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Original Article

BRCA1 and BRCA2 Germline Mutation Screening in Western Algeria using High Resolution Melting Analysis (HRM)

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Abstract

Breast cancer is the leading cause of cancer deaths in Algerian women. Our aim is to analyze BRCA1 and BRCA2 genes mutations in 100 Algerian patients with a family history suggestive of genetic predisposition to breast cancer. BRCA1 and BRCA2 mutations were searched by High–Resolution Melting (HRM) analysis, followed by direct sequencing, and Multiplex Ligation–Dependent Probe Amplification (MLPA) for large deletions or duplications. An unclassified variant c.5117G>C, p.Gly1706ALA and a pathogenic mutation c.2125_2126insA, p.Phe709TyrfX3 were detected in the BRCA1 gene. No large deletions or duplications were detected with MLPA. One deleterious mutation c.250C>T, p.Gin84X, and one unclassified variant c.9364G>A, p. Ala3122Thr were identified in BRCA2 gene. The pathological significance of this variant has to be specified and analysis of its segregation in the family and differs from those provided in the literature. Although on a limited cohort, our findings suggest a higher frequency of BRCA1/2 mutations in Algeria and it would be of interest to search for the presence of these pathogenic mutations in other family member for preventing the risk of cancer.

Keywords: Breast cancer, BRCA1, BRCA2, Western Algeria, High Resolution Melting Analysis, HRM

Introduction

Breast cancer is still a major cause of death by cancer among Algerian women[(1)]. Studies of breast cancer in North Africa (including Morocco, Algeria, Tunisia, and Libya) have shown BC patterns similar to those in the Middle East. Over the region, BC accounts for the majority of cancers in women. Age–standardized incidence rates (ASRs) per 100,000for BC in 2002 was 23.5 in Algeria and 29 in Tunisia, to nearly 37 in Morocco[(2–4)]. The size and grade of breast tumors in the Maghreb are increased, while the median age of onset (48) in Algeria, Morocco, and Tunisia, is more than ten years earlier than the European/North American median of 61. The size and grade of breast tumors are also higher in North Africa than elsewhere [(5)].

Linkage studies conducted in the 1990 led to the discovery that mutations in the BRCA1 and BRCA2 tumor suppressor genes conferred a high risk of breast cancer [(4, 5)]. These genes also predispose to ovarian cancer and a substantial percentage of families with breast and ovarian cancer harbor mutations in BRCA1 and/or BRCA2 gene [(6–8)].

Breast cancer incidences increased rapidly in early adulthood until ages 30 to 40 years for women who carry BRCA1 mutation and until ages 40 to 50 years for BRCA2 carriers, then remained at a similar, constant incidence (20–30 per 100 person–years) until age 80 years. For contralateral breast cancer, the cumulative risk 20 years after breast cancer diagnosis was 40% for BRCA1 and 26% for BRCA2 carriers. Breast cancer risk increased with increasing number of first– and second–degree relatives diagnosed as having breast cancer for both BRCA1 and BRCA2 [(9)].

To date, very few reports have been published about the spectrum of BRCA1 and BRCA2 mutations in Algerian women [(10–13)] and about 11% of breast cancer cases in Algeria occur in women of less than 35 years of age, and 55% of cases at less than 50 [(14)]. The young age at

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onset and high grade suggest the influence of genetic factors such as mutation of BRCA. The knowledge about the identification of BRCA1 and BRCA2 mutations in Algerian familial breast/ovarian cancer will lead to better understanding of genetic risk factors of this disease.

Materials and Methods

Participants

Patients in this study were referred through the military teaching hospital of Oran. Two hundred and fifty (250) patients with a breast and/or ovarian cancers were selected. 100 index cases were chosen with familial breast cancer. Data on the occurrence of tumours, histopathological characteristics and pedigree data were collected. In addition, age at initial diagnosis was recorded. The selection criteria for the families were based on: (a) Women with a history of two or more relatives on the same side of the family with breast and ovarian cancer; (b) Two or more cases of breast and ovarian cancer in first–degree relatives; (c) Cases of bilateral breast cancer; (d) Breast or ovarian cancer before the age of 40; (e) Male relative with breast cancer. Prior to collecting blood, all selected patients and relatives were informed about the objectives of the study and that their DNA samples would be analyzed for mutations in genes associated with hereditary breast cancer. Only 49 females and one male agreed to the protocol of this study and provided written informed consent. Ethical approval was obtained from appropriate institution.

Mutation analysis

Blood DNA was obtained from each of the index cases. Genomic DNA was extracted from peripheral blood collected on EDTA, in accordance with the manufacturer's protocols[15]. The mutation analysis approach used is based on BRCA1 and BRCA2 mutations screening by High–Resolution melting (HRM) curve analysis followed by direct sequencing. Samples for which no pathogenic mutation was found in BRCA1 gene were analyzed by Multiplex Ligation dependent Probe Amplification (MLPA)[16]. All coding exon of BRCA1 and BRCA2 including flanking intronic regions were submitted to pre–screening with HRM curve analysis. The PCR and HRM assays were performed as described elsewhere[17],using the Light Cycler 480 instrument (Roche Diagnostics Gmbh, Mannheim, Germany). HRM were performed in a 20µl final volume with 20 ng DNA, 200µl probe master (LC480, Roche, ref. 04887301), 20µl Resolight Dye (LC480, Roche, ref. 04909640), 40µl forwards, 40µl reverse, 60µl H2O. PCR was performed in a light cycler 480 High Resolution melting Master Kit (Roche Diagnostics Gmbh, Mannheim, Germany). Touchdown–PCR reactions were used: 10–min hold at 95°C, followed by 37 cycles at 95°Cfor 10 sec, touchdown annealing step (decreasing 1°C/cycle) by 65°C to 55°C for 10 sec and 72°C for 20 sec. The melting curves were normalized and temperature shifted to allow samples to be directly compared. Difference plots were generated by selecting a negative control, converting the melting profile to a horizontal line and normalizing the melting profiles of the other samples against this sample. Significant differences in fluorescence from the horizontal baseline were indicative of mutations. Differences were judged as significant if the replicates fell outside the range of variation seen in the wild type samples.

PCR product was purified using the filter plate Multiscreen Millipore (ref. MANU03050). The sequencing reactions are carried out from about 10ng of purified PCR product (generally 1µl of PCR product), using Mix Big Dye Terminator® (Kit 100 reactions Applera EC–4337450). The reaction sequence was performed in thermal cycler 9700, then purified over Sephadex G50(Amersham Pharmacia Biotech ref. 17–0041–01).20µl of deionized formamideHI–FI (Applied Biosystems ref. 4311320) was added. The sequences obtained for each PCR product is read via the software "Sequencing Analysis" (Applied Biosystems ABI3100). Then they are compared with reference sequences using the Seqscape software.

Results

Overall, 4 mutations were identified among four unrelated patients, 2 in BRCA1 and 2 in BRCA2. Table1 shows the clinical features of BRCA1/2 mutation–positive patients. A distinct germline missense mutation in BRCA1 was located in exon 18 (Figure1). A nucleotide change (c.5117G>C) was identified in codon 1706, that causes an amino acid change Gly>Ala. The C–terminal region is a highly conserved structure containing two BRCA1 C–terminal (BRCT) tandem repeat domains. Missense changes within this motif cause protein folding defects[18–20] and inhibit transcriptional transactivation.

The G1706A variant is located in the first BRCT domain (amino acids1650–1736) and has been partially characterized by predictive modelling and some functional assays[21].

Protein structure modelling analysis conducted by Lovelock and collaborators (2006)[22] predicted that the substitution of an alanine at position 1706 would cause a moderate increase in hydrophobicity and size. The glycine at position 1706 occurs within an α–helix structure running from leucine 1701 to isoleucine 1707. Substitution of glycine with a larger alanine at 1706 was predicted to disrupt a bend in the helix, possibly with functional consequences if normal protein function is reliant on the

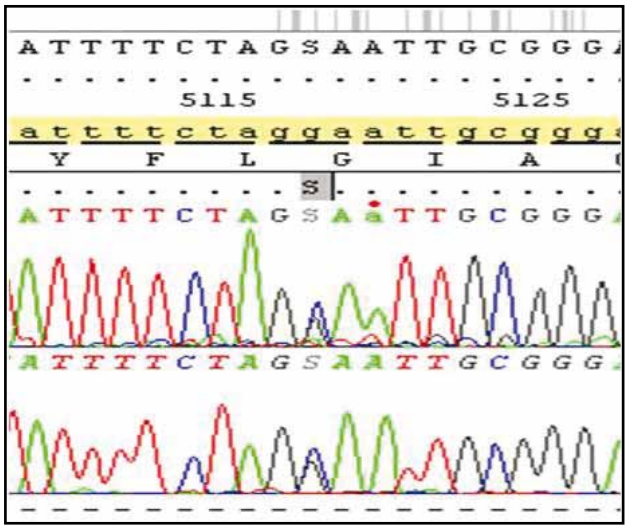


Figure1: Genomic DNA sequence analysis of exon 18 of the BRCA1 gene in patient number one. Transition G>C on nucleotide 5117.25x25mm

The electropherogram indicates the position of the mutation (bold) within the wild type BRCA1 DNA sequence (NCBI NM–007294.2). Sequencing profile for subject 1 with BRCA1 c.5117G>C mutation, DNA from blood sample, heterozygous for mutation.

maintenance of this conformation. The substitution was predicted to cause clashes between the side chains of valine 1687 and valine 1713, which were not relieved by changes in the side chain rotamers of each residue.

The histopathology analysis of the tumour from the index case indicated an infiltrative ductal carcinoma with ER/PR/HER2–negative (Table 2).

The second pathogenic mutation in BRCA1 gene is: c.2125–2126insA (Figure2). It's a germline frameshift mutation located in exon 11b leading to the productionof a premature truncated proteins. This mutation was found in woman diagnosed at the age of 38 for breast cancer and has a personal and strong family history suggestive of genetic predisposition to breast cancer. Histological analysis indicated infiltrative ductal carcinoma (Table2). No mutation was detected in BRCA2 gene.

The third deleterious mutation is c.250C>T (Figure3). The substitution C>T in codon 84 causes an early end of translation of the protein. This mutation was detected in a man with breast cancer diagnosed at the age of 63. The

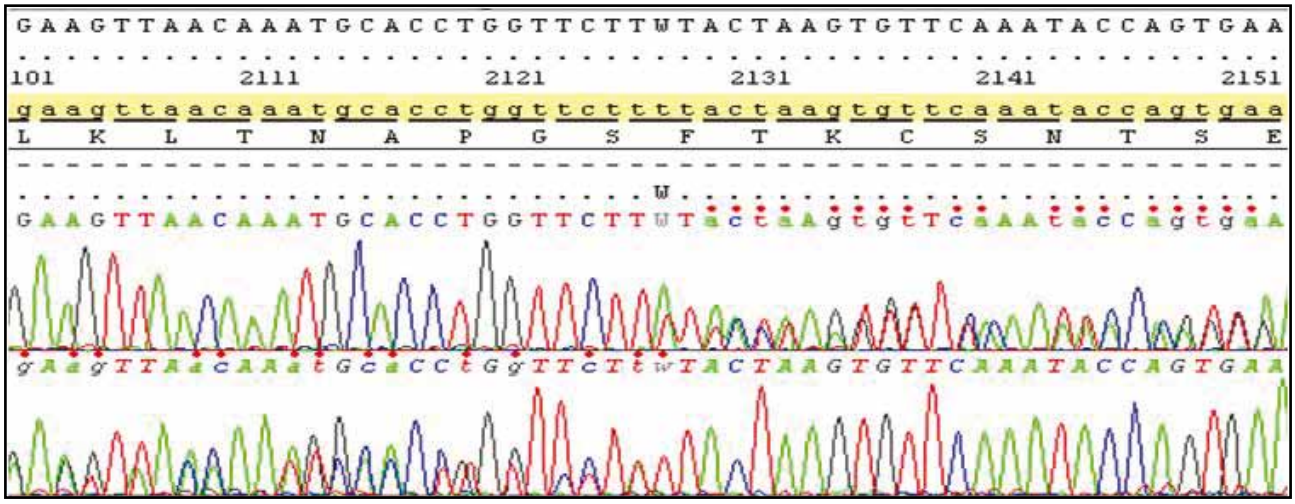


Figure 2: Genomic DNA sequence analysis of exon 11b of the BRCA1 gene in patient number two (c.2125–2126 ins A).

The electropherogram indicates the position of the mutation (bold) within the wild type BRCA1 DNA sequence (NCBI NM_007294.2). Sequencing profile for subject 2 with BRCA1 c.2125–2126insA mutation, DNA from blood sample, heterozygous for mutation.

Subject No.	Exon	Codon	Designation	Hgvs Cdna	Hgvs Proteine	Mutation Type	Pheno-type	Family History
	BRCA1							
1	18	1706	G1706E	c.5117G>C	Gly1706Ala	M	BC	2 BC
2	11	709	2244insA	c.2125_2126insA	Phe709TyrfsX3	F	BC	2BC/3UC
	BRCA2							
3	3	84	Q84X	c.250C>T	P.Gin84x	N	MBC	2BC/PC
4	25	3122	-	c.9364G>A	P.Ala3122Thr	M	BBC	2BC

Table 1: BRCA1/BRCA2 germline mutations identified in Algerian families with a Hereditary History of Breast Cancer.

Acronyms: BC=breast cancer; MBC=male breast cancer; BBC=bilateral breast cancer; M=missense mutation; F=Frame shift mutation; N=nonsense mutation.

Subject No.	Sequence variant HGVS nomenclature	Proband	Age at diagnosis	Histological type	SBR grade	ER status	PR status	Her2
	BRCA1							
1	c.5117G>C	BC	28	IDC	II	-	-	-
2	c.2125_2126insA	BC	47	IDC	III	+	+	-
	BRCA2							
3	c.250C>T	MBC	69	IDC	II	+	+	-
4	c.9364G>A	BBC	31	IDC	III	+	+	-

Table 2: Clinicopathological characteristics of breast cancer patients with BRCA1/BRCA2 mutations.

Acronyms: BC=breast cancer; MBC=male breast cancer; BBC=bilateral breast cancer; IDC= infiltrative ductal carcinoma; ER=estrogen receptor; PR=progesterone receptor; Her2= human epidermal growth factor receptor 2

presence of a male proband or a male relative diagnosed with breast cancer is suggestive of BRCA2 mutation. There were two other cases of cancer in the family, a brother of the affected male who had suffered from prostate cancer, diagnosed when he was 63 but was not alive at the time of the study, and his daughter,who had breast cancer diagnosed at the age of 32. Histopathological study of both tumours revealed an invasive ductal carcinoma and a positive steroid receptor status (Table2).

The variant located in exon 25 c.9364G>A seats in the BRCT domain of the protein (Figure 4). This substitution A>G in codon 3122 causes an amino acid change Ala>Thr. We detected this mutation in woman with bilateral breast cancer diagnosed at the age of 31. It has been classified as rare or unclassified variant. It involves an amino acid conserved in inter–species comparisons. The in silico study of the variant is in favor of a pathogenic alteration. The PolyPhen2 program indicated that this mutation is predicted to be damaging with a score of 1.000. The feature of this variant is unknown at this time. The pathological significance of this variant has to be specified through a familial study and co–segregation analysis.

Discussion

The prevalence and spectrum of BRCA mutations in North African breast cancer (BC) and/or ovarian cancer (OC) families have not yet been thoroughly studied. Three studies have investigated BRCA gene mutation in BC patient with affected relatives in Tunisia[23–26]. In Morocco, three recent studies have investigated BRCA germline mutation in BC patient[27–29]. To date, few molecular genetic studies of BRCA1/BRCA2 germline mutation have been reported in the Algerian population. To the best of our knowledge, our report is the first study using HRM and MLPA analysis of a population from western Algeria. Thirteen index cases and one male with breast cancer

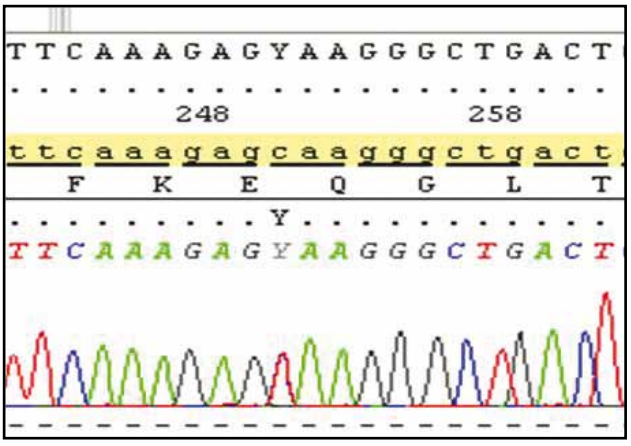


Figure 3: Genomic DNA sequence analysis of exon 3 of the BRCA2 gene in patient number three. Transition C>T on nucleotide 250.

The electropherogram indicates the position of the mutation (bold) within the wild type BRCA2 DNA sequence (NCBI NM_000059.3. Sequencing profile for subject 3 with BRCA2 c.250C>T mutation, DNA from blood sample, heterozygous for mutation.

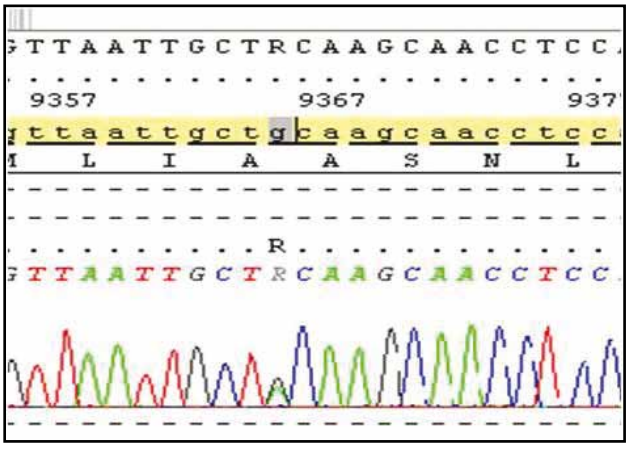


Figure 4: Genomic DNA sequence analysis of exon 25 of the BRCA2 gene in patient number four. Transition G >A on nucleotide 9364.

The electropherogram indicates the position of the mutation (bold) within the wild type BRCA2 DNA sequence (NCBI NM_000059.3. Sequencing profile for subject 4 with BRCA2 c.9364G>A mutation, DNA from blood sample, heterozygous for mutation.

have been examined for germline mutation in the BRCA1/ BRCA2 genes. Four pathogenic mutations have been identified (2in BRCA1and2 in BRCA2) in 4 of 50 families. DNA sequences of sporadic cases arising before the age of 38 and familial cases revealed five deleterious mutations in BRCA1 among 51 early–onset sporadic cases, and four mutations among 11 families[10]. In their study, Cherbal and collaborators (2010)[11] described analysis of the BRCA1gene in 86 individuals from 70 families from an Algerian cohort with a personal and familial history suggestive of genetic predisposition to breast cancer. Three distinct pathogenic mutations were detected in BRCA1 and two pathogenic mutations were detected in BRCA2. Recently, three deleterious mutations were found in the BRCA1 gene and four mutations in the BRCA2 gene in an eastern Algerian population[13].

These mutations in Algerian patients with breast cancer were previously described in the BIC database. We note that one mutation in BRCA2 gene (c. 250C>T) has been found in a male with breast cancer and he had also a family history of cancer (brother with prostate cancer and daughter with breast cancer at early onset). According to Arena et al, Diez et al and Tournier et al[30–32], the presence of a male with breast cancer is suggestive of BRCA2 mutation and all men with breast cancer should be regarded as being possibly inherited and should be fully investigated. This mutation was already described in the UMD database but only identified in women with breast cancer and is more frequent in Western European patients[33].

Our results show that the c.5117G>C mutation in BRCA1 gene was detected in the patient with early onset breast cancer (28–years old) with ER–, PR– and Her2– profiles. This patient had a triple negative disease, suggesting a relationship between breast cancer "triple negative" and breast cancer with BRCA1 gene mutation, as reported by Reis–Filho and Tutt, (2008)[34]; Turner and Reis–Filho, (2006)[35]. We note that sequencing or high resolution melting (HRM) analysis of the coding and flanking intronic regions of BRCA1 and BRCA2 in this patient revealed no other variation. Three Algerian pedigrees were ascertained to be carriers of the G1706A variant but the histopathology data of the tumours from this patient are not available[12]. The G1706A has been classified as a missense mutation by BIC. Analysis of the G1706A variant in functional assays conducted by Lovelock and collaborators, (2006)[22]suggests that it is unlikely to be associated with significant risk of breast cancer and may be classified as benign according to the criteria of Goldgar et al, (2004)[36]. The result from a meta–analysis conducted by Tun and collaborators (2013)[37] suggest that among the selected breast cancer patient population with high–risk features, women with TNBC are approximately 5.6 times more likely to have BRCA1 mutation compared with non–TNBC phenotype, and approximately two in nine women with TNBC harbor BRCA1 mutation. Our findings suggest that this variant could account for the disease and highlight the importance to use this indication for routine testing for BRCA1 gene in Algerian breast cancer families with a triple negative disease. In addition, this unclassified variant is the most frequently detected in Moroccan[27] and Tunisian population[24] but has been observed in conjunction with a deleterious mutation in a Tunisian population. These findings reflect the genetic heterogeneity of these populations.

We note that the frameshift mutation c.2125_2126insA identified in BRCA1 gene was identified in women with a strong breast cancer history (3 members first–degree). There is a significant evidence to suggest that this variant is a dominant high–risk pathogenic mutation so, it encodes an illegitimate codon stop (UMDDATABASE). The missense mutation (c.9364G>A) identified in the BRCA2 gene not reported in BIC, was found in one patient with bilateral breast cancer. However, it was seen only in the French population. The in silico study of the variant is in favor of a likely pathogenic character. In addition, this mutation is predicted to be damaging with a score of 1.000 by PolyPhen–2 prediction program. The feature of this variant is unknown at this time.We note that these pathogenic mutations described in our study differ from those provided by the literature and also differ from those seen in Tunisian and Moroccan populations. These findings could suggest a large BRCA1 and BRCA2 mutations spectrum not only in Algerian population but also in North African populations.

Conclusion

In our experiment, HRM was used to identify BRCA1 and BRCA2 mutations in western Algerian patients with a family history suggestive of genetic predisposition to breast cancer. Our findings could suggest a larger frequency of BRCA1/2 mutations in Algeria. We hope that it would be interesting to develop a BRCA1 and BRCA2 mutation database for Algerian and North Africanpopulation. To explain the role of these genes in Algerian breast cancer, further studies are needed with a larger samples size. Detailed examination of the clinical data and pedigree findings are important for counselling patient with familial breast cancer and for the eligibility for genetic testing as routine test. It would be of interest to search this pathogenic mutation in the other family members and adopting a targeted prevention strategy. Finally, we suggest that the national public health of Algerian population should develop a breast cancer

genetic screening program and individual testing for women with breast cancer disease.

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