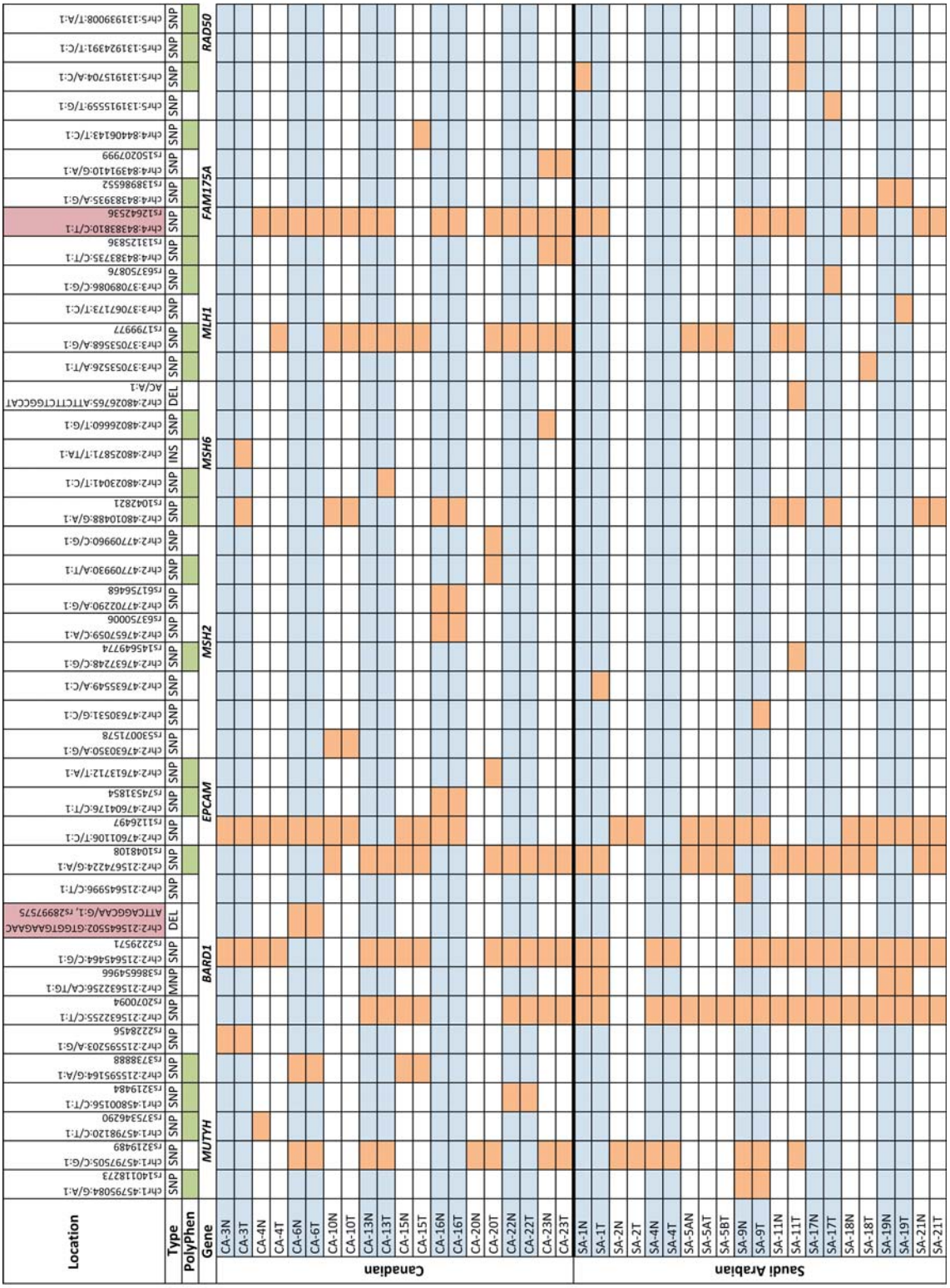


Table III. *Distribution of variants detected in 10 Canadian and 10 Saudi Arabian breast cancer patients. Single nucleotide variants (SNV), multi-nucleotide variants (MVN), insertions (INS) and deletions (DEL) in each sample are shown in orange. Mutations described in this article are shown in red. The SNVs predicted to be 'probably' or 'potentially' deleterious by PolyPhen-2 (www.genetics.bwh.harvard.edu/pph2/). are shown in green. All non-coding variants and synonymous mutations were excluded. "N" indicates normal samples and "T" indicates tumor samples.*

Table III. *Continued*

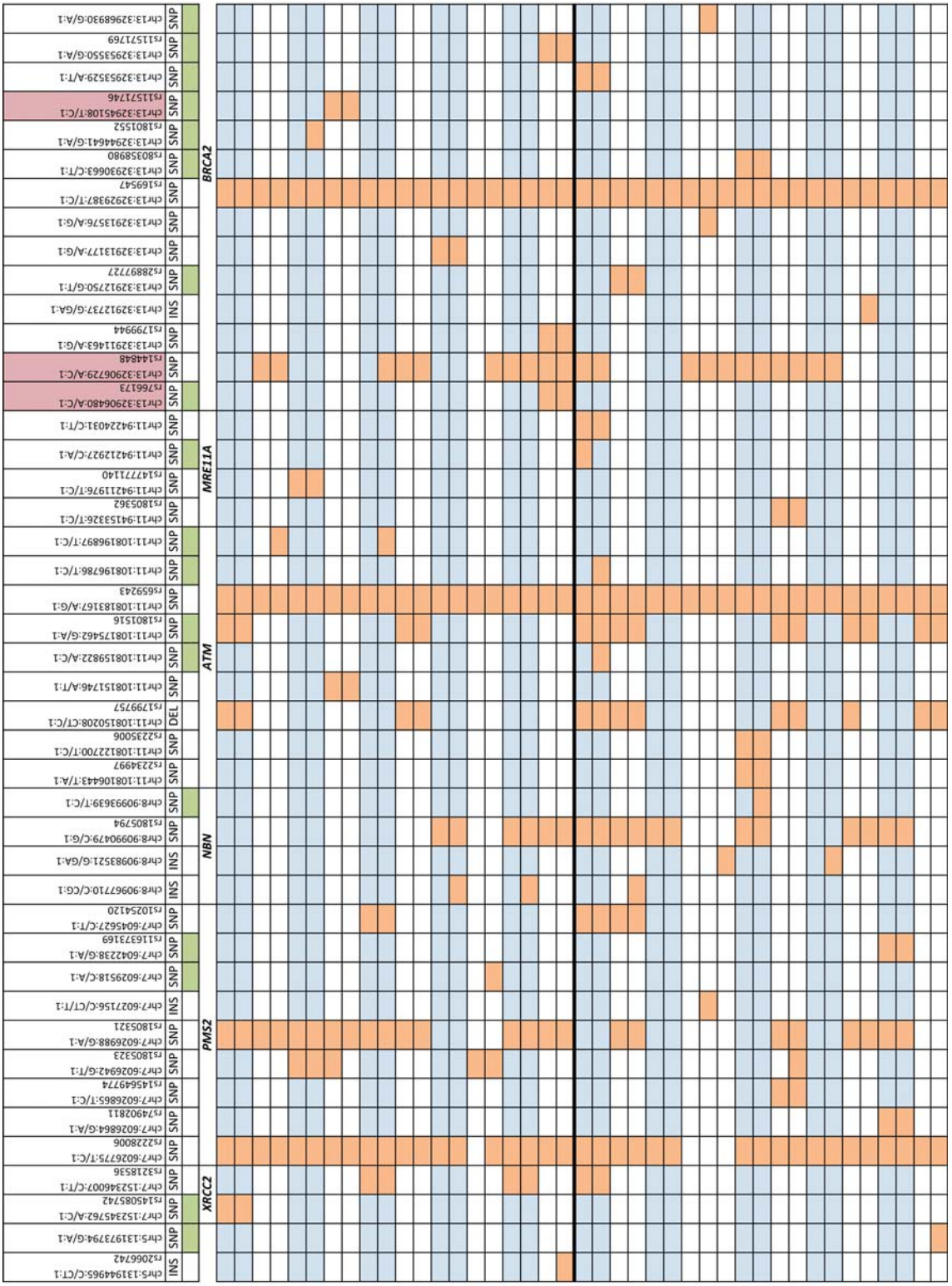


Table III. *Continued*

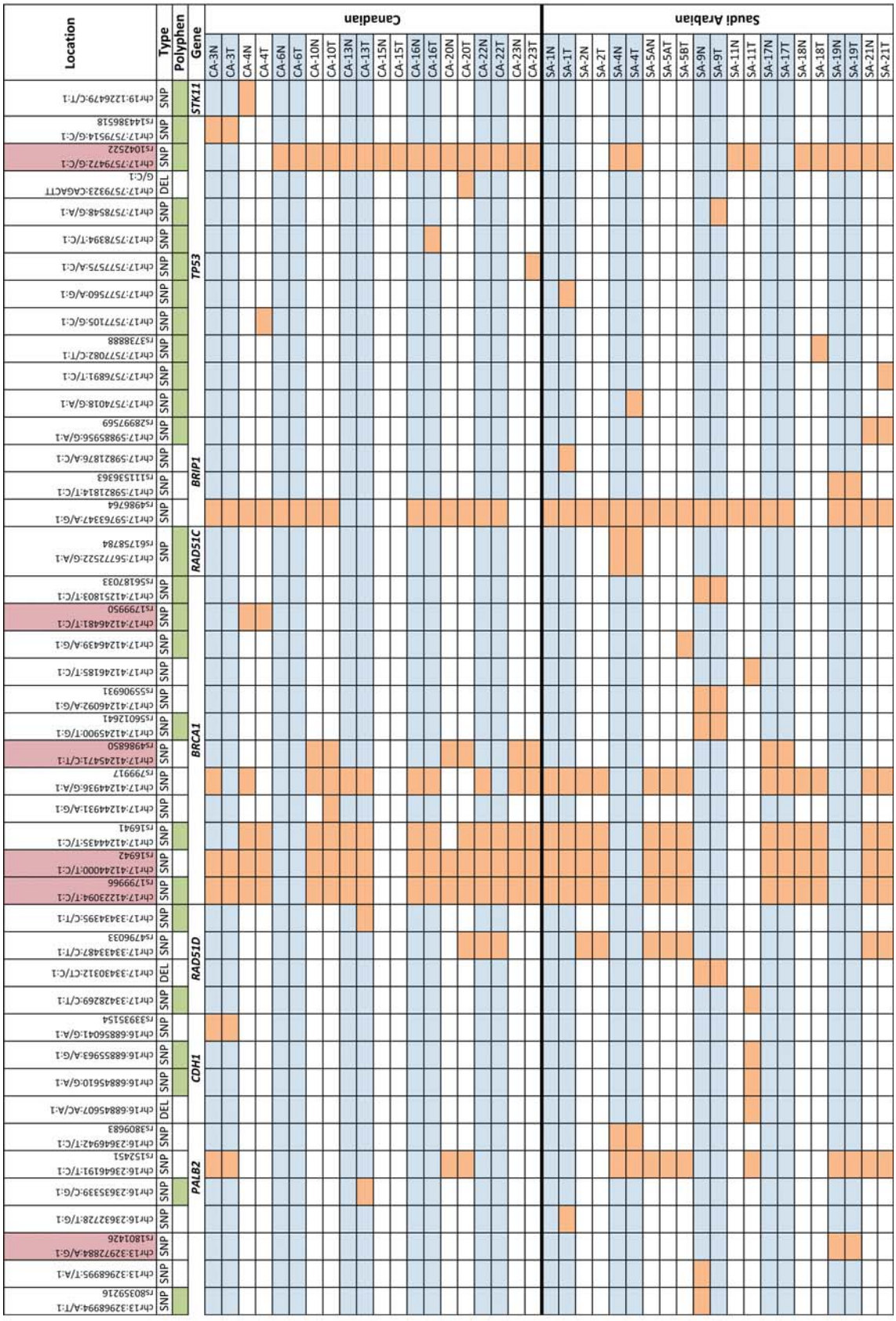


Table III. *Continued*

Table IV. *Summary of mutations in each population. The number of mutations found in either Canadian or Saudi Arabian or both populations is shown. The column "Polyphen" shows the number of mutations predicted by Polyphen2 as probably or possibly damaging the structure and/or function of the protein. The column rsID (reference SNP) indicates number of known mutations in dbSNP database.*

Gene Name	Chromosome	Canadian	Saudi Arabian	Both	PolyPhen	rsID
MUTYH	1	2	1	1	3	4
BARD1	2	3	2	3	2	7
EPCAM	2	2	0	1	2	2
MSH2	2	5	3	0	2	4
MSH6	2	3	1	1	3	1
MLH1	3	0	3	1	3	2
FAM175A	4	3	1	1	4	4
RAD50	5	1	5	0	4	1
XRCC2	7	1	0	1	1	2
PMS2	7	1	4	4	2	7
NBN	8	0	2	2	1	1
ATM	11	2	4	3	4	5
MRE11A	11	1	3	0	1	2
BRCA2	13	6	9	2	9	11
PALB2	16	1	2	1	1	2
CDH1	16	1	3	0	2	1
RAD51D	17	1	2	1	2	1
BRCA1	17	2	5	5	6	9
RAD51C	17	0	1	0	1	1
BRIP1	17	0	3	1	1	3
TP53	17	5	5	1	10	4
STK11	19	1	0	0	1	0
CHEK2	22	0	0	0	0	0
Total		41	59	29	65	74

We found a total of 129 non-synonymous mutations consisting of 116 single nucleotide variants (SNVs), one multiple nucleotide variant, six deletion variants and six insertion variants that were within the coding regions of the 22 genes in the Breast and Ovarian Cancer Predisposing panel but not in the *CHEK2* gene (Table III, https://activenet.sunnybrook.ca/docs/D0113327.pdf). The average number of non-synonymous germline mutations detected in the Canadian patients was 16.8±1.3 compared to 17.2±1.2 mutations in the EPSA patients, not statistically different by the t-test. Moreover, the average number of somatic variants occurring in tumour tissue was evaluated as 19.1±1.1 and 20.5±1.6 variants for the Canadian and EPSA patients, respectively. While overall the average number of non-synonymous somatic variants was higher in the EPSA population, the difference was not statistically significant by *t*-test.

Out of the 129 mutations, only 74 had previously been reported in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) (Table III, and IV). The most frequently altered genes in the 23 gene panel were *BRCA2* with 17 mutations, followed by *BRCA1* (12 mutations) and *TP53* (11 mutations). A total of 41 unique mutations were found in the Canadian patients and 59 unique mutations were found within the Saudi Arabian patients. Additionally, there were 29 mutations common to both populations (Table III and IV). The frequency of these mutations were well congruent with global MAF data obtained from the 1000 genome project. However, the frequency of the SNP at position chr2:215595164 in *BARD1* gene in the Canadian population was significantly higher (two out of 10 patients, allelic frequency of 10%) than that of the global MAF (1%) (Table V). Furthermore, the majority of mutations were found to occur both within the tumour samples and the normal adjacent tissue, indicating germline as opposed to acquired somatic mutations.

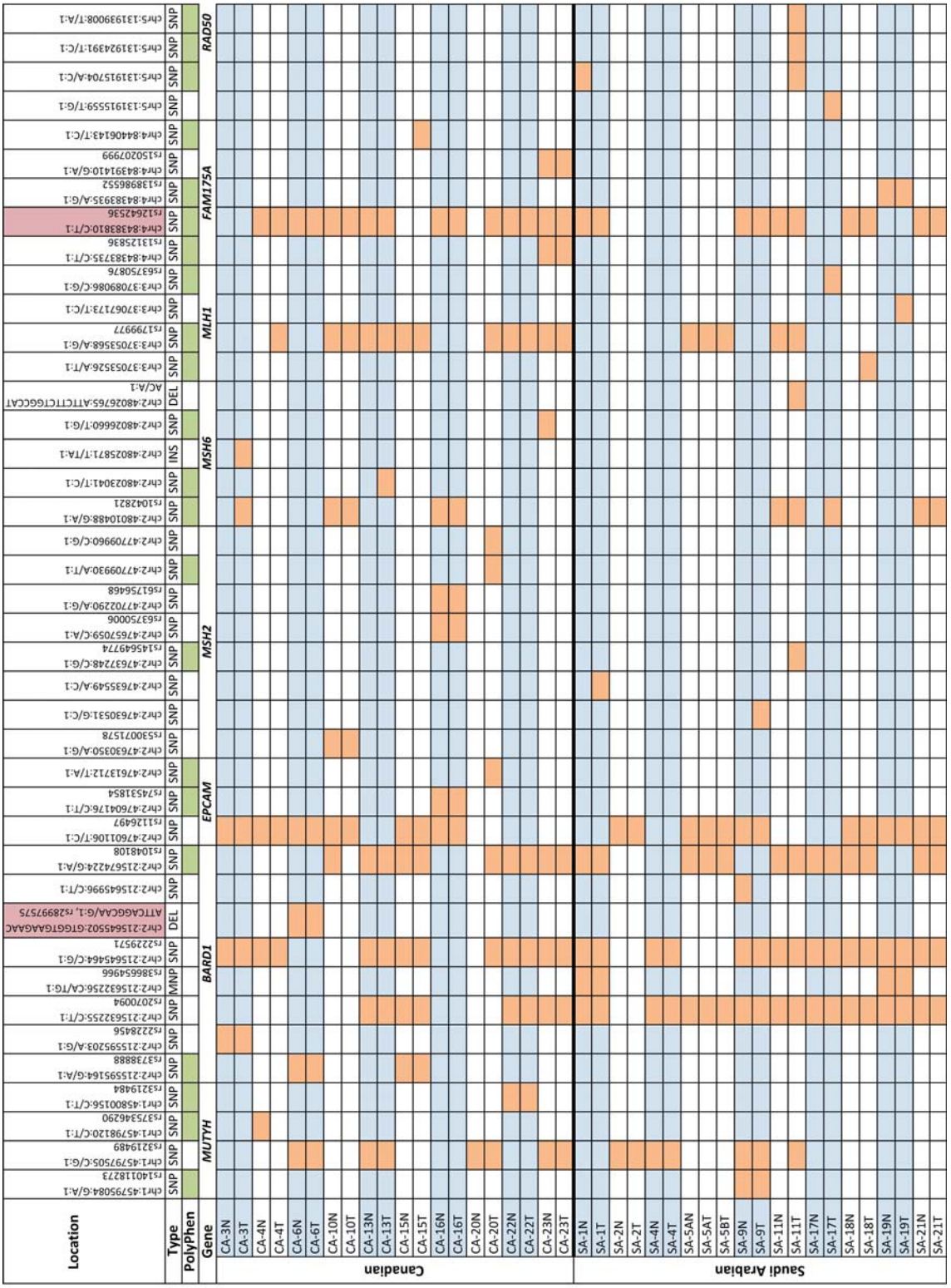
PolyPhen2 analysis revealed 65 out of 116 SNPs were predicted to be potentially damaging. Fifty-one SNVs unlikely to have a deleterious effect were excluded from further analysis. Potentially damaging mutations in *EPCAM*, *XRCC2*, *PALB2* and *STK11* genes were found only in the Canadian patients. Similarly potentially damaging mutations in *MRE11A, RAD51C* and *BRIP1* genes were found only in the EPSA patients (Table III and IV).

An SNP in *FAM175* gene (chr4:84383810, rs12642536) was found by PGM sequencing in seven out of 10 Canadian and five of 10 Saudi Arabian samples, and was predicted to be deleterious by PolyPhen2 analysis of the associated amino acid substitution (Ala348Thr). The SNV was confirmed in six Canadian samples by Sanger sequencing (Figure 1). CA-3 and CA-15 of *FAM175* did not carry the SNV but CA-4 and CA-22 had homozygous mutations in this location, while CA-6 and C-10 showed heterozygous mutations. A 21-nucleotide deletion at chr2:215645502 (rs28997575) in *BARD1* gene in one Canadian sample (CA-6) was also validated by Sanger sequencing.

Discussion

Previous reports have indicated that patients from EPSA with breast cancer have a different gene expression profile from that known for their age-matched North American counterparts (1). Herein we used next-generation exome-targeted sequencing technologies to update our understanding over these molecular differences. Breast cancer samples from Canadian and Saudi Arabian women were analysed for SNPs, as well as small INDEL variants, using the Breast and Ovarian Cancer Predisposing panel, which targets 395 coding exons of 23 genes, in 823 amplicons, of DNA repair and other genes linked with breast cancer susceptibility (4). Average age of onset for the Saudi patients was 40.4 years of age. This is similar to earlier reports on age of onset in the Saudi patients. It is in stark contrast to the 57.5 year average age of onset in the Canadian patients.

Table V. *Comparison of the mutation frequency with global Minor Allelle Frequency (MAF) in 1000 genome phase 1 population. The mutations from 10 Canadian patients (20 alleles) and 10 Saudi Arabian patients (20 alleles) were compared with 1000 genome phase 1 population Minor Allele Frequency (MAF) data from 1,088 people (2,176 alleles). Only mutations found in at least 2 patients were included. SNVs predicted to be 'probably' or 'potentially' deleterious by PolyPhen-2 (www.genetics.bwh.harvard.edu/pph2/) are shown in green.*



We found a total of 129 non-synonymous mutations which were within coding regions: 41 mutations in 18 genes were unique to the Canadian population and 59 mutations in 20 genes were unique to the Saudi Arabian population; 55/129 unique mutations in 22 genes have not been previously reported in the database; 29 mutations in 16 genes were common to both populations. All common mutations in both populations were previously reported. The most frequently mutated gene in both patient populations was *BRCA2*, followed by *BRCA1* and *TP53* gene.

Earlier work revealed an increased risk of disease with increasing numbers of variant alleles for 25 SNPs in *BRCA1*, *BRCA2*, *ATM*, *TP53* and *CHEK2* (8). Nine of these 25 SNPs were also found in our study, four in *BRCA2* (rs766173, rs144848, rs11571746 and rs1801426), four in *BRCA1* (rs1799966, rs16941, rs4986850 and rs1799950) and one (rs1042522) in *TP53*. Interestingly, every patient in our study carried at least one of these mutations.

With the exception of a few genes, *e.g. RB* in retinoblastoma, there are few cancer types that can be predicted from a single SNP. Even in the case of *BRCA1* and *BRCA2* mutations, penetrance is 65%, meaning that up to 35% of women with this mutation will not experience a *BRCA1/-2*-related cancer in their lifetime (9).

Unique to this work is the identification of mutations frequently found in the Saudi Arabian population that are rare in the Canadian population. This work will allow for direction of genetic analytical resources toward the clinical needs of each particular population. Given the prophylactic interventions available to women with significant lifetime risk of breast cancer compared to the vast medical, psychological and social costs associated with breast cancer treatment, finding new effective screening options is critical, as is identifying mutations in specific populations to facilitate screening, as with the Ashkenazi Jewish mutation and current cancer care screening guidelines.

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