BRIEF COMMUNICATION

Altered Expression of Telomere-Associated Genes in Leukocytes among BRCA1 and BRCA2 Carriers†

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Abstract

Telomere dysfunction resulting from telomere shortening and deregulation of shelterin components has been linked to the pathogenesis of age-related disorders, including cancer. Recent evidence suggests that BRCA1/2 (BRCA1 and BRCA2) tumor suppressor gene products play an important role in telomere maintenance. Although telomere shortening has been reported in BRCA1/2 carriers, the direct effects of BRCA1/2 haploinsufficiency on telomere maintenance and predisposition to cancer development are not completely understood. In this study, we assessed the telomere-associated and telomere-proximal gene expression profiles in peripheral blood leukocytes from patients with a *BRCA1* or *BRCA2* mutation, compared to samples from sporadic and familial breast cancer individuals. We found that 25 genes, including *TINF2* gene (a negative regulator of telomere length), were significantly differentially expressed in *BRCA1* carriers. Leukocyte telomere length analysis revealed that BRCA1/2 carriers had relatively shorter telomeres than healthy controls. Further, affected *BRCA1/2* carriers were well differentiated from unaffected *BRCA1/2* carriers by the expression of telomere-proximal genes. Our results link BRCA1/2 haploinsufficiency to changes in telomere length, telomere-associated as well as telomere-proximal gene expression. Thus, this work supports the effect of BRCA1/2 haploinsufficiency in the biology underlying telomere dysfunction in cancer development. Future studies evaluating these findings will require a large study population. This article is protected by copyright. All rights reserved

Introduction

Multiple studies suggest that the "one-hit" effects of *BRCA1/2* (*BRCA1* and *BRCA2*) occur even before loss of the remaining wild-type allele and precede development of cancer (so called "haploinsufficiency") [1-5]. For instance, cultured epithelial cells from heterozygous carriers of *BRCA1/2* mutations exhibit genomic instability characterized by gene copy number loss and decreased homology-directed repair capacity *in vitro* [5]. Other work suggests that this genomic instability may be due to deregulation of genes involved in the G_2/M cell cycle transition and DNA damage response in *BRCA1* heterozygous mutant cells, while *BRCA2* heterozygous mutant cells show deregulation of genes involved in the G_1/S cell cycle checkpoint [6-8]. Interestingly, *BRCA1*, but not *BRCA2*, heterozygous epithelial cultured cells show upregulation of the secretoglobin family of genes and expression profiles similar to those seen in stem and progenitor cells [1]. This finding corroborates a study suggesting a role for BRCA1 in regulating stemness and differentiation in breast progenitor cells [9]. A separate study demonstrated increased proliferation and clonogenic capacity, coupled with epidermal growth factor receptor (EGFR) activation, in primary mammary epithelial cells from *BRCA1* mutation carriers [10].

Telomere dysfunction has been hypothesized to account for the unstable phenotype in cells derived from heterozygous *BRCA1/2* mutation carriers [4,11-14]. Telomere dysfunction including telomere shortening has been linked to a variety of human age-related ailments, including cardiovascular disease, neurodegenerative disease, chronic inflammatory disease, and cancer [15]. An analysis of case control studies revealed an association between short telomeres in peripheral blood leukocytes (PBL) and elevated risk for cancers, including bladder, head and neck, lung, and kidney cancers [16-22]. Genetic anticipation, or earlier age of onset and

sometimes severity of disease in successive generations, has been attributed to telomere shortening in familial cancer syndromes such as Li-Fraumeni and dyskeratosis congenita [23,24]. An association has been reported between genetic anticipation and shortened telomeres in hereditary breast cancers with BRCA1/2 mutations, but not in sporadic breast cancers [14]. A similar study of ovarian cancer found significantly shorter PBL telomeres in both sporadic and hereditary cases compared to healthy controls by the same group [25]. Such studies are largely association-based, and the factors driving telomere shortening and their contributions to disease development remain largely unknown. Therefore, efforts to shed light on the mechanisms driving telomere dysfunction in cancer, though limited, have offered helpful clues.

To understand the predisposition of BRCA1/2 carriers to cancer development, this pilot study focused on identifying genes that contribute to telomere dysfunction in hereditary cancer with BRCA1/2 mutations by comparing to other breast cancers (i.e., familial and sporadic cancers). In addition, we determined the association between leukocyte telomere length and expression of telomere-proximal genes in BRCA1/2 mutation carriers. This study sheds light on the distinct role between BRCA1 and BRCA2 in telomere maintenance, as well as, provides insight into further investigations on the role of BRCA1/2 haploinsufficiency in the biology underlying telomere dysfunction in cancer development.

Materials and Methods

Sample Collection

All BRCA1/2, sporadic, and familial blood samples were obtained from Indiana University Simon Cancer Center (IUSCC), along with the approved Institutional Review Board (IRB) protocol (IRB #1011003798); 10 mL of peripheral blood was collected from total 40 women (BRCA1, n = 10; BRCA2, n = 10; hereditary breast cancer without BRCA1/2, n = 9; and sporadic breast cancer, n = 11). Five BRCA1 and 4 BRCA2 samples were from women who had not developed cancer. We also obtained freeze-dried PBL DNA of 10 healthy women (25<age<70, median age = 49) from the Komen Tissue Bank at the IUSCC, along with IRB approval (IRB # 1206009001).

Gene array analysis

The high-quality total RNA from blood samples was purified using the QIAamp RNA blood kit (Qiagen) according to manufacturer's instructions. The Whole-Genome DASL Assay was used intact total RNAs in concentrations ranging from 20 – 100 ng/µl. Following RNA extraction and quality assessment, Illumina® Whole-Genome DASL™ microarray (Human Ref-8 BeadChips) analysis was performed by Indiana University School of Medicine core facility. The raw data was normalized and analyzed using Partek® Genomic Suite. Differentially expressed genes were identified using ANOVA analysis. The Indiana University Bioinformatics Core conducted the gene array analysis and assisted with identification of differentially expressed genes. To identify altered expression in subtelomeric genes associated with telomere shortening, we compared our microarray data with the GSE6799 dataset from the public Gene Expression Omnibus repository,

as first described in Lou et al. 2009 [26]. Additionally, DAVID 6.8 bioinformatics resource was used for grouping genes based on functional similarity in the data [27].

Telomere length measurement

Genomic DNA was extracted from PBL cells using the DNeasy blood and tissue kit **(**Qiagen, #69504). DNA quality was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). For the southern blotting, TeloTAGGG assay was used according to the manufacturer's protocol (Roche, # 12 209 136 001). Mean telomere length was calculated using ImageQuant software and TELORUN Excel spreadsheet program [28]. For telomere qPCR, singleplex qPCR was performed for each sample using telc/telg and hbgu/hbgd primers [29,30]. A five-point standard curve (3-fold serial dilutions from 50 to 0.62 ng) of normal female diploid DNA (Promega, #G1521) was included on all plates. A relative measurement of the telomere length of each sample was calculated based on the standard curves by dividing the quantity of telomeric DNA (T) by the quantity of single-copy-gene DNA (S). The reference DNA has a T/S ratio of 1 (7.9 – 8.2 kb in telomere length determined by southern blot analysis). All samples were run in triplicate three times and the average Ct was used for the analysis. Control male diploid DNA (Promega, #G1471) was run on all plates to assess the plate-to-plate variations. Through the analyses, *P*-values < 0.05 were considered significant.

Quantitative Reverse Transcription PCR (qRT-PCR)

mRNA was extracted using the TRIzol reagent (Thermo Fisher Scientific, #15596026). cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, #4368814). qPCR was performed with SYBR Select Master mix (Thermo Fisher Scientific, #4472908) on a QuantStudio 6 Flex real-time PCR system (Applied Biosystems).

Each sample was tested in triplicate in three independent experiments. Relative mRNA level was analyzed by the comparative Ct method using *ACTB* as a reference gene and cDNA generated from blood of healthy woman as the reference cDNA. All primers sequences were shown in Supplement Table S4. Through the PCR analysis, *P*-values < 0.05 were considered significant.

Statistical analysis

Unsupervised hierarchical clustering was used to construct heat maps by the Indiana University Bioinformatics Core.

Results and Discussion

Telomere-associated genes were deregulated among BRCA1 and BRCA2 carriers

We collected peripheral blood leukocytes (PBL) from 10 individuals who have a BRCA1 mutation (i.e., BRCA1 carrier), 10 individuals who have a BRCA2 mutation (i.e., BRCA2 carrier), 9 familial cancer patients (with no identified BRCA1/2 mutation but a family history of breast cancer), and 11 sporadic cancer patients. Characteristics of subjects used in this study are shown in Supplemental Table S1. Using mRNA purified from these PBL samples, we first performed the Illumina whole-genome DASL® (cDNA-mediated Annealing, Selection, Extension and Ligation) gene expression assay to identify any differently expressed telomere-associated genes that play a role similar to telomere-binding proteins in diverse ways. Unsupervised hierarchical clustering analysis revealed that there were three distinct clusters of gene expression among telomere-associated genes: (i) those upregulated in both BRCA1 and BRCA2 carriers (ii) those upregulated only in BRCA1 carriers, and (iii) those not differentially expressed among BRCA1/2 carriers and sporadic individuals (Figure 1A). Based on *P*-value

between groups, we found a total of 46 differentially expressed genes in BRCA1 carriers compared with all other individuals (i.e., BRCA2 carriers, familial, and sporadic individuals) (Supplemental Table S2). One of two downregulated genes, *IGFBP2* gene encodes a protein of insulin-like growth factor binding protein 2. BRCA1 has been shown to directly interact with the insulin-like growth factor signaling and the *IGFBP2* gene has been reported as a potential disease modifier in BRCA2 carriers [31]. The other downregulated gene, *SERPINE1* gene's product interacts with p53 and is also likely involved in the urokinase-type plasminogen-mediated signaling pathway in breast cancer [32,33]. Based on *P*-values, the top three hits were the *PPP2R5C, MX1,* and *TINF2* genes. The *PPP2R5C* gene product belongs to the phosphatase 2A (PP2A) regulatory subunit B family (1.43-fold change) and has the capability to interact with BRCA1 [34]. The *MX1* gene encodes for interferon-induced GTP-binding protein and was previously reported as a potential downstream gene regulated by BRCA1 (1.40-fold change) [35]. Notably, the *TINF2* gene, which encodes for a component of the shelterin protein complex TIN2, was upregulated with a fold change of 1.54.

We next compared the above 46 genes by the resulting differential gene list from the comparison between BRCA1/2 carriers *vs*. non-BRCA1/2 carriers, 25 out of the 46 differentially regulated genes were noted to be specifically associated with the BRCA1 carriers (Figure 1B and Table 1). According to DAVID 6.8, the top three deregulated functional annotation charts were: (i) telomere/chromosome maintenance, (ii) cell cycle, and (iii) DNA binding (Supplemental Figure S1). qRT-PCR confirmed that the genes with the highest fold changes (*TINF2, MX1*, and *PPP2CB*) and plasminogen activator inhibitor-encoding gene (*SERPINE1*) were differentially expressed between BRCA1 and BRCA2 individuals (Figure 1C). The *PPP2CB* gene encodes a

component of PP2A, one of four major serine/threonine phosphatases [36,37]. Because PP2A regulates autophosphorylation of ATM [38], altered gene expression of PP2A subunits could affect the telomere damage signaling pathway. When BRCA2 carriers were compared with all other groups, only 8 genes were identified as being significantly downregulated in BRCA2 carriers (Supplemental Table S3).

Telomere length was shorter in BRCA1/2 carriers

Because the microarray analysis revealed differential expression of negative regulators of telomere length (*TINF2, TERF1, and TRER2*), between BRCA1 and BRCA2 carriers, we hypothesized that the altered expression of these genes would affect telomere length among those individuals. The telomere qPCR results did not indicate significant differences in leukocyte telomere length between BRCA1 and BRCA2 carriers (Figure 2A, $P = 0.24$); instead, we observed that both two carriers had relatively shorter telomeres compared to healthy controls (Figure 2B, $P = 3.9 \times 10^{-7}$). Because the telomere-related expression profiles were different between BRCA1 and BRCA2 carriers, we speculate that these two carriers could undergo different molecular processes (or pathways) to cause telomere shortening. It was reported that BRCA1 and BRCA2 tumor suppressor gene products play fundamentally distinct roles in overlapping biological processes: BRCA2 functions to load RAD51 recombinase at the sites of DNA damage, while BRCA1 is required during the initial steps of the DNA damage signal amplification [39-42]. Another distinction was observed that a majority of BRCA1 breast tumors were basal-like while BRCA2 breast tumors were mainly luminal B [43,44]. Tumors with BRCA1 mutations are generally negative for both estrogen and progesterone receptors, whereas most tumors with BRCA2 mutations are positive for these hormone receptors. These differences

may facilitate our understanding of how *BRCA1* and *BRCA2* mutations induce separate molecular pathways through telomere shortening (or loss) and contribute to breast cancer development.

Our findings support a previous report [14] that BRCA1/2 germline mutation carriers results in shorter overall telomere length in PBL at baseline (Figure 2B, $P = 8.0 \times 10^{-5}$). In addition, Sedic et al. reported that human mammary epithelial cells (HMECs) from BRCA1-mutation carriers (BRCA1mut/+) exhibited increased genomic instability and rapid telomere erosion [45]. However, other previous studies reported longer telomeres in PBL from BRCA1/2 carriers, or no association was found [46,47]. These distinct conclusions are suspected to be due, in part, to the different analysis procedures. For example, Cawthon originally reported that telomere qPCR was analyzed by the standard curve method [30], while most epidemiological telomere studies use the comparative C_t method. Moreover, each study uses a different control DNA, and the detailed information was often not described well in each report.

We also compared telomere length in unaffected BRCA1/2 carriers with affected BRCA1/2 carriers and found a trend that affected BRCA carriers had shorter telomeres than unaffected BRCA1/2 carriers (Figure 2B, $P = 0.062$). Although the *P*-value was not significant due to the small sample size, this trend may explain that hematopoietic stem-like cells suffer telomere damage in the course of disease development as well as during therapy. In addition, leukocyte telomere homeostasis could be influenced by additional environmental factors (e.g., immunologic response to cancer) during carcinogenesis. In this case, leukocyte telomere length may remain short, even among BRCA1/2 patients in clinical remission. To rule out the

possibility of technical bias against the qPCR-based results, we carried out classical southern bot analysis using the same DNA samples (Figure 2C). The results from the southern blot analysis were highly correlated with the results from telomere length qPCR (P = 0.022), indicating that the variation between two different methods was relatively small and there was no technical bias in this study (Figure 2D).

Genes within one megabase of the telomere were expressed differently between affected and unaffected BRCA1/2 carriers.

Next, we determined whether expression of subtelomeric genes was associated with telomere shortening. To do this, we compared our microarray data with the GSE6799 dataset [26]. The dataset contains 1,323 subtelomeric (within 1 Mb from telomere) genes representing all telomere ends, along with 92 random control genes, 12 housekeeping genes and 198 other telomere-associated genes. Using this dataset, it has been reported that expression of three genes, *ISG15*, *DSP*, and *C1S*, located at three different subtelomeric ends (chromosome 1p, 6p, and 12p, respectively) were increased in fibroblasts and myoblasts with telomere shortening [26,48]. In general, we found that clusters of upregulated genes were more evident in BRCA1/2 carriers as compared to sporadic or familial individuals (Supplemental Figure S2). When we compared genes in the GSE6799 dataset with BRCA1 individuals who had developed breast cancer *versus* those who had not, twenty genes were deregulated in affected BRCA1 carriers (n = 5) compared to unaffected BRCA1 carriers (n = 5). Almost all of them (19 out of 20 genes) were downregulated and only *SERPINE1* gene expressed highly in affected BRCA1 carriers (Table 2). A recent study indeed reported a role of *SERPINE* gene product in longevity. The study demonstrated that carriers of the null *SERPINE1* allele had a longer life span along with longer

leukocyte telomere length [49]. Out of the 20 genes, 12 genes were localized within 1 Mb of the telomere (*blue arrowhead with asterisk* in Figure 3A). Both *ZNF10* and *CHFR* are located on chromosome 12q24.33, and both *DLL1* and *FAM120B* are located on chromosome 6q27, indicating that these subtelomeric regions could be influenced by telomere shortening [48,50]. Our qRT-PCR results confirmed that the genes analyzed were indeed downregulated in the affected BRCA1 group compared to the unaffected BRCA1 group (Figure 3B). A comparison of BRCA2 carriers who developed breast cancer *versus* those who did not develop breast cancer yielded 14 differentially expressed genes, including 10 downregulated (Table 3). Out of the 14 genes, 7 genes (*TUBB3*, *HBZ*, *ING1*, *CYP2E1*, *UBE2J2*, *TXNL4A*, and *RPL23AP82*) were localized to subtelomeric regions (*red arrowhead with asterisk* in Figure 3A). We confirmed by qRT-PCR that the genes assayed have significantly altered expression levels between the affected and the unaffected BRCA2 groups (Figure 3C). These findings suggest that altered expression of telomere-proximal genes may be associated with the differences in telomere length among affected and unaffected BRCA1/2 carriers. Although the detailed mechanism of gene downregulation at subtelomeres due to telomere shortening remains unknown, a recent report has shown that telomeric repeat-containing RNA (TERRA) are accumulated when telomeres are critically shortened [51]. Hence, we speculate that one possible mechanism of the gene downregulation might be involved in TERRA expression levels. When TERRA expression increases and is accumulated at short telomeres, TERRA might modulate the expression level of telomere-proximal (subtelomeric) genes. Whatever the case, the gene expression changes specific to BRCA1/2 carriers with telomere shortening may become potential molecular indicators for capturing the early onset of BRCA1/2 breast cancer. One of limitations in this

study is the small sample size for analysis. Therefore, further investigation is required to validate our findings with a larger sample size.

In summary, we presented significantly different gene expression patterns between BRCA1 and BRCA2 carriers. The present study is unique because we focused on identifying changes in telomere-associated genes as well as telomere-proximal genes associated with BRCA1/2 mutation status. This pilot study sheds light on previously uncharacterized differences between BRCA1/2 carriers with relation to telomere-associated genes.

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Conflict of Interest Statement

The authors declare no conflicts of interest related to this study.

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Legends to Figures

Figure 1 Telomere-associated genes express differently among BRCA1 and BRCA2 carriers.

(A) Unsupervised hierarchical clustering of expression of telomerase-associated genes reveals three distinct gene clusters. Distinct gene clusters are denoted by boxes outlined in *blue dot-line*. From left to right, the first cluster shows genes not differentially expressed among sporadic, familial, and BRCA1/2 carrier individuals. The second cluster highlights genes overexpressed in BRCA1 and BRCA2 carrier individuals, and the third cluster highlights genes overexpressed only in BRCA1 carrier individuals. Up-regulation, down-regulation, and no significant change are indicated by *red*, *green*, *black*, respectively. **(B)** Schematic diagram representing the microarray results of the differentially regulated genes in BRCA1/2 carriers. **(C)** Validation of microarray data with quantitative RT-PCR. *ACTB* (actin beta) gene was used to normalize the data. Data represent mean values ± standard deviations of the means from three independent experiments performed in triplicate. $n = 4$.

Figure 2 BRCA1/2 mutation is associated with shorter telomere length.

(A) No difference in age-adjusted telomere length between BRCA1 and BRCA2 carriers. Dash line represents the mean of relative telomere length (T/S ratio) determined by qPCR (0.73 in BRCA1, 0.62 in BRCA2). *P*-value is not significant. **(B)** Shorter telomeres in BRCA1/2 carriers than controls. Age-adjusted telomere length was plotted ($n = 10$ in each group). *Dash line* represents the mean of relative telomere length (T/S ratio) determined by qPCR (0.60 in affected BRCA1/2, 0.77 in unaffected BRCA1/2, and 1.31 in controls). **(C)** Representative southern blot of telomere length analysis. The southern blot shows telomere lengths from leukocyte of normal control (N), sporadic cancer (S), familial cancer (F), BRCA1 carrier (B1) and BRCA2 carrier (B2) individuals. kb, kilobases. **(D)** Correlation between relative T/S ratios determined by qPCR and mean TRF lengths determined by Southern blot analysis. Pearson's correlation coefficient was 0.790.

Figure 3 Telomere-proximal genes were expressed differently between affected and unaffected BRCA1/2 carriers.

(A) Genes loci of differentially expressed genes. Twenty genes (*blue arrowhead*) from GSE6799 express differently between BRCA1 individuals with cancer vs BRCA1individuals without cancer. Fourteen genes (*red arrowhead*) from GSE6799 express differently between BRCA2 individuals with cancer vs BRCA2 individuals without cancer, respectively. *Asterisk* (*) represents a gene within 1 Mb from telomere. **(B)** and **(C)** qRT-PCR of telomere-proximal genes in BRCA1 carriers (B) and BRCA2 carriers (C). *ACTB* (actin beta) gene was used to normalize the data. Data represent mean values ± standard deviations of the means from three independent experiments performed in triplicate. $n = 4$.

Table 1 Summary of 25 telomere-associated genes deregulated in BRCA1 carriers.

Gene	Fold Change	P value
TINF2	1.54	4.0E-03
MX1	1.40	3.0E-03
PPP2CB	1.38	9.5E-03
JUNB	1.38	2.1E-02
TTC21B	1.35	2.4E-02
ZNF34	1.35	3.1E-02
TERF1	1.35	4.2E-02
RPS11	1.34	2.6E-02
CHFR	1.33	8.0E-03
ZNF324	1.31	1.5E-02
TERF2	1.30	1.6E-02
BIN1	1.29	4.1E-01
FN3KRP	1.28	7.8E-03
CCND3	1.27	1.4E-02
CTDP1	1.27	3.1E-02
BARD1	1.27	1.8E-02
ZBTB45	1.26	2.8E-02
TRDMT1	1.23	3.2E-02
SIP1	1.23	2.8E-02
MFNG	1.23	3.4E-02
FYTTD1	1.22	3.8E-02
UPF3A	1.21	7.5E-03
PPP2R2A	1.16	3.8E-02
SERPINE1	-1.23	2.1E-02
IGFBP2	-1.24	3.8E-02

Table 2 Summary of 20 genes from GSE6799, which express differently in affected BRCA1 individuals compared to unaffected BRCA1 individuals.

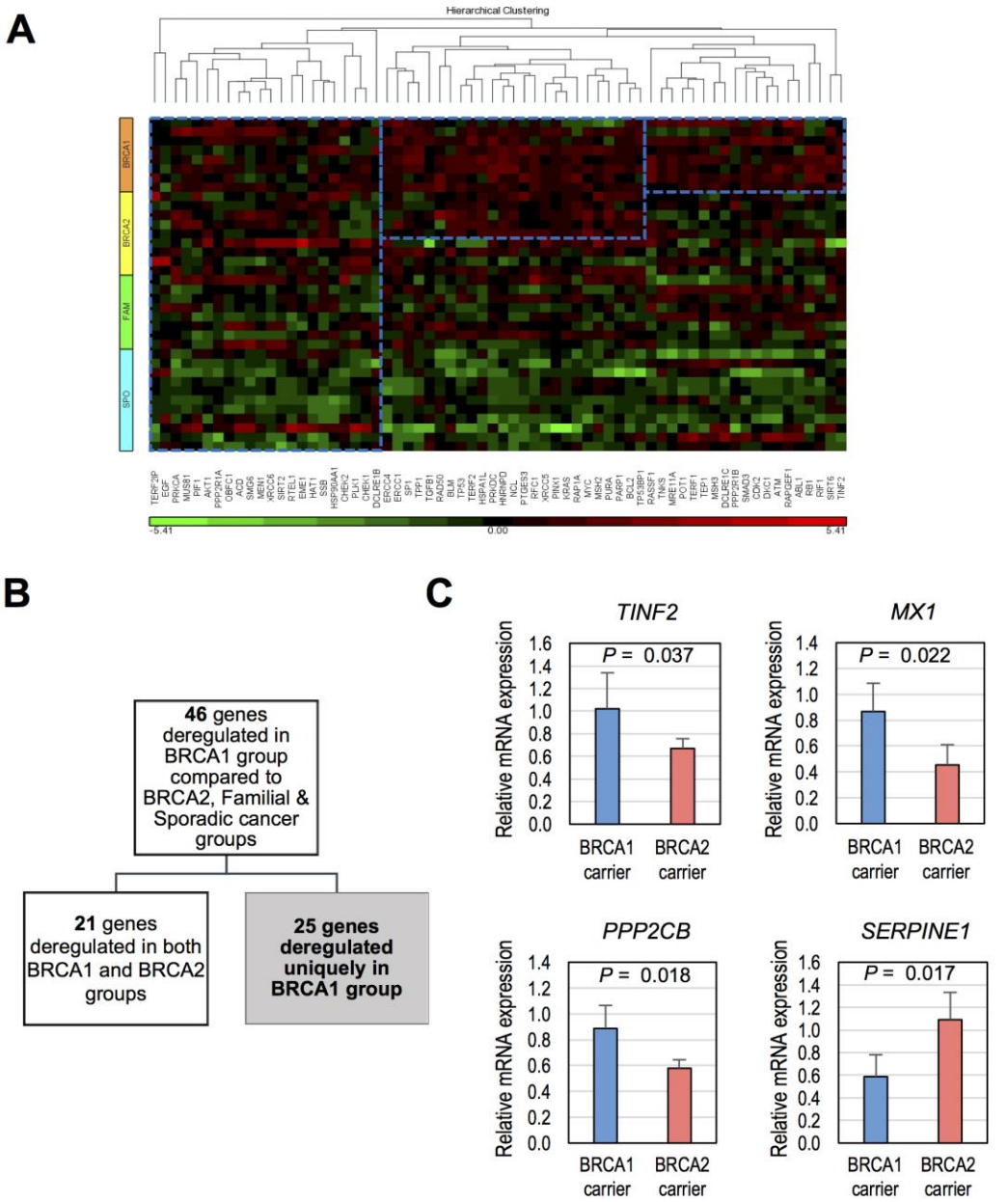
Gene	Gene locus	Fold change	P value
SERPINE1	7q22.1	1.37	4.1E-02
CECR5	22q11.1	-1.42	4.7E-02
PSPH	7p11.2	-1.45	2.8E-02
ZCCHC3	20p13	-1.48	4.8E-02
ZNF10	12q24.33*	-1.50	1.4E-02
NPAS2	2q11.2	-1.51	7.0E-03
CHFR	12q24.33*	-1.52	2.5E-02
SFRS17A	Xp22.33*/Yp11.2	-1.55	3.4E-02
RPH3AL	17p13.3	-1.57	4.3E-02
CCR7	17q21.2	-1.58	1.1E-02
ID2	2p25.1	-1.59	1.5E-02
DLL1	6q27*	-1.61	1.8E-02
FAM120B	6q27*	-1.61	4.7E-02
SH3YL1	2p25.3	-1.62	2.3E-02
CYP2E1	10q26.3*	-1.63	7.0E-03
ZNF324	19q13.43*	-1.63	1.1E-02
MFNG	22q13.1	-1.65	4.2E-03
RGS19	20q13.33*	-1.66	1.1E-02
ZNF34	8q24.3*	-1.79	1.8E-02
MEN1	11q13.1	-1.84	8.9E-03

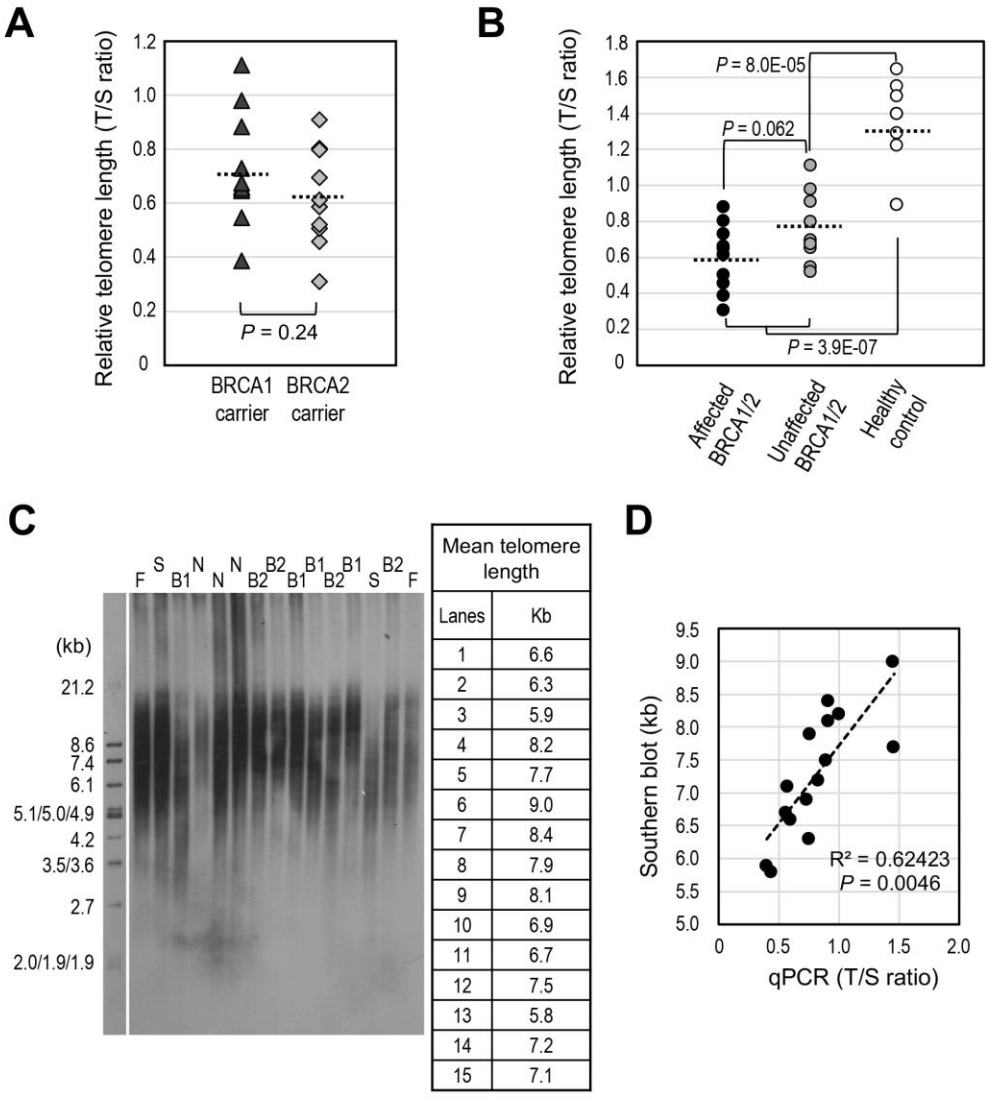
*telomere-proximal locus

Table 3 Summary of 14 genes from GSE6799, which express differently in affected BRCA2 individuals compared to unaffected BRCA2 individuals.

Gene	Gene locus	Fold change	P value
TUBB3	16q24.3*	1.73	3.4E-04
CCNE1	19q12	1.49	1.0E-02
CDKN2A	9p21.3	1.41	2.5E-02
HBZ	16p13.3*	1.39	2.2E-02
BLM	15q26.1	-1.27	4.0E-02
ING1	13q34*	-1.28	3.1E-02
PSMD11	17q11.2	-1.33	4.5E-02
MYC	8q24.21	-1.37	2.5E-02
CYP2E1	10q26.3*	-1.41	3.6E-02
UBE2J2	1p36.33*	-1.46	1.8E-02
CDA	1p36.12	-1.49	2.2E-02
TXNL4A	18q23	-1.55	3.3E-02
RPL23AP82	22q13.33*	-1.64	2.5E-02
DNMT3A	2p23.3	-1.68	1.5E-03

*telomere-proximal locus





Mean telomere length	
Lanes	Kb
1	6.6
2	6.3
3	5.9
4	8.2
5	7.7
6	9.0
7	8.4
8	7.9
9	8.1
10	6.9
11	6.7
12	7.5
13	5.8
14	7.2
15	7.1