GERMLINE *ATM* MUTATIONAL ANALYSIS IN *BRCA1/BRCA2* NEGATIVE HEREDITARY BREAST CANCER FAMILIES BY MALDI-TOF MASS SPECTROMETRY

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Abstract

Biallelic inactivation of *ATM* gene causes the rare autosomal recessive disorder Ataxia-Telangiectasia (A-T). Female relatives of A-T patients have a two-fold higher risk of developing breast cancer (BC) compared with the general population. *ATM* mutation carrier identification is laborious and expensive, therefore, a more rapid and directed strategy for *ATM* mutation profiling is needed. We designed a case-control study to determine the prevalence of 32 known *ATM* mutations causing A-T in Spanish population in 323 *BRCA1/BRCA2* negative hereditary breast cancer (HBC) cases and 625 matched Spanish controls. For the detection of the 32 *ATM* mutations we used the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique. We identified one patient carrier of the c.8264_8268delATAAG *ATM* mutation. This mutation was not found in the 625 controls. These results suggest a low frequency of these 32 A-T causing mutations in the HBC cases in our population. Further case-control studies analyzing the entire coding and flanking sequences of the *ATM* gene are warranted in Spanish BC patients to know its implication in BC predisposition.

Keywords

Hereditary Breast Cancer, ataxia-telangiectasia, *ATM*, *BRCA1*, *BRCA2*, case-control study, MALDI-TOF mass spectrometry.

Abbreviations

BC: Breast cancer

HBC: hereditary breast cancer

PI3K: phosphatidylinositol 3-kinase

A-T: Ataxia-Telangiectasia

RR: relative risk

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer among western women. Approximately 7% of BC cases are of hereditary origin [1]. To date, several BC susceptibility alleles have been identified and categorized into three groups according to their population frequency and the risks conferred: rare high-penetrance alleles, moderate-penetrance alleles, and common low-penetrance alleles [2]. The *BRCA1* and *BRCA2* genes are the two major predisposition high penetrance genes to BC conferring a risk that is 10-to 30-times the risk among women in the general population. However, germline mutations in these genes only account for up to 16% of the hereditary breast cancer (HBC) burden [3]. Mutations in other BC predisposing high-penetrance genes such as *TP53*, *STK11*, *CDH1*, *PTEN* and lately *RAD51C* [4] are also associated with HBC, but each one has a small contribution to familial BC (1%) except for patients with specific features or in the context of rare cancer syndromes [5,6]. Recently published genome-wide association studies provide evidence for low-penetrant BC susceptibility loci that confer a small relative risk up to 1.5-fold and an overall 8% of the HBC risk [7]. A third group known as moderate-penetrance BC genes confers increased risks of two to fourfold compared to the 10% risk in the general population. Thus far, five moderate-risk BC genes have been convincingly identified: *CHEK2*, *BRIP1*, *PALB2*, *NBS1*, and *ATM* [8].

The *ATM* gene (ataxia telangiectasia mutated; MIM#607585) encodes a lipid kinase phosphatidylinositol 3-kinase (PI3K) expressed in a wide range of tissues. It is the key player of a signaling cascade that detects and repairs DNA double-strand breaks, a cascade in which the BRCA1 and BRCA2 proteins are involved [9]. Biallelic inactivation of *ATM* gene causes the rare autosomal recessive disorder Ataxia-Telangiectasia (A-T), a neurodegenerative disorder characterised by progressive cerebellar ataxia and dysarthria, associated with ocular telangiectasia, immunodeficiency, sensitivity to ionising radiation, and an increased risk of cancers, especially leukaemias and lymphomas [10]. Most of the patients with A-T are compound heterozygotes (inheriting distinct *ATM* mutations from each parent) and over 70% of these mutations are base substitutions, small insertions or deletions and splicing alterations that generate premature termination codons which in turn result in a truncated and unstable ATM protein (see http://chromium.liacs.nl/LOVD2/home.php?select_db=ATM) [11]. The prevalence of such *ATM* mutations has been shown to be as high as 0.5–1% in Western general populations [12]. The link between A-T and BC arouse from clinical and epidemiological studies observing that female relatives of A-T patients had an increased risk of BC [12-15]. This association

was definitively confirmed in a case-control study by Renwick *et al* [16]. The authors analyzed 443 cases of familial BC and 521 controls, and found 12 women carrying an *ATM* mutation in the case group (2.70%) and two in the control group (0.38%). They estimated that *ATM* mutation heterozygosity was associated with a BC relative risk (RR) of 2.37 (95% CI: 1.51–3.78). Our group [17] reported the first results of *ATM* germline mutations in Spanish population with early-onset BC and showed that one of 43 patients (2.3%) had a deleterious truncating *ATM* mutation (c.3802delG) that causes A-T in the homozygous state.

Despite all this evidence, the large size of *ATM* (it contains 63 exons spanning approximately 150 kb of genomic DNA) and its very heterogeneous mutational spectrum makes mutation carrier identification laborious and expensive. Hence a rapid and more directed strategy for *ATM* mutation profiling is needed. Founder effects for *ATM* mutations have been reported in different countries, including Spain [18-20]. All the 13 *ATM* mutations identified by Renwick *et al* [16] were predicted to cause A-T, and nine of those identified in cases have previously been reported in A-T families, including the two most common mutations in the UK, c.5762ins137 and c.3802delG. More recently, a study of Bogdanova *et al* [21] provided evidence for the association of an *ATM* founder mutation (c.5932G>T; p.E1978X, a common A-T causing mutation) with BC in Eastern European populations. These findings suggest that it is possible to approach carrier testing by first identifying the most common mutations in selected populations and then developing rapid assays that use small amounts of genomic DNA and less costly methods.

The goal of our study was to confirm the implication of *ATM* as a susceptibility gene in *BRCA1*/*BRCA2* wild-type HBC Spanish families. We designed a case-control Spanish HBC study to determine the prevalence of known *ATM* mutations causing A-T in Spanish populations using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique.

Patients and Methods

Patient samples

Genomic DNA was available from index cases of 323 *BRCA1* and *BRCA2* wild-type Spanish HBC families from two distinct populations of Spanish origin: Catalonian and Galician. The 228 Catalonian cases were selected from high risk families with at least three cases of BC among relatives of 1st and 2nd degree and no cases of ovarian cancer or male BC. The families were recruited from the

cancer genetics clinics of the Catalan Institute of Oncology, University Hospital Vall d'Hebron, and Santa Creu i Sant Pau Hospital from Barcelona, Catalan Institute of Oncology of Girona and Sant Joan University Hospital of Tarragona. The 95 Galician HBC cases had previously been studied by Blanco *et al* for germline mutations in *TP53* and *PTEN* [22]. All participants provided written informed consent and the study was approved by the institutional review boards. DNA samples from up to 625 geographically matched Caucasian Spanish controls (426 from Catalonia and 199 from Galicia) without personal or familial antecedents of any cancer were also analysed to establish the frequency of the 32 selected mutations in each respective population. DNA was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and diluted to a final concentration of 20 ng/ul for genotyping.

ATM mutation selection

We analyzed 32 *ATM* mutations responsible for A-T in Spanish population previously described by Mitui M. *et al* [18], Castellví-Bel *et al* [20] and García Pérez *et al* [23], together with the *ATM* mutation and the likely deleterious variants reported previously by our group in early-onset BC Spanish patients negative for *BRCA1/2* mutations [17] (Table1).

Genotyping

Mutation screening of the 32 mutations was performed at the Spanish National Genotyping Center in the node of Santiago de Compostela by MALDI-TOF MS using the Sequenom MassArray System and iPLEX Gold genotyping chemistry. The design of oligonucleotides was carried out according to the guidelines of Sequenom and performed using MassARRAY Assay Design software (version 1.0). Mutations detected were confirmed by bi-directional sequencing with "Big Dye Terminator v3.1 Cycles Sequencing kit" (Applied Byosistems, Warrington, UK) on ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems) in accordance with the manufacturer's instructions. Primer sequences are available on request. Sequences were examined using Staden Package software (Open Source Technology Group, Inc.).

Results and Discussion

In the current study we analyzed the presence of 32 *ATM* mutations in *BRCA1/BRCA2* negative Spanish HBC samples using MALDI-TOF mass spectrometry technology. The genotyped mutations were selected since were known to be responsible for A-T in Spanish populations.

We identified the mutation c.8264_8268delATAAG in heterozygous state in a Catalonian woman with BC at the age of 34 (Figure 1A-B). The patient had two first degree relatives diagnosed with BC at the ages of 58 and 66, but none sample was available from these patients. The mutation was confirmed by direct sequencing (Figure 1C). No other mutations were found in control or case samples. Interestingly, c.8264_8268delATAAG had previously been identified in one Spanish, one Costa Rican, and three Brazilian A-T patients with different STRs and SNPs haplotypes, suggesting that these represent independent mutational events or a hot-spot in the *ATM* gene [18].

The utility of genetic testing of BC genes has comprehensively been shown for *BRCA1* and *BRCA2* genes [24]. The demonstration that *ATM* mutations predispose to BC [16] could also be used in risk stratification of women to allow them to make changes on their medical surveillance and lifestyle habits to reduce their risk of BC. Nevertheless, it is not yet known what determines which women with *ATM* mutations will develop BC (around 15 per cent of female *ATM* carriers) [25] and this limits the clinical utility of identifying such mutations. Moreover, the significance of most *ATM* missense variants is unknown and only a few of them have been considered to be pathogenic. Recently, Fletcher *et al* [26] showed that the combined effects of five missense *ATM* SNPs were associated with a small increased risk of BC, explaining an estimated 0.03% of the excess familial risk of BC. Tavtigian *et al* [27] used an *in silico* missense-substitution analysis to assess the contribution to BC risk of rare *ATM* variants described in seven published *ATM* case-control studies and from their own mutation screening data of additional case-control analyses. They provided evidence that a subset of rare missense *ATM* substitutions confer increased BC risk. An analysis on a subset of these rare variants lead them to propose the hypothesis that the missense mutations conferring increased risk of BC are more concentrated in the last third of the ATM protein (FAT, kinase, and FATC domains) and that a subset of these missense substitutions actually confer higher risk of BC than do protein-truncating variants on average. Moreover, if *ATM* mutations act multiplicatively with other genetic BC factors it may be possible to identify women with combinations of BC susceptibility alleles, which all together give risks similar to those of *BRCA1/2*.

ATM mutation status may also be of relevance for the treatment of BC due to its influence on response to radiation, as it has recently been suggested in a nested case-control study within a cohort of 52,536 survivors of unilateral BC. Women who carried deleterious *ATM* variants and who were treated with radiation had a statistically significantly higher risk of contralateral BC [28].

Together, these data reveal that *ATM* truncating variants, as well as a number of missense variants confer BC risk. However, their prevalence is usually low and their variety may differ widely among populations from different geographical or ethnic origins. Therefore, taking the complexity and size of the gene into account, specific analysis approaches are necessary by using reliable, flexible, and rapid methodologies.

Several methods have previously been used to screen for *ATM* mutations in order to overcome the complexity of analyzing *ATM* gene, such as a non-isotopic RNase cleavage-based assay (NIRCA) [29], Denaturating Gradient Gel Electrophoresis (DGGE) [30], single-strand conformational polymorphism (SSCP) [31], denaturing high-performance liquid chromatography (DHPLC) [32] and more recently, enhanced mismatch mutation analysis (EMMA) [33]. Despite the availability of all these techniques for *ATM* analysis, Sanger sequencing of the entire coding and flanking sequences of *ATM* gene remains standard making *ATM* mutation identification expensive and labour intensive.

We analyzed in *BRCA1/BRCA2* negative Spanish HBC samples a panel of 32 *ATM* mutations previously found in A-T Spanish patients using MALDI-TOF mass spectrometry. In our hands, this methodology was technically successful to study this set of *ATM* selected mutations. Nevertheless, the predictive power of our approach is low due to the small frequency of the 32 mutations found in the 323 *BRCA1* and *BRCA2* wild-type Spanish women with HBC with a prevalence of 0.31% (1/323; 95% CI 0.008-1.71). The practicability and flexibility of this technology allow the addition of new *ATM* mutations in the design, which could improve its efficiency in the future. Hence, further case-control studies are warranted analysing the whole coding sequence of *ATM* in Spanish BC patients.

Future steps in moderate-risk BC genes mutation profiling, such as *ATM*, will probably require a comprehensive strategy by full sequencing of the genome applying massive parallel “next generation” sequencing procedures. Preliminary reports [34] suggest that this sequencing technology would enable accurate and cost-effective full screening of a large number of predisposition genes involved in HBC.

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Conflicts of Interest

There are no potential conflicts of interest

Figure Legends

Figure 1: Analysis of c.8264-8268delATAAG *ATM* mutation.

A. Genotyping calls for c.8264-8268delATAAG. Sample carrying the deletion in heterozygous state is indicated by a green square, while those without the deletion are displayed as blue triangles.

B. Sequenom mass spectrum for the mutation c.8264-8268delATAAG. The top figure indicates the wild-type allele, while the bottom one indicates both, wild-type and c.8264-8268delATAAG mutation alleles.

C. Direct sequencing of the *ATM* c.8264_8268delATAAG carrier sample. The arrow shows the start point of the nucleotides deletion.

Table Legends

Table 1: *ATM* mutations (n=32) previously described in A-T Spanish patients and genotyped in our study using the platform MALDI-TOF mass spectrometry by Sequenom.

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Table 1.

ATM mutations (n=32) previously described in Spanish population and genotyped in our study using the platform MALDI-TOF mass spectrometry by Sequenom,

Nomenclature in the original reference	cDNA reference sequence NM_000051.3	Protein reference sequence NP_000042.3	Reference for Spanish population
640delT	c.640delT	p.Ser214ProfsX16	18
715delT	c.716delT	p.Phe239SerfsX16	18
IVS15-2A>C	c.1899-2A>C	p.Cys633X	23
2250G>A	c.2250G>A	p.Glu709_Lys750del	18
2413C>T	c.2413C>T	p.Arg805X	18
IVS21+1G>A	c.2921+1G>A	p.(?)	18
IVS21+1G>CA	c.2921+1delinsCA	p.Tyr947GlnfsX9	23
3712_3716delTTATT	c.3712_3716delTTATT	p.Leu1238LysfsX6	18
3763T>G	c.3763T>G	p.Leu1255Val	17
3802delG	c.3802delG	p.Val1268X	17
3836G>A	c.3836G>A	p.Trp1279X	18
3894_3895insT	c.3894dup	p.Ala1299CysfsX3	18
IVS28+1711del3450	c.3994-1415_4270	p.(?)	18
IVS33+2T>C	c.4776+2T>C	p.(?)	18
5188C>T	c.5188C>T	p.Arg1730X	18
5644C>T	c.5644C>T	p.Arg1882X	18
6314G>C	c.6314G>C	p.Arg2105Thr	17
6342_6343insC	c.6342dup	p.Val2115ArgfsX12	18
6347_6348delGG	c.6347_6347+1del	p.Ser2116ThrfsX4	18

7653T>C	c.7653T>C	Synonymous	17
8100A>T	c.8100A>T	p.Lys2700Asn	18
8103_8104delAA	c.8103_8104delAA	p.Ile2702ArgfsX15	18
8156G>A	c.8156G>A	p.Arg2719His	17
8177C>T	c.8177C>T	p.Ala2726Val	18,20
8264_8268delATAAG	c.8264_8268delATAAG	p.2718_2756del39	18
8283_8284delTC	c.8283_8284delTC	p.Gln2762AlafsX6	18
IVS62+1G>A	c.8786+1G>A	p.Gly2891AspfsX9	23
8875_8878delGACT	c.8875_8878delGACT	p.Asp2959GlyfsX3	18
8977C>T	c.8977C>T	p.Arg2993X	18
c.9008del28	c.9008_9035del	p.Lys3004TyrfsX5	23
c.9010_9037del28	c.9010_9037del	p.Lys3004TyrfsX5	18,20
c.9170_9171delGA	c.9170_9171delGA	p.X3057PhefxX5	18