University of Pisa

PhD COURSE in

“Molecular, metabolic and functional exploration
of the nervous system and the sense organs”

Effects on human transcriptome of two
BRCA1-BRCT mutations: M1775R and A1789T

Candidate

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Abstract

BRCA1 (breast cancer 1, early onset) mutations confer a high risk of breast and ovarian cancer. Most of BRCA1 cancer-predisposing mutations originate truncated proteins, but missense mutations have been also detected in familial breast and ovarian cancer patients. These variants are rare and their role in cancer predisposition is often difficult to ascertain. In the present work I studied the molecular mechanisms affected in human cells by two BRCA1 missense variants, M1775R and A1789T, both located in the second BRCT domain. These variants have been isolated from familial breast cancer patients and their effect on cell transcriptome has been previously investigated in yeast cells. Here I compared the expression profiles of HeLa cells transfected with one or the other variant and HeLa cells transfected with BRCA1 wild-type.

Microarray data analysis was performed by three comparisons: M1775R versus wild-type
(M1775RvsWT-contrast), A1789T versus wild-type (A1789TvsWT-contrast) and the mutated BRCT domain versus wild-type (MutvsWT-contrast), obtained by considering the two variants as a whole. I found 201 differentially expressed genes in M1775RvsWT-contrast, 313 in A1789TvsWT-contrast and 173 in MutvsWT-contrast. Most of these genes participate in cell processes that are often deregulated in cancer, such as cell cycle progression and DNA damage response and repair. These results represent the first molecular evidence of the pathogenetic role of M1775R, already known by functional studies, and give support to a similar role for A1789T, first hypothesized based on yeast cell experiments.
1. Introduction

1.1 BRCA1 and breast cancer

*BRCA1* (breast cancer 1, early onset) has been the first breast cancer susceptibility gene to be cloned (Hall, et al. 1990; Miki, et al. 1994). Along with *BRCA2* (breast cancer 2, early onset) it is the most important disease-associated gene for inherited breast cancer. It acts as a tumor suppressor gene whose truncating or inactivating germline mutations confer a cumulative risk of breast cancer of up to 80%. *BRCA1* germline mutations also cause a risk of 30-40% of developing ovarian cancer (O'Donovan and Livingston 2010). From an histopathological point of view, *BRCA1* germline mutation carriers usually develop high grade Invasive Ductal Carcinomas of No Special Type, negative for Estrogen receptor, Progesterone receptor and Receptor tyrosine-protein kinase erbB-2 (triple negative). In addition, in approximately 15% of sporadic breast cancers there is loss of nuclear BRCA1 expression, mainly due to
epigenetic inactivation or post-transcriptional downregulation (Vargas, et al. 2011).

1.2 BRCA1 in the cell cycle control

In eukaryotic cells, following DNA damage, cell cycle checkpoints are activated to arrest cells at certain stage during cell cycle, giving them enough time to repair damaged DNA before resuming cell cycle progression (Latif, et al. 2001). Cell cycle checkpoints are critical for the maintenance of genomic integrity as they prevent cells from duplication of damaged DNA and passage of it to daughter cells. Consistently, dysfunction of proteins involved in cell cycle checkpoints often results in genomic instability and tumorigenesis (Dasika, et al. 1999). According to the different stages in which they function, cell cycle checkpoints can be categorized into G1/S, S-phase and G2/M checkpoints. BRCA1 is involved in all of these checkpoints activations.
1.2.1 BRCA1 in the G1/S checkpoint

The G1/S checkpoint is defective in BRCA1-depleted cells. BRCA1 is indeed required for TP53 (tumor protein p53) phosphorylation mediated by ATM/ATR (ataxia telangiectasia mutated and ataxia telangiectasia and Rad3 related) in response to DNA damage by ionizing or ultraviolet irradiation (Fabbro, et al. 2004). This phosphorylation of TP53 leads to cell cycle arrest through the activation of the promoter of CDKN1A (cyclin-dependent kinase inhibitor 1A) (el-Deiry, et al. 1994). BRCA1 has also been shown to increase TP53-mediated activation of CDKN1A promoter (Chai, et al. 1999; Zhang, et al. 1998)

1.2.2 BRCA1 in the S-phase checkpoint

BRCA1 is specifically required for the S-phase checkpoint mediated by ATM in response to ionizing irradiation (Xu, et al. 2002). In addition, BRCA1 may also regulate ATM activation following DNA damage in S phase, as it interacts and colocalize with the MRN complex (MRE11A/RAD50/NBN: meiotic
recombination 11 homolog A (S. cerevisiae), nibrin, RAD50 homolog (S. cerevisiae)) (Zhong, et al. 1999), which directly activates ATM (Lee and Paull 2004). Evidence has also been presented that BRCA1 is involved in the ATR-mediated S-phase checkpoint activated by stalled replication forks, which can be induced by treatment of cells with UV or hydroxyurea (Tibbetts, et al. 2000). Both BRCA1-ATM and BRCA1-ATR interactions produce the phosphorylation of BRCA1 on specific Ser/Thr residues (Tibbetts, et al. 2000; Xu, et al. 2002).

1.2.3 BRCA1 in the G2/M checkpoint

The phosphorylation of BRCA1 by ATM is required for cell cycle arrest in G2 induced by ionizing irradiation (Xu, et al. 2001). In addition, loss of BRCA1 abolishes the G2/M checkpoint activation, as BRCA1 regulates CHEK1 (CHK1 checkpoint homolog (S. pombe)) kinase activity during G2/M checkpoint activation (Yu and Chen 2004).
1.3 BRCA1 in DNA repair

In response to DNA damage, different DNA repair processes utilize different repair machineries. However, the two predominant repair pathways are homologous recombination and non-homologous end-joining. Non-homologous end-joining is the most common form of DNA repair in cells and constitutes a relatively error-prone type of repair as it functions without a template. Homologous recombination faithfully repairs damaged DNA as it occurs only during S and G2 phase of the cell cycle when sister chromatids are present and therefore can be used as a template (Jackson 2002).

BRCA1, along with the MRN complex and other tumor suppressors and DNA damage repair proteins, forms a super complex called BASC (BRCA1 Brca1 Associated genome Surveillance Complex). The BASC coordinates multiple activities related to the maintenance of genomic integrity, having an important function in DNA damage sensing as well as in DNA repair (Wang, et al. 2000). In particular, BRCA1, the MRN complex and RBBP8 (retinoblastoma

In addition, BRCA1 has been reported to stimulate the base excision repair mechanism by inducing the activity of key enzymes of this pathway (Saha, et al. 2010).

### 1.4 BRCA1 and global heterocromatin state

BRCA1 deficiency leads to global DNA hypomethylation and chromatin abnormalities related to cancer. Consistently, it has been shown that *DNMT1* (DNA (cytosine-5-)-methyltransferase 1), the methylation maintenance enzyme, is a transcriptional target of *BRCA1* (Shukla, et al. 2010).

The most recent hypothesis on BRCA1 concerns a role in maintaining global heterochromatin integrity. It has been proved that BRCA1 deficiency disrupts gene silencing at the tandemly repeated DNA regions,
probably through the loss of ubiquitylation of histone H2A (Zhu, et al. 2011).

1.5 BRCA1 protein structure

BRCA1 consists of different functional domains: a N-terminal RING finger domain, two nuclear localization signals, a “SQ” cluster, a branched DNA binding domain and a C-terminal domain containing two BRCT (BRCA1 C-Terminus) repeats (Linger and Kruk 2010). The RING domain of BRCA1 has E3 ubiquitin ligase activity and facilitates protein ubiquitination, which occurs when BRCA1 heterodimerizes with BARD1 (BRCA1 associated RING domain 1) (Baer and Ludwig 2002). BRCT repeats have been found in many other proteins that regulate DNA damage response and have a crucial role for their function (Callebaut and Mornon 1997). BRCT repeats have been also described as phosphopeptide-interacting motifs, facilitating the assembly of DNA damage signaling complexes following checkpoint kinases activation (Rodriguez, et al. 2003).
Consistently, the BRCT repeats of BRCA1 have been found to be essential for its targeting to sites of DNA damage, due to the BRCT-mediated interaction of BRCA1 with other proteins involved in DNA repair (Wang, et al. 2007). BRCT domains are also involved in the transcriptional activity of BRCA1 and the second BRCT repeat (aa 1760-1863) is critical for the activation of the CDKN1A promoter (Chai, et al. 1999). Finally, a recent paper reported that BRCA1 tumor suppression depends on BRCT phosphoprotein binding (Shakya, et al. 2011).

Due to the relevance of this region for BRCA1 function, the study of mutations located in this region appears of particular interest.

### 1.6 BRCA1 mutations

A complete list of the mutations detected in BRCA1 is reported in the BIC (Breast Cancer Information Core) database (http://research.nhgri.nih.gov/bic/). Most of the risk-associated BRCA1 variants are frameshift or nonsense
mutations that result in a truncated protein. However, missense mutations associated with breast cancer risk have also been reported. Some of them have not been studied or even completely characterized because of their rarity and/or the difficulty of attributing functional significance to this type of mutation.

1.7 Aim of the present work

Aim of this work was to investigate the effects on human cell transcriptome of two BRCA1 missense variants, M1775R and A1789T, both located within the second BRCA1 BRCT domain and isolated from familial breast cancers. In a previous work we examined the expression profiles induced by these two mutations in yeast cells (Di Cecco, et al. 2009). We found alterations of molecular mechanisms critical for cell proliferation control and genome integrity, suggestive of a putative role of these two variants in breast cancer pathogenesis. Here, I compared the expression profiles of HeLa cells transfected with one
or the other *BRCA1* variant with that of HeLa cells transfected with *BRCA1* wild-type.
2. Materials and methods

2.1 BRCA1 missense variants

Both *BRCA1* variants are located within the second BRCT domain and, while M1775R has widely been described as deleterious (Kawai, et al. 2002; Miki, et al. 1994; Nikolopoulos, et al. 2007; Olopade, et al. 2003; Varma, et al. 2005; Williams and Glover 2003), A1789T has been studied only by our group. In yeast cells both these mutations reverted the growth suppression (small colony) phenotype, but only M1775R induced homologous recombination (Caligo, et al. 2009). In HeLa cells A1789T significantly altered the non-homologous end-joining activity as compared to BRCA1 wild-type (Guidugli, et al. 2011).
2.2 HeLa cells transfection

Five aliquots of the same clone of HeLa G1 cells were transiently transfected with the pcDNA3-BRCA1wild-type vector, five with the pcDNA3-BRCA1-M1775R derivative vector and five with the pcDNA3-BRCA1-A1789T derivative vector as described by Guidugli et al. [Guidugli et al., 2011].

Twenty-four hours after transfection, cells were washed twice in PBS 1X, pelleted and immediately used to extract RNA or proteins. The increased expression of BRCA1 was assessed by Western Blot analysis as indicated below.

2.3 Microarray

Gene expression was investigated by Whole Human Genome Oligo Microarrays 4x44k G4112F (Agilent Technologies, Palo Alto, CA, USA) containing 4 arrays with 45220 60-mer oligonucleotide probes representing 34392 known and 6608 unknown human
transcripts. A reference design was adopted using as reference a pool of all the RNA samples from the wild-type clones. Non-reference samples were labeled with Cy3 and the reference sample with Cy5.

Total RNA was extracted and DNase purified with PerfectPure RNA Cultured Cell Kit (5 PRIME) (Eppendorf, Hamburg, Germany). All RNAs, measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, Del, USA), displayed a 260/280 OD ratio > 1.9. The RNA integrity was verified by 1.2% agarose-formaldehyde gel electrophoresis.

Total RNA samples were amplified and labeled with the Quick-Amp Labeling kit (Agilent Technologies, Palo Alto, CA, USA). One hundred µl of In Situ Hybridisation Kit Plus mix (Agilent Technologies, Palo Alto, CA, USA) containing 825 ng of Cy3 -labelled aRNA (ranging from 11 to 14 Cy3 pmoles) and 825 ng of Cy5-labelled aRNA (18 Cy5 pmoles) were hybridized to each array at 65°C for 17 h under constant rotation. The arrays were then washed 1 min at RT in 6X SSPE, 0.005% TritonX-102; 1 min at 37°C in 0.06X SSPE, 0.005% Triton X-102; 30 sec at RT in Acetonitrile solution (Agilent...
Technologies, Palo Alto, CA, USA) and 30 sec at RT in Stabilization and Drying solution (Agilent Technologies, Palo Alto, CA, USA).

Microarray images were acquired by the Agilent scanner G2565BA and intensity raw data were extracted by the software Feature Extraction V10.5 (Agilent Technologies, Palo Alto, CA, USA). Data preprocessing and statistical analysis were performed by LIMMA (LInear Model of Microarray Analysis) (Smyth 2005) tool. The intensity raw data were background-subtracted by the normexp method and normalized within-arrays with the LOESS and, for each non-reference sample classes, between-arrays with the scale methods.

The contrast matrix was set to evaluate three comparisons: M1775R versus wild-type (M1775RvsWT-contrast), A1789T versus wild-type (A1789TvswT-contrast) and Mutated BRCT domain versus wild-type (MutvsWT-contrast), considering the two variants as a whole in the latter case. Statistical significance to each gene in each comparison was
assigned by B-statistic (Lonnstedt and Speed 2002) and only genes with B-statistic>0 were included.

The pathway analysis was done by Pathway-Express ((Draghici, et al. 2007) http://vortex.cs.wayne.edu/projects.htm). The Gene Ontology terms were collected by Onto-Express (Khatri, et al. 2002); http://vortex.cs.wayne.edu/projects.htm). The network of biological interactions among differentially expressed genes and relevant biological terms was observed by Coremine (http://www.coremine.com/medical).

2.4 RT-qPCR

RT-qPCR was performed by the iCycler iQ instrument (Biorad, Hercules, CA, USA) and the iQ SYBR Green Supermix (Biorad, Hercules, CA, USA). Total RNAs were reverse transcribed by the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA). PCR primers (listed in Table 1) were designed by Beacon Designer 4.0 (Premier
RT-qPCR experiments were performed according to MIQE guidelines (Bustin, et al. 2009). Primer amplification efficiency (90%-110%) and correlation coefficient (>0.98) were tested. Four housekeeping genes, ACTB (actin, beta), HPRT1 (hypoxanthine phosphoribosyltransferase 1), TBP (TATA box binding protein) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), tested for stability by geNorm (M<1.5 and V_{3/4}<0.07) (Vandesompele, et al. 2002), were used to normalize the differential expression values of target genes. The analysis was performed considering the variants separately for the M1775R vs WT- and the A1789T vs WT- contrasts, but as a whole for the Mut vs WT-contrast. One-tailed Wilcoxon signed rank test was applied to evaluate the statistical significance of results adopting a threshold of 0.05.
2.5 Western Blot

Western Blot was performed as previously reported (Guidugli, et al. 2011).

The expression of BRCA1 protein was assessed using the anti-BRCA1 monoclonal antibody Ab4 diluted 1:100 (Calbiochem-Novabiochem Corp., Gibbstown, NJ, USA) that recognizes aa 1005–1313 in the exon 11 of BRCA1.

The level of protein expression was analyzed for: GPR56 (anti-GPR56 rabbit polyclonal antibody H-93: sc-99089, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:1000 ), MRE11A (anti-MRE11A mouse monoclonal antibody 18: sc-135992, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:500); NFKB1 (anti-NFKB1 mouse monoclonal antibody E-10: sc-8414, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:100 ) and PML (anti-PML mouse monoclonal IgG2b clone 36.1-104, Upstate Biotechnology, Inc., Waltham, MA, USA, dilution 1:500).
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>actin, beta</td>
<td>F: 5'-AACGTGAACGGTGAAAGGTGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GACTTCTGTAACAGCAGTCTC-3'</td>
</tr>
<tr>
<td>HPRT1</td>
<td>hypoxanthine phosphoribosyltransferase 1</td>
<td>F: 5'-ACATCTGGAGTCTATGACATCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-TAAAACAAATGCGCCCAAAGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>F: 5'-GTGAAAGGCTGAGCTAAGC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GGTGAAGAGCGCAATGGAATC-3'</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
<td>F: 5'-GGTGTTTGTGAAAGATGAGGTCG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CCAGATAGCAGCAGGGTATGAG-3'</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>F: 5'-ACTAGGCGGTTGAATGAGGTT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-CAGTCTGAGTGCAGGAAAGG-3'</td>
</tr>
<tr>
<td>EDN1</td>
<td>endothelin 1</td>
<td>F: 5'-CCAACCATCTTCAGCCTCC-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GTCAGACACAAACACTCCCTAG-3'</td>
</tr>
<tr>
<td>EEF1E1</td>
<td>eukaryotic translation elongation factor 1 epsilon 1</td>
<td>F: 5'-TGCGGGAGGCTTTGCTCTG-3'</td>
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<td></td>
<td></td>
<td>R: 5'-CTGTGATAGCTTTGACCAATGGTGTG-3'</td>
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<td>GPR56</td>
<td>G protein-coupled receptor 56</td>
<td>F: 5'-CTACAGCCAGAAGATGGTTGACT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GCAGAAGCCAGGATGGTTGACT-3'</td>
</tr>
<tr>
<td>MRE11A</td>
<td>MRE11 meiotic recombination 11 homolog A (S. cerevisiae)</td>
<td>F: 5'-GATGATGAAATGCGGCTATG-3'</td>
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<tr>
<td></td>
<td></td>
<td>R: 5'-TGGTTGTGCTCTGAGATGC-3'</td>
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<tr>
<td>NFKB1</td>
<td>nuclear factor of kappa light polypeptide gene enhancer in B-cells 1</td>
<td>F: 5'-CCGTGGGAATGTTGAGGTC-3'</td>
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<td></td>
<td>R: 5'-TGAGAATGAGGTTGAGATTG-3'</td>
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<td>OBFC2B</td>
<td>oligonucleotide/oligosaccharide-binding fold containing 2B</td>
<td>F: 5'-GACGTGTTGAGCTACT-3'</td>
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<td></td>
<td></td>
<td>R: 5'-TGGCTCAGTTGTT-3'</td>
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<td>PML</td>
<td>promyelocytic leukemia</td>
<td>F: 5'-CCAAGGCAGTCTCACAC-3'</td>
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<tr>
<td></td>
<td></td>
<td>R: 5'-TTCGGCCATCTGAGCTTCC-3'</td>
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<tr>
<td>SOD2</td>
<td>superoxide dismutase 2, mitochondrial</td>
<td>F: 5'-GGGTCCAAGGCTAGGTTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: 5'-GTCCTCACCACATCAATCCC-3'</td>
</tr>
</tbody>
</table>

Table 1: Primer sequences.
3. Results

3.1 Microarray results

The M1775R vs WT-contrast showed 201 differentially expressed genes, 129 downregulated and 72 upregulated, while the A1789T vs WT-contrast showed 313 differentially expressed genes, 128 downregulated and 185 upregulated and the Mut vs WT-contrast showed 173 differentially expressed genes, 100 downregulated and 73 upregulated. Twenty-four of these genes were differentially expressed with the same fold change direction in all the comparisons (Fig. 1).

Complete information about the microarray experiments and results can be retrieved from the ArrayExpress database at the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/arrayexpress/) by using the following accession number: E-MTAB-761.
Fig. 1: Venn diagram showing the numbers of differentially expressed genes shared by the three comparisons.

Pathway analysis mapped 40 genes in 58 KEGG pathways for M1775R vs WT-contrast, 52 genes in 62 KEGG pathways for A1789T vs WT-contrast and 27 genes in 37 KEGG pathways for Mut vs WT-contrast. In
all the three comparisons many pathways with high impact factor were involved in cancer.

Twenty-eight pathways were in common among the three comparisons as indicated in Fig. 2.

![Venn diagram showing the numbers of pathways shared by the three comparisons.](image)

Coremine identified 3594 and 2045 genes linked to biological terms concerning “Cell Proliferation” and “DNA damage and repair” processes, respectively.
Intersections among these two lists and the three lists of differentially expressed genes are shown in Fig. 3.
Fig. 3: Intersections among the lists of differentially expressed genes and “Cell Proliferation” (a) and “DNA damage and repair” (b).
3.2 Microarray data validation

The differential expression of nine transcripts (Table 1) among those identified by microarray analysis was validated by RT-qPCR: CDKN1A (cyclin-dependent kinase inhibitor 1A (p21, Cip1)), EDN1 (endothelin 1), EEF1E1 (eukaryotic translation elongation factor 1 epsilon 1), GPR56 (G protein-coupled receptor 56), MRE11A (MRE11 meiotic recombination 11 homolog A (S. cerevisiae)), NFKB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), OBFC2B (oligonucleotide/oligosaccharide-binding fold containing 2B), PML (promyelocytic leukemia) and SOD2 (superoxide dismutase 2, mitochondrial) (Fig. 4).

The differential expression was consistently confirmed for all the thirteen validations.

The differential expression of GPR56, MRE11A, NFKB1 and PML proteins was also confirmed by Western Blot analysis.
Fig. 4: Microarray and RT-qPCR log2-Fold changes for the 9 validated genes. All the log2-Fold changes are statistically significant (p-value<0.05).
4. Discussion

Aim of this study was to analyze the effects on human cell transcriptome of two *BRCA1* missense variants located in the BRCT domain of the protein, M1775R and A1789T. Specifically, the gene expression profiles of HeLa cells transfected with one or the other variant were compared with that of HeLa cells transfected with *BRCA1* wild-type. We also analyzed the combined outcome of the two BRCT mutations in comparison to the wild-type. To this purpose, three different statistical contrasts were performed: M1775R *versus* wild-type (M1775RvsWT-contrast), A1789T *versus* wild-type (A1789TvsWT-contrast) and Mutated BRCT domain *versus* wild-type (MutvsWT-contrast) (see Materials and methods). Pathway analysis retrieved many pathways involved in cancer onset and progression as well as linked to specific tumors. Ontological and data-mining analyses highlighted three functional categories: cell cycle regulation, apoptosis and DNA damage response and repair, typically deregulated in cancer cells. Cell cycle and apoptosis deregulation leads to aberrant cell
proliferation, while an impaired DNA damage response and repair causes genomic instability. All these processes are closely connected, as apoptosis, constituting a defense from anomalous proliferation, is linked to cell cycle block and is activated in response to DNA damage.

4.1 Aberrant cell proliferation

Cancer cells abnormally proliferate. In these cells occurs overexpression of mitogenic factors, such as cell cycle positive regulators, as well as impairment of mechanisms ensuring correct cell division, including apoptosis, as reviewed by Strobl et al., Zafonte et al. and Vermeulen et al., among many (Strobl, et al. 1995; Vermeulen, et al. 2003; Zafonte, et al. 2000).

In our data, a considerable number of differentially expressed genes is strictly linked to cell proliferation (Table 2).
<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Contrast</th>
<th>log2 (Fold Change)</th>
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<tbody>
<tr>
<td>Cell cycle arrest impairment</td>
<td>CDKN1A</td>
<td>cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>M1775R vs WT</td>
<td>-0.3066647</td>
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<td>CEBPA</td>
<td>CCAAT/enhancer binding protein (C/EBP), alpha</td>
<td>M1775R vs WT, Mut vs WT</td>
<td>-0.3728651, -0.3190284</td>
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<td>SMAD3</td>
<td>SMAD family member 3</td>
<td>A1789T vs WT, M1775R vs WT, Mut vs WT</td>
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<td>CCND1</td>
<td>cyclin D1</td>
<td>A1789T vs WT</td>
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<td>RASSF1</td>
<td>Ras association (RalGDS/AF-6) domain family member 1</td>
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<td>Cell proliferation enhancement</td>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
<td>A1789T vs WT, M1775R vs WT, Mut vs WT</td>
<td>0.4515777, 0.4020256, 0.4365775</td>
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<td>DUSP1</td>
<td>dual specificity phosphatase 1</td>
<td>A1789T vs WT, M1775R vs WT, Mut vs WT</td>
<td>0.3844494, 0.7606655, 0.5060076</td>
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<td>DUSP2</td>
<td>dual specificity phosphatase 2</td>
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<td>EDN1</td>
<td>endothelin 1</td>
<td>M1775R vs WT, Mut vs WT</td>
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<td>SKP1</td>
<td>S-phase kinase-associated protein 1</td>
<td>A1789T vs WT</td>
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<td>GPR56</td>
<td>G protein-coupled receptor 56</td>
<td>A1789T vs WT, M1775R vs WT, Mut vs WT</td>
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<td>Apoptosis blocking</td>
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<td>TNFRSF10B</td>
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<td>DYRK2</td>
<td>dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2</td>
<td>M1775R vs WT</td>
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<td>PLEKHF1</td>
<td>pleckstrin homology domain containing, family F (with FYVE domain) member 1</td>
<td>Mut vs WT</td>
<td>-0.2374774</td>
</tr>
</tbody>
</table>

Table 2: Genes linked to aberrant cell proliferation.
4.1.1 Cell cycle arrest impairment

*CDKN1A*, downregulated by M1775R, is a main effector of cell cycle arrest in response to DNA damage and a promoter of apoptosis (Cazzalini, et al. 2010). Its expression is usually activated by BRCA1 (Chai, et al. 1999).

Cell cycle can be also arrested by the cooperation of *CDKN1A* with *CEBPA* (Harris, et al. 2001) that is in turn downregulated by M1775R.

*CDKN1A* expression is normally activated also by *SMAD3*, a known transcription factor that acts as an effector of the TGF-beta pathway (Moustakas and Kardassis 1998; Pardali, et al. 2005), downregulated in all the three comparisons. The overexpression of *SMAD3* in a breast cancer cell line causes cell cycle arrest (Tian, et al. 2003), while in *SMAD3*-/ mammary epithelial cells, both TGF-beta-induced growth inhibition and apoptosis are lost (Kohn, et al. 2010).

*SMAD3* also contributes to the 3-indole-induced G1 arrest in cancer cells (Huang, et al. 2011) and its inhibition depends on CCND1-CDK4 (cyclin-dependent
kinase 4) action in breast cancer cells overexpressing **CCND1** (Zelivianski, et al. 2010), upregulated by A1789T. The loss or reduction of BRCA1 expression, moreover, significantly reduces the TGF-beta induced activation of SMAD3 in breast cancer cells (Li, et al. 2009).

Considering this, the downregulation of **CDKN1A**, **CEBPA** and **SMAD3** might constitute a key element in carcinogenesis predisposition caused by the considered variants. The downregulation of other four genes further supports the hypothesis of a deficiency in cell cycle arrest: **PML** (in M1775RvsWT) which codifies for a phosphoprotein localized in nuclear bodies, involved in TP53 (tumor protein p53) - mediated cell cycle arrest in G1 (Chan, et al. 1997) as well as in recognition and/or processing of DNA breaks by recruiting TP53 and MRE11A (Carbone, et al. 2002); **RUVBL1** (in M1775RvsWT) that encodes a highly conserved ATP-dependent DNA helicase which is part of chromatin-remodeling complexes and plays a role in apoptosis and DNA repair (Ikura, et al. 2000; Jha, et al. 2008; Makino, et al. 1998); **TXNIP** (in A1789TvsWT) that encodes a transcriptional repressor acting as a tumor suppressor, as its transfection
induces cell-cycle arrest in G0/G1 phase and is downregulated in human tumors (Han, et al. 2003) and \textit{RASSF1} (in A1789T vs WT), a tumor suppressor that blocks cell cycle progression by inhibiting CCND1 accumulation. It is epigenetically inactivated at high frequency in a variety of tumors, including breast cancer (Burbee, et al. 2001; Shivakumar, et al. 2002).

\subsection*{4.1.2 Cell proliferation enhancement}

The transcription factor \textit{FOS}, upregulated in all the three comparisons, is a well known protooncogene that positively regulates cell cycle progression (Shaulian and Karin 2001) and is induced in human breast cancer cell cultures, being part of the mitogenic signal transduction typical of these cells, as reviewed by Strobl et al. (Strobl, et al. 1995).

\textit{DUSP1}, upregulated in all the three comparisons, and \textit{DUSP2}, upregulated in Mut vs WT, belong to a subfamily of tyrosine phosphatases that regulate the activity of Mitogen-Activated Protein Kinases (MAPKs). MAPKs are key effectors for cell growth control and survival in physiological and pathological conditions, including cancer. DUSPs have therefore
been proposed as potential targets for anticancer drugs, as reviewed by Nunes-Xavier et al. (Nunes-Xavier, et al. 2011). DUSP1 inhibits apoptosis in human mammary epithelial and breast carcinoma cells (Small, et al. 2004). Moreover, DUSP1 expression was found upregulated in many breast cancers (Wang, et al. 2003). The overexpression of DUSP2 in ovarian cancers has been correlated with poor outcome (Givant-Horwitz, et al. 2004).

**EDN1**, upregulated by M1775R and in MutvsWT, is a vasoconstrictor that has also co-mitogenic activity, potentiating the growth factors effects. Altered EDN1 signalling is involved in carcinogenesis by modulating cell survival and promoting invasiveness (Bagnato and Rosanò 2008).

**SKP1**, upregulated by A1789T, is a component of the SCF complex that mediates the ubiquitination of cell cycle proteins promoting cell cycle progression (Bassermann and Pagano 2010).

**ZWILCH**, upregulated by A1789T, is an essential component of the mitotic checkpoint that prevents cells from exiting mitosis prematurely (Kops, et al. 2005; Williams and Glover 2003).
**GPR56**, downregulated in all the three contrasts, is a G protein-coupled receptor involved in adhesion processes that participates in cytoskeletal signaling, cellular adhesion and tumor invasion. It is downregulated in melanoma cell lines, while its overexpression suppresses tumor growth and metastasis (Xu, et al. 2006).

### 4.1.3 Apoptosis blocking

**NFKB1**, downregulated by M1775R, is a pleiotropic transcription factor involved in many biological processes like inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. Whether NFKB activation contributes or not to cancer is controversial, as reviewed by Shishodia and Aggarwal (Shishodia and Aggarwal 2004), as it regulates the expression of both antiapoptotic (Rayet and Gélinas 1999) and proapoptotic genes (Kühnel, et al. 2000; Shetty, et al. 2005).

Interestingly, **TNFRSF10B**, that was in turn downregulated by M1775R, is one of the proapoptotic genes upregulated by NFKB (Shetty, et al. 2005). TNFRSF10B is one of the two apoptosis-activating receptors binding the receptor TNFSF10 (tumor
necrosis factor (ligand) superfamily, member 10) (Sheridan, et al. 1997). Upon binding, an adaptor protein called FADD (Fas(TNFRSF6)-Associated via Death Domain) is recruited to the death receptors, forming a signaling complex that leads to apoptosis through caspases activation (Suliman, et al. 2001). The downregulation of both *NFKB1* and *TNFRSF10B* in our data suggests an inhibition of TNF-mediated apoptosis.

Two other proapoptotic genes, *DYRK2* and *PLEKHF1*, resulted as downregulated in our data. DYRK2, downregulated by M1775R, is a protein kinase that regulates TP53 to induce apoptosis in response to DNA damage downstream of ATM (Taira, et al. 2007), and PLEKHF1, downregulated in MutvsWT, is a recently discovered lysosome-associated protein that activates caspase-independent apoptosis (Chen, et al. 2005) by interacting with the TP53 transactivation domain (Li, et al. 2007).
4.2 Genomic instability

An improper reaction to genotoxic stress causes genomic instability, leading to tumorigenesis. Deficiencies in DNA damage signaling and repair pathways are thus fundamental to the etiology of cancer (Khanna and Jackson 2001).

A number of differentially expressed genes takes part in genotoxic stress response. Among these genes, some were downregulated causing an increase in genomic instability, while others were upregulated (Table 3). Many tumors, including BRCA1-deficient breast cancers, show an overexpression of genes linked to DNA repair that correlates with chemoresistance and poor prognosis (Martin, et al. 2007; Saviozzi, et al. 2009). Moreover, an increased nuclear staining of DNA repair proteins has been recently observed in tissue sections of breast cancers carrying the M1775R mutation, suggesting a new mechanism of tumorigenesis involving an enhance of homologous recombination (Dever, et al. 2011).
<table>
<thead>
<tr>
<th>Biological Process</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Contrast</th>
<th>log2 (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA damage response and repair downregulation</td>
<td>EEF1E1</td>
<td>eukaryotic translation elongation factor 1 epsilon 1</td>
<td>A1789T vs WT</td>
<td>-0.4309041</td>
</tr>
<tr>
<td></td>
<td>SMC1A</td>
<td>structural maintenance of chromosomes 1A</td>
<td>A1789T vs WT</td>
<td>-0.2754507</td>
</tr>
<tr>
<td></td>
<td>PPP1CC</td>
<td>protein phosphatase 1, catalytic subunit, gamma isozyme</td>
<td>A1789T vs WT</td>
<td>-0.4286825</td>
</tr>
<tr>
<td></td>
<td>AHNAK</td>
<td>AHNAK nucleoprotein</td>
<td>A1789T vs WT</td>
<td>-0.3988113</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M1775R vs WT</td>
<td>-0.3103867</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs WT</td>
<td>-0.3940570</td>
</tr>
<tr>
<td></td>
<td>SOD2</td>
<td>superoxide dismutase 2, mitochondrial</td>
<td>M1775R vs WT</td>
<td>-0.3376169</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs WT</td>
<td>-0.2502831</td>
</tr>
<tr>
<td>DNA damage response and repair upregulation</td>
<td>MRE11A</td>
<td>MRE11 meiotic recombination 11 homolog A (S. cerevisiae)</td>
<td>A1789T vs WT</td>
<td>0.3293561</td>
</tr>
<tr>
<td></td>
<td>TERF1</td>
<td>telomeric repeat binding factor (NIMA-interacting) 1</td>
<td>Mut vs WT</td>
<td>0.2790907</td>
</tr>
<tr>
<td></td>
<td>OBFC2A</td>
<td>oligonucleotide/oligosaccharide-binding fold containing 2A</td>
<td>M1775R vs WT</td>
<td>0.3666172</td>
</tr>
<tr>
<td></td>
<td>OBFC2B</td>
<td>oligonucleotide/oligosaccharide-binding fold containing 2B</td>
<td>A1789T vs WT</td>
<td>0.4070777</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mut vs WT</td>
<td>0.3417360</td>
</tr>
</tbody>
</table>

Table 3: Genes linked to genomic instability.

### 4.2.1 DNA damage response and repair downregulation

**EEF1E1**, downregulated by A1789T, first discovered as associated with a macromolecular tRNA synthetase complex, is a key factor for ATM/ATR-mediated TP53 activation in response to DNA damage (Park, et al. 2005).

**SMC1A**, downregulated by A1789T, encodes an evolutionarily conserved chromosomal protein, component of the cohesin complex (Sumara, et al. 2000). It is involved in the ATM/NBN-dependent S-
phase checkpoint pathway, associating with BRCA1 and being phosphorylated in response to ionizing radiations (Yazdi, et al. 2002).

**PPP1CC**, downregulated by A1789T, is the catalytic subunit of the gamma isoform of PP1 which is a component of a signaling complex, PPP1R1A/PPP1R15A/PPP1CC (protein phosphatase 1, regulatory (inhibitor) subunit 1A, protein phosphatase 1, regulatory subunit 15A, protein phosphatase 1, catalytic subunit, gamma isozyme) that positively regulates apoptosis in response to various stresses, including growth arrest and DNA damage (Connor, et al. 2001).


**SOD2**, downregulated by M1775R and in MutvsWT, is a member of the iron/manganese
superoxide dismutase family that acts as a free radical scavenger, thus protecting from oxidative damage. It is a candidate tumor suppressor gene as the loss of heterozygosity of its region on chromosome 6 has been found in about 40% of human malignant melanomas (Oberley and Oberley 1997) and the deletion of chromosome 6 long arm has been identified in SV40 transformed human fibroblast (Bravard, et al. 1992). In addition, SOD2 overexpression suppresses the tumorigenicity of breast cancer cells (Li, et al. 1995).

4.2.2 DNA damage response and repair upregulation

*MRE11A*, upregulated by A1789T, encodes a component of the BASC that specifically promotes non-homologous end-joining (see Introduction) (Wang, et al. 2000; Zhang and Powell 2005). The nuclease activity of MRE11A is required for non-homologous end-joining mediated by the MRN complex (see Introduction), and, interestingly, BRCA1 suppresses this activity *in vitro* (Paull, et al. 2001). Interestingly, the A1789T variant altered the non-

**TERF1**, upregulated in MutvsWT, is a telomere-associated protein, member of the telomere nucleoprotein complex that interacts with various macromolecular complexes including MRN (Kuimov 2004).

**OBFC2A**, upregulated by M1775R, and **OBFC2B**, upregulated by A1789T and in MutvsWT, encode single-stranded DNA-binding proteins essential for a variety of DNA metabolic processes, including replication, recombination and damage detection and repair. OBFC2B, in particular, as an early participant in DNA damage response, is critical for genomic stability (Richard, et al. 2008).
5. Concluding remarks

This work was focused on two BRCA1 BRCT missense variants, M1775R and A1789T, both isolated from familial breast cancers. M1775R has widely been described as deleterious (Kawai, et al. 2002; Miki, et al. 1994; Nikolopoulos, et al. 2007; Olopade, et al. 2003; Varma, et al. 2005; Williams and Glover 2003), while A1789T has been studied only by our group. In yeast cells both of these mutations reverted the growth suppression (small colony) phenotype (Caligo, et al. 2009), showing a characteristic behaviour of cancer-predisposing missense BRCA1 BRCT mutations (Coyne, et al. 2004). M1775R also induced homologous recombination in yeast cells (Caligo, et al. 2009). In HeLa cells A1789T significantly altered the non-homologous end-joining activity as compared to BRCA1 wild-type (Guidugli, et al. 2011).

As previously observed in yeast cells (Di Cecco, et al. 2009), also in human cells the BRCA1 variants M1775R and A1789T affect the expression of many genes critical for cell proliferation and genome integrity maintenance. Our results represent a
molecular confirm of the pathogenetic role of M1775R and give support to a similar role of A1789T that we first hypothesized on the basis of the experiments in yeast cells.
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