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Article

Characterization of new ATM deletion associated with Hereditary Breast Cancer.

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Abstract: Next Generation Sequencing based cancer risk screening with multigene panels has become the most successful method for programming cancer prevention strategies. ATM germ-line heterozygosity has been described as able to increase tumor susceptibility. In particular, families that carry heterozygous germ-line variants of ATM gene show a 5- to 9-fold risk of developing breast cancer. Recent studies identified ATM as the second most mutated gene after CHEK2 in BRCA-negative patients. Nowadays, more than 170 potential missense variants and several truncating mutations have been identified in ATM gene. Here we present the molecular characterization a new ATM deletion, identified thanks to the CNV algorithm implemented in the NGS analysis pipeline. An automated workflow implementing the SOPHiA Genetics' Hereditary Cancer Solution (HCS) protocol was used to generate NGS libraries that were sequenced on Illumina MiSeq Platform. NGS data analysis allowed to identify a new inactivating deletion of exons 19-27 of ATM gene. DNA breakpoint was characterized both at DNA and RNA level

Keywords: next-generation sequencing; Breast Cancer, ATM, Homologous Recombination Repair, Hereditary Cancer Syndromes, Clinical Genomics, Molecular Diagnostics

1. Introduction

Breast cancer is one of the most common tumors in the female population, and 5-10% of cases arise as hereditary breast cancer (HBC) [1]. Next Generation Sequencing based cancer risk screening with multigene panels has become the most successful method for programming cancer prevention strategies [2-4]. Although BRCA1 and BRCA2 remain the most frequently mutated genes, mutations in other DNA repair genes account for a significant fraction (~25%) of HBC cases [5] ATM germ-line heterozygosity occurs in about 1% of the population and has been described as able to increase tumour susceptibility. In particular, families carrying heterozygous germ-line variants of ATM gene show a 5- to 9-fold risk of developing breast cancer, particularly in women younger than 50 years [6]. Approximately, the 3% of the families affected by hereditary breast and ovarian cancer harbors a loss-of-function ATM mutation [7]. Recent studies identified ATM as the second most mutated gene after CHEK2 in BRCA-negative patients. Nowadays, more than 170 potential missense variants and several truncating mutations have been identified in ATM

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gene. Little data are available on the deletions and rearrangements of the ATM gene, especially since not all analytical pipelines of the NGS data implement specific algorithms for predicting CNVs. The artificial intelligence-based NGS data analysis platform, SOPHiA DDM software, incorporates a specific algorithm to study CNVs from NGS data generated using the SOPHiA Genetics' Hereditary Cancer Solution (HCS) kit. Here we present the molecular validation and characterization both at DNA and RNA level of a new ATM deletion identified with diagnostic NGS for hereditary cancer risk screening.

2. Materials and Methods

2.1. Subjects

Written informed consent was obtained from all subjects. Total genomic DNA was extracted from blood samples using Maxwell 16 Blood DNA Purification Kit and Maxwell 16 MDx AS3000 instrument (Promega, Madison, Wisconsin USA).

2.2. NGS Sequencing

Two hundred ng of genomic DNA has been processed with the Hereditary Cancer Solution (HCS) kit (SOPHiA GENETICS, Saint-Sulpice, Switzerland). The capture-based target enrichment, of 26 cancer related genes (*ATM, APC, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, EPCAM, FAM175A, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PIK3CA, PMS2, PMS2C, PTEN, RAD50, RAD51C, RAD51D, STK11, TP53, XRCC2*) and the library construction protocols were carried out exclusively with the automated procedure implemented on the STARlet platform (Hamilton). Library quantification was carried out with fluorometric quantitation using Qubit dsDNA High Sensitivity kit (Thermofisher Scientific). Quality control was performed by analyzing the profile of each sample obtained with Bioanalyzer DNA 1000 (Agilent Technologies).

Sequencing was achieved onto a 600-cycle format V3 flow-cell, via Illumina MiSeq DX platform according to their and SOPHiA Genetics' protocols. Sequencing data were processed for single nucleotide variants (SNVs), indels, and copy number variations (CNVs) via SOPHiA DDM platform based on SOPHiA Artificial Intelligence (AI).

2.3. Multiplex Ligation-dependent Probe Amplification (MLPA) Analysis

One hundred ng of genomic DNA of the proband was analyzed for CNVs detection in ATM gene with SALSA MLPA P041 and P042 probemix, (MRC Holland, Amsterdam, Netherlands) according to manufacturer's instructions on ABIPrism 3130XL Genetic Analyzer (Thermo Fisher Scientific). The data were analyzed with Coffalyzer software (MRC Holland, Amsterdam, Netherlands).

2.4. Long-range PCR of the gene region including the deletion.

The deletion, detected by NGS sequencing and MLPA, was confirmed with Long-range PCR using the Fast Start PCR Master Mix (Roche, Penzberg, Germany). FastPCR Software (Primer Digital Ltd, Helsinki, Finland) was used to design the following primers:

ATM-e18-4FW: 5′-GTGTAACTACTGCTCAGACCAA-3′

ATM-e28-8R: 5′-CAGACCAATACTGTGTCCTTTAGGGCA-3′

DNA was amplified using the following PCR conditions: an initial denaturation at 94°C for 3′, followed by 10 cycles at 94°C for 20″, 59°C for 30″, 68°C for 7′, and by other 20 cycles at 94°C for 20″, 59°C for 30″ and 68°C for 7′ with an increase of 10″ for every PCR cycle, and a final extension at 68°C for 13′.

2.5. Characterization of the deletion breakpoint

The PCR product was sequenced with ABIPrism 3130XL Genetic Analyzer (Thermo Fisher Scientific), using the following primers:

ATM-e18-10FW: 5′-CTACCAAATCCCTCCACCTGCAT-3′

ATM-E28-7R: 5′-GAGCAGGATCCAAATCCCTAACAGAGT-3′

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The nucleotide sequence was compared to wild type ATM genomic sequence [LRG_135, NG_009830.1]. Intronic repeated sequences were found with Repeat Masker software [http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker].

3.1. Analysis of ATM mRNA

Total RNA was extracted with Maxwell 16 Total RNA Purification Kit and Maxwell 16 MDx AS3000 instrument (Promega). 500 ng of total RNA was reverse transcribed using MMLV Reverse Transcriptase (Thermo Fisher Scientific) as previously described [8]. PCR amplification of the gene region including the deletion was performed as described above. Then, the PCR fragment harboring the deletion was extracted from agarose gel and sequenced as previously described [9]. The nucleotide sequence of the amplified cDNA fragment was compared to the wild type sequence of ATM mRNA [NM_000051.3].

3. Results

3.1. Identification of genomic variant by NGS analysis

The proband had been referred for genetic testing based on a family history of breast cancer at early onset. Her mother was affected by monolateral breast cancer, diagnosed at the age of 45, positive to hormone receptors with metastases in axillary lymph nodes. The subject was previously tested for the mutational status of only BRCA1 and BRCA2 genes and reported as negative for pathogenic variants (data not shown). In order to identify any further variants that could explain the phenotype the subject was analyzed using the CE-IVD Hereditary Cancer Solution (HCS) assay by SOPHiA Genetics. The results showed a wide heterozygous deletion of exons from 19 to 27 of ATM gene.

3.2. MLPA analysis and breakpoint characterization

In order to confirm the deletion of the region spanning from exon 19 to exon 27 of the ATM gene, MLPA analysis was performed on the genomic DNA of the proband with two different mix of probes in order to examine the whole gene. The results confirmed this deletion in heterozygous state (Supplementary Fig. 1). To further characterize the deletion, we employed a long-range PCR design to amplify the breakpoint region in proband DNA and, both in his mother and sister, for verifying the segregation. This assay confirmed the presence of the deletion, showing a 2.5 Kb PCR product in proband and in his mother (Fig. 1). In order to try to explain the possible causal mechanism of the deletion we analyzed the sequence using the Repeat Masker software that is a program that screens DNA sequences for interspersed repeats and low complexity DNA sequences. The output showed several repetitive genomic elements, as ALU sequences, in particular in intron 18. Then, to definitely characterize the exact breakpoint we sequenced the PCR amplicon by Sanger sequencing that revealed a small six-nucleotide element, GGCTCA, overlapping the two introns involved in the rearrangement (Fig. 2) and allowed to exactly describe the deletion at DNA level (c.2838+2162_4110-292del).

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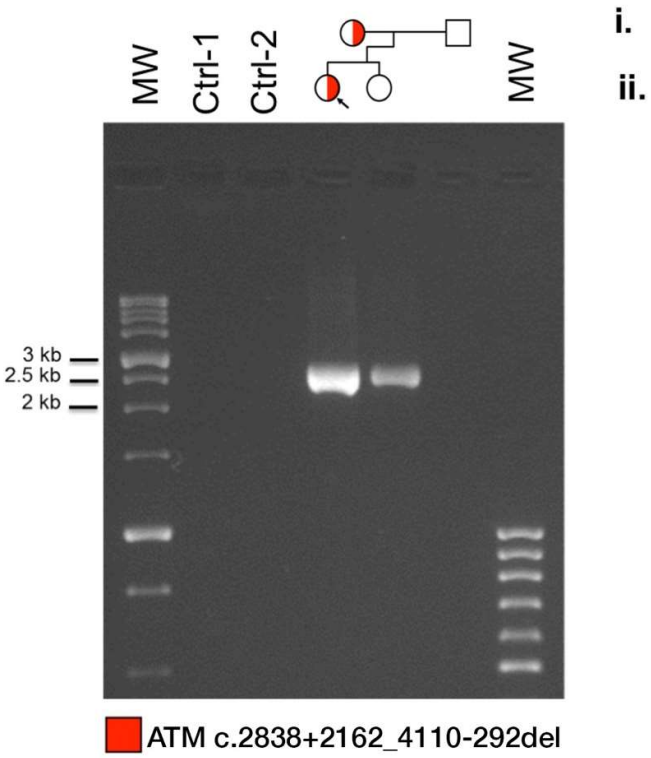

Figure 1. Breakpoint characterization of ATM exon 19-27 deletion: long-range PCR confirmed the presence of the deletion, showing a 2.5 Kb PCR product in proband DNA. The analysis of other family members confirms the presence of the deletion in proband's mother (lanes 4 and 5). Two DNAs from healty subjects were amplified as control (lanes 2 and 3). Molecular weight markers (MW) are loaded in lanes 1 and 7.

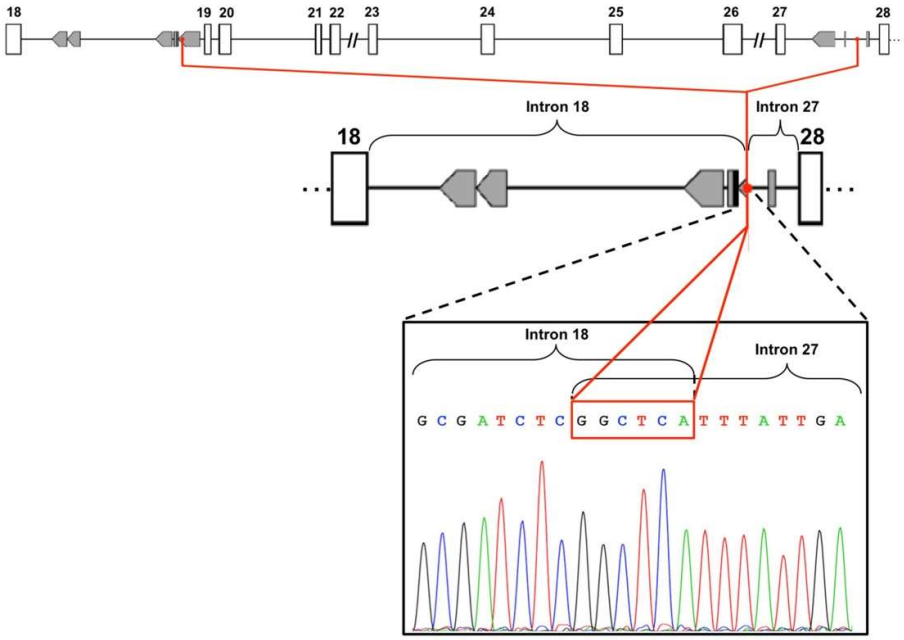

Figure 2. Analysis of repetitive genomic elements and Sanger sequencing: upper and middle part of the figure show the analysis of genomic sequences performed through Repeat Masker software that revealed several repetitive genomic elements, in particular ALU sequences (in gray boxes), in

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intron 18. Lower part of the figure, Sanger sequencing of the 2.5Kb PCR product shows the breakpoint region of the rearrangement, characterized by a small six-nucleotide element, GGCTCA, overlapping the two introns

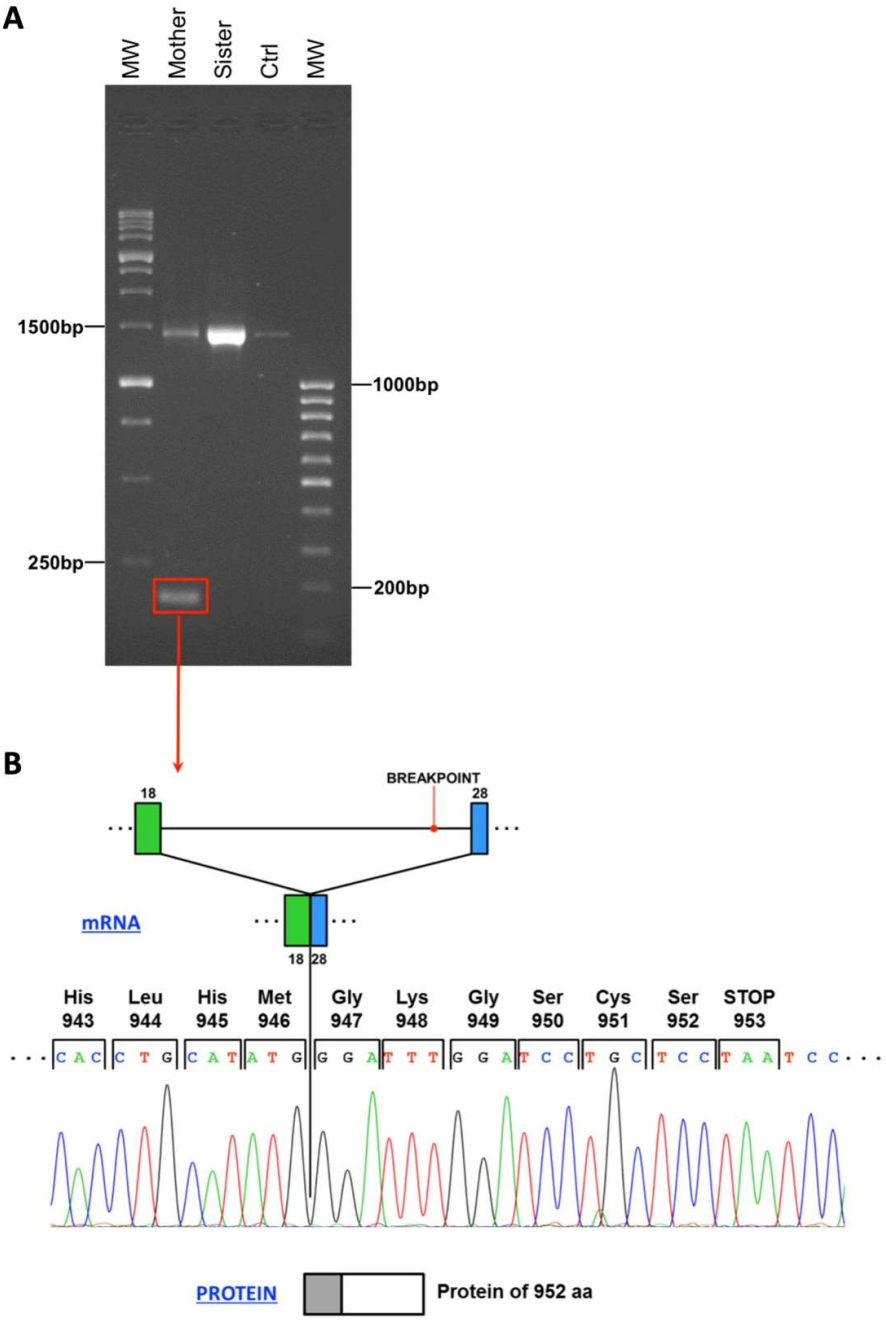

Figure 3. Characterization of mRNA sequence resulting from the deletion: RNA derived from proband's mother and sister showed a 200 bp PCR product represented in Panel A that underwent Sanger sequencing, displayed in Panel B. This analysis allowed us to demonstrate that this variant generates a premature stop codon that encodes for a truncated protein of 952 aa, instead of the wild type 3056 aa ATM protein.

3.3. Characterization of the mRNA sequence

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To characterize the RNA transcript derived from the deleted allele, we reverse-transcribed the proband's mother and sister RNA because the proband's RNA was no longer available. The cDNA was then amplified and the gene region carrying the deletion was sequenced. As shown in Fig. 3A the mother cDNA originated both the wild type allele (1500 bp) and the mutated one (200 bp). In the mutated isoform, exon 28 is juxtaposed to exon 18 (Fig. 3B). This variant disrupts the reading frame of the mRNA generating a premature stop codon that encodes for a truncated protein of 952 aa, instead of the wild type 3056 aa ATM protein. (p.Tyr947Glyfs*7).

4. Discussion

The characterization of the mutational status of genes involved in homologous recombination repair pathway is absolutely the most important approach for designing the prevention strategies for inherited cancer syndromes. A large number of missense and truncating ATM variants have been detected in BRCA1- or BRCA2-negative patient [10].

SOPHiA Genetics HCS gene panel allowed us to detect a novel ATM CNV in a BRCA-negative HBC patient. The deletion of exons 19 to 27 of ATM gene, was detected in an Italian woman who received a diagnosis of monolateral breast cancer at the age of 34. Her mother had ER + monolateral breast cancer, at the age of 45 with axillary lymph node involvement. The NGS detected CNV was firtly validated by means of MLPA assay. Then, long-range PCR and Sanger sequencing were employed in order to characterize the breakpoint at DNA level (c.2838+2162_4110-292del) in proband DNA and to study the segregation also in his mother and in two sisters.

A further characterization at RNA level on the proband's mother and sister identified the presence of both the wild-type and the mutant allele in the mother's sample. In the mutated isoform, exon 28 is juxtaposed to exon 18, disrupting the mRNA reading frame and generating a premature stop codon. The predicted aberrant ATM protein consist of 952 amino acids instead of 3056 amino acids of wild type ATM. This abnormal protein lacks the domain at amino acid positions 1373-1382, required for c-Abl protein interaction and crucial to mediate cell cycle arrest in G1 phase [11]. In addition, at least 3 other important domains are lost from the ATM protein due to the deletion such as FAT (FRAP-ATM-TRRAP), PIKK (phosphatidylinositol 3-kinase-related kinase domain) and FATC (FAT C-terminal domain) domains, mediating the majority of ATM functions [12,13].

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Institutional Review Board Statement: Ethical review and approval were waived for this work by following the guidelines of our Regional Ethics Committee which reports that the evaluation of a single case report or case series (up to 5 cases) whose sole purpose is reporting, i.e. the purpose of which is to inform the scientific community by describing some unusual or new cases, is not within the competence of the Ethics Committee.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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