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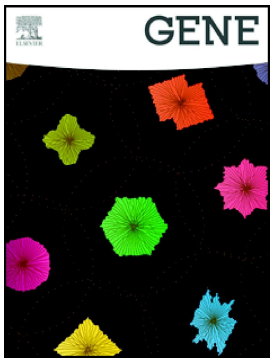

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Expression analysis of the *MCPH1/BRIT1* and *BRCA1* tumour suppressor genes and telomerase splice variants in epithelial ovarian cancer

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Abstract

Aims: The aim of this study was to explore the correlation of *hTERT* splice variant expression with *MCPH1/BRIT1* and *BRCA1* expression in epithelial ovarian cancer (EOC) samples.

Background: Telomerase activation can contribute to the progression of tumours and the development of cancer. However, the regulation of telomerase activity remains unclear. *MCPH1* (also known as *BRIT1,* BRCT-repeat inhibitor of *hTERT* expression) and *BRCA1* are tumor suppressor genes that have been linked to telomerase expression.

Methods: qPCR was used to investigate telomerase splice variants, *MCPH1/BRIT1* and *BRCA1* expression in EOC tissue and primary cultures.

Results: The wild type α+/β+ *hTERT* variant was the most common splice variant in the EOC samples, followed by α+/β- *hTERT,* a dominant negative regulator of telomerase activity. EOC samples expressing high total *hTERT* demonstrated significantly lower *MCPH1/BRIT1* expression in both tissue (p=0.05) and primary cultures (p=0.03). We identified a negative correlation between *MCPH1/BRIT1* and α+/β+ hTERT (*p*=0.04), and a strong positive association between *MCPH1/BRIT1* and both α-/β+ hTERT and α-/β- hTERT (both *p*=0.02). A positive association was observed between *BRCA1* and α-/β+ *hTERT* and α-/β- *hTERT* expression (*p*=0.003 and *p*=0.04, respectively).

Conclusions: These findings support a regulatory effect of *MCPH1/BRIT1* and *BRCA1* on telomerase activity, particularly the negative association between *MCPH1/BRIT1* and the functional form of *hTERT* (α+/β+).

1. Introduction

Telomeres are regions of repetitive nucleotide sequences at each end of a chromatid. Their function is to preserve genome stability by constructing protein-DNA complexes containing *TRF1, TRF2, POT1, Rap1,* and *TPP1*. This complex, known as the shelterin complex, is critical for both the maintenance and function of the telomere [1]. In humans, telomeres are composed of a highly repetitive (1000-2000 times) hexameric TTAGGG sequence. In somatic cells, the telomere gets shorter with each cell cycle because of the end replication problem [2].

In germ cells, stem cells and cancer cells, telomerase – a ribonucleoprotein enzyme – offsets this shortening process by adding repetitive nucleotide sequences to the ends of the DNA, thus preventing damage to the chromosome ends. Telomerase consists of a number of sub-units, including an RNA moiety (*hTR*), which is expressed universally, and a telomerase reverse transcriptase (*hTERT*). *hTERT* is composed of 16 exons, which results in a mRNA transcript of approximately 4 kb [3].

Alternative splicing of *hTERT* has been shown to affect telomerase activity. To date, fourteen alternative splice variants have been identified, the most common variants being α, β and γ deletions. These variations contribute to a range of different disease-specific transcripts [4-7]. Splice variants have been identified in a variety of tumors, including thyroid [8], gastrointestinal system tumors [9] and myelodysplastic syndromes [10]. The α-/β+ splice variant results from a 36-bp in-frame deletion of exonic sequences in exon 6 and the α+/β- splice variant results from a 183-bp deletion in which exons 7 and 8 are lost, resulting in a truncated protein. Splicing at these sites may

happen in combination or independently to create 3 alternatives from the full-length α+/β+ (WT): α+/β-, α-/β+, and α-/β- [6].

The activation of telomerase has also been shown to contribute to the progression of tumors and the development of cancer [11]. For example, increased telomerase activity has been detected in 92% of malignant ovarian tumors [12], and the activity of telomerase has been connected to survival rates and the aggressive development of ovarian tumors [13-14]. Telomerase activity has therefore been proposed as a marker for predicting ovarian cancer relapse following treatment with chemotherapy [13,15]. Furthermore, inhibition of telomerase activity in ovarian cancer cells inhibits cell growth and induces apoptosis [16]. Taking into account these findings, Meng and others have suggested using an inhibitor of telomerase activity, BIBR1532, combined with classic chemotherapy, to treat ovarian cancer [14].

Telomerase activity has been shown to be regulated by the tumor suppressors MCPH1 and BRCA1 [17-18]. *MCPH1*, which was first identified as *BRIT1* (BRCT-repeat inhibitor of hTERT expression*)* [17]*,* interacts directly with the *hTERT* proximal promoter and inhibits *hTERT* expression, thus resulting in reduced telomerase activity [19].

This study has investigated the relationship of *MCPH1/BRIT1* and *BRCA1* with *hTERT* and *hTERT* splice variant expression in EOC tissue and primary cultures. *MCPH1/BRIT1* and total *hTERT* expression displayed an inverse association. A positive association between *MCPH1/BRIT1* and *BRCA1* with α-/β+ *hTERT* and α-/β- *hTERT* was also identified. This study provides new insight into the tumor suppressor functions of *MCPH1/BRIT1*

and *BRCA1*, linking them with the inactivation of the functional form of *hTERT* and the activation of dominant negative splice variants of *hTERT*.

2. Materials and Methods

2.1 Sample collection and ethical approval

Four ovarian cancer cell lines (OVCA433, SKOV-3, TR175, 1847) were obtained from Cancer Research UK, and 2 immortal primary EOC cultures were established from ascites samples from patients with high-grade EOC (1015-A1 and 1016-A1) as previously described [20]. Patient information and clinical data for 20 tissue samples used in this study (15 EOCs, 4 benign tumours and 1 normal ovarian sample from an adjacent tumor) and 40 primary EOC cultures are presented in Supplementary Table 1. Ethical approval was obtained from the Local Research Ethics Committee of the Leeds Teaching Hospitals NHS Trust, Leeds, UK, (REC reference 09/H1306/96).

2.2 Cell culture

Cells were maintained in RPMI1640 + Glutamax™ medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100µg/ml; Gibco Carlsbad, CA, USA). Cells were incubated in 25–75 cm^2 flasks under standard culture conditions in a 5% CO_2 incubator at 37℃.

2.3 Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity levels were measured using the TRAP assay. Harvested cells grown to 80-90% confluence in a 75cm^2 flask were lysed in 400µl of 1X CHAPS lysis buffer on ice for 30 min then spun at 12,000 g for 20 min at 4˚C. The supernatant was stored in aliquots at -80˚C. The protein concentration was measured using the DC protein reagent set (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. A 25µl PCR reaction mixture containing 1 X TRAP$_{EZE}$® XL Reaction Mix, 2 units TITANIUM™ *Taq* DNA polymerase (Clontech, Mountain View, CA, USA), and 100 ng of protein extract was incubated at 30℃ for 30 min. The PCR was performed with an ABI PRISM 7900 Sequence Detector System using the following conditions: 95℃ for 2 min, followed by 45-cycle amplification (94℃ for 15 seconds, 59℃ for 1 min and 45℃ for 35 seconds). A positive control containing 500 telomerase active cells was prepared for each assay. A negative control sample lacking telomerase, a no template control and heated negative controls for each sample were also included. The generated data were analyzed with 7900 sequence detector software (Version 1.2.3; PE Applied Biosystems, Carlsbad, CA, USA). The amount of telomerase activity was calculated based on a standard curve using TSR8 supplied in the kit.

2.4 RNA extraction and reverse transcriptase reaction

Frozen tissue samples were embedded in Optimal Cutting Temperature (OCT) embedding medium (Lamb Inc.) on cork discs. A Leica CM3050 S cryostat was used to cut the embedded tissue into 10–20µm sections at -20˚C. Ten sections from each sample were placed into a 1.5ml

Eppendorf tube. Tissue was washed twice with PBS to remove all OCT residues. RNA was extracted from frozen tissue samples, cell lines and primary EOC cultures using the TRIzol™ reagent according to the manufacturer's protocol. Tissue samples were resuspended in 1ml of TRIzol reagent and homogenized on ice using a P200 pipette (Gilson, Middleton, WI, USA).

2.5 Quantitative real-time PCR (qRT-PCR)

Complementary DNA (cDNA) was used as the DNA template in PCR reactions. cDNA synthesis was performed using Superscript III (Invitrogen, Paisley, Scotland, UK). Briefly, a 14µl reaction contained 1500ng total RNA, 1µg random hexamers, and 1µl dNTPs (10mMol) were heated to 65°C for 5 min and cooled on ice for 2 min. 6µl of a master mix containing 4µl 5x first-strand buffer, 1µl 0.1M dithiothreitol (DTT), and 1µl Superscript III (200 U/µl) was added and incubated at 25°C for 5 min before heating at 50°C for 50 min and finally at 70°C for 15 min.

Duplicate real-time PCR analysis was performed using the ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA) for both SYBR Green (*MCPH1/BRIT1* and *BRCA1* amplifications) and TaqMan reactions (*hTERT* amplification) [21-22]. The *36B4* gene was used as an endogenous control to normalize gene expression. Standard curves were performed using serial dilutions of cDNA from human fetal brain for *MCPH1/BRIT1* amplification, normal human ovary (Clontech, Mountain View, CA, USA), for *BRCA1,* and human MCF7 breast adenocarcinoma cells for *hTERT* amplification. PCR reactions were carried out in a 96-well plate using

a 25µl reaction volume using the following conditions: 2 min at 50˚C followed by 10 min at 95˚C and 45 cycles of denaturation at 95˚C for 15 seconds and annealing/extension at 60˚C for 1 min. Total *hTERT* primers were designed to detect all splice variants types in one run. Details of the primer sequences for qRT-PCR are listed in Supplementary Table 2. Mann-Whitney (MW) tests were performed to determine statistical significance. Kaplan-Meir survival curve analysis was performed followed by a false discovery rate detection ajustment generating q values due to the small sample size of the study requiring $q<0.05$ (95% confidence level) to be considered significant (GraphPad Prism 7).

2.6 Expression of α-/β+ hTERT

The effect of increased α-/β+ *hTERT* expression on *MCPH1/BRIT1* expression was measured in the 4 ovarian cancer cell lines (OVCA433, SKOV-3, TR175, 1847) and human embryonic kidney HEK 293 cells (ATCC, Middlesex, Uk). An α-/β+ *hTERT* construct was kindly provided by Professor RR Reddel (Children's Medical Research Institute, University of Sydney, Australia) in the pIRESneo vector (Clontech) [23]. HEK 293 cells were seeded at 2 x 10^5 cells/ml and allowed to grow until 70–80% confluence, and then transfected using Lipofectamine 2000 (Invitrogen, USA) based on the manufacturer's protocol. A ratio of 1:4 lipofectamine to α-/β+ *hTERT* plasmid DNA concentration (µl/µg) was used after 48 hours. The expression levels of α-/β+ *hTERT* and *MCPH1/BRIT1* were evaluated using qRT-PCR as described above.

3. Results

3.1 Expression profiles of total hTERT and hTERT splice variants in ovarian cancer cell lines

Total *hTERT* and its splice variants were examined in the OVCA433, SKOV-3, TR175 and 1847 cell lines. Telomerase activity was detected in all lines, with levels ranging from 0.086 to 3.38 log10 TPG units. The relative expression of *hTERT* variants was evaluated as the percentage of each *hTERT* splice variant in relation to total *hTERT* expression (Fig. 1, Supplementary Table 3). The expression level of α-/β+ h*TERT* was higher than for other deletion splice variants (Fig. 1). We then investigated whether the expression levels of different *hTERT* splice variants were linked with telomerase activity; however, no statistically significant correlations were detected.

We next measured *hTERT* expression in normal ovary as well as EOC tissue and primary EOC cultures. We did not detect any *hTERT* expression in the 5 benign or normal samples examined. By contrast, *hTERT* was detected in 60% (12/15) of EOC tissues and 52% (21/40) of cultured samples. Samples were classified as expressing high or low total *hTERT* based on the mean expression levels of EOC tissue (Fig. 2A) and primary cultures (Fig. 2B). We then examined the expression of the different *hTERT* splice variants (α+/β+, α- /β+, α+/β- and α-/β).

All EOC tissue samples (12/12) and 81% of primary cultures (17/21) expressing total *hTERT* were also positive for wild type (α+/β+) *hTERT*. The α+/β- *hTERT* splice variant, a dominant negative regulator of telomerase activity, was identified in both EOC tissue (75%, 9/12) and primary cultures

(86%, 18/21) expressing total *hTERT*. α-/β+ and α-/β- *hTERT* splice variants were detected in 50% (6/12) of EOC tissues (Fig. 2C). The α-/β- *hTERT* splice variant was detected in 43% (9/21) of primary cultures and the α-/β+ *hTERT* splice variant was detected in 38% (8/21) of primary cultures (Fig. 2D). All EOC tissue samples and primary cultures showed expression of at least 1 splice variant, except for one. Although this expressed low levels of total *hTERT,* none of the 4 splice variants investigated here were detected.

3.2 Expression profiles of MCPH1/BRIT1 and BRCA1

The expression levels of both *MCPH1/BRIT1* and *BRCA1* were examined in normal ovary, EOC tissue and primary cultures. Samples were classified as high or low expressing based on the mean of the EOC tissues and primary cultures respectively. MCPH1/BRIT1 expression was detected in normal ovary and all EOC tissue samples and primary cultures. Ten of 17 EOC tissue samples (59%) and 25/37 primary culture samples (68%) showed low *MCPH1/BRIT1* mRNA expression (Fig. 2E and 2F). BRCA1 expression was detected in all EOC tissues (17/17) and 18/31 (58%) primary cultures. Ten of 17 (59%) and 14/18 (78%) patients expressed low BRCA1 in EOC tissues (Fig. 2G) and in primary cultures (Fig. 2H), respectively.

3.3 Comparison of total hTERT with MCPH1/BRIT1 and BRCA1

Samples were categorized into 3 groups (negative, low and high) based on expression levels of total *hTERT*, then correlated to the expression levels of *MCPH1*/BRIT1 and *BRCA1*. Unexpectedly, *MCPH1/BRIT1* expression was lower in total *hTERT* negative samples than that in samples

with low total *hTERT* and was similar to samples with high total *hTERT* (p=0.01) in EOC tissues (Fig. 3A). A similar trend was noted in primary cultures though this result was not significant (p=0.10). Investigation of *MCPH1/BRIT1* mRNA expression in total *hTERT* positive EOC tissue samples revealed a significant inverse association between high total *hTERT* and low *MCPH1/BRIT1* (p=0.05; Fig. 3A). A similar inverse association was observed in primary cultures (p=0.03; Fig. 3B).

BRCA1 expression levels were comparable in EOC tissue samples with negative or low or high total *hTERT* expression (Fig. 3C). However, in primary cultures lower levels of *BRCA1* were observed in samples with negative total *hTERT* compared to samples with positive total *hTERT* expression (p=0.04; Fig. 3D).

3.4 Comparison of hTERT splicing variants with MCPH1/BRIT1 expression

In EOC tissues, the correlation of *hTERT* splice variants with *MCPH1/BRIT1* expression revealed a strong negative association between *MCPH1/BRIT1* and the functional form α+/β+ *hTERT,* such that samples expressing high α+/β+ *hTERT* exhibited a reduction in *MCPH1/BRIT1* expression (p=0.04; Fig. 4A). High *MCPH1/BRIT1* expression was associated with high levels of α-/β+ (p=0.02; Fig. 4B) and α-/β- (p=0.02; Fig. 4C) *hTERT* expression. There was no significant association between the α+/β- *hTERT* variant and *MCPH1/BRIT1* expression levels (data not shown).

We also performed relative expression analysis, which has previously been used to interpret telomerase expression [24]. The percentage of each *hTERT* isoform was calculated in relation to the overall expression and then

correlated with *MCPH1/BRIT1* expression. Samples with low *MCPH1/BRIT1* expression expressed exclusively α+/β+ *hTERT* or α+/β+ and α+/β- *hTERT*, whereas α-/β+ or α-/β- *hTERT* or both were detected in high *MCPH1/BRIT1*-expressing samples only (Fig. 4D). A high percentage α+/β+ *hTERT* was identified in low *MCPH1/BRIT1* expression samples (p=0.01; Fig. 4E). No α-/β+ or α-/β- *hTERT* expression was detected in low *MCPH1/BRIT1* samples, only in high *MCPH1/BRIT1* expression samples ((p=0.02); Fig. 4F) and (p=0.02; 4G). No significant difference in the percentage of α+/β- *hTERT* expression in samples with low or high *MCPH1/BRIT1* expression was identified (data not shown). We did not observe any statistical differences between *hTERT* variants (absolute and relative expression values) and *MCPH1/BRIT1* in primary cultures (data not shown). The correlation between high *MCPH1/BRIT1* expression and α-/β+ *hTERT* expression was further supported using α-/β+ *hTERT* constructs in cancer cell lines. We observed increased *MCPH1/BRIT1* expression in 3/5 (67%) of cell lines transfected with an α-/β+ *hTERT* construct (Fig. 4H). These findings confirm our observations in EOC tissues.

3.5 Comparison of hTERT splicing variants with BRCA1 expression

Similar to *MCPH1/BRIT1*, *BRCA1* expression was correlated with absolute and relative values of *hTERT* splice variants in EOC tissues. We found that 6/7 samples expressing low levels of *BRCA1* were negative for α-/β+ *hTERT* expression (p=0.003; Fig. 5A). Samples with low levels of *BRCA1* were also negative for α-/β- *hTERT* expression*;* however, this association was not significant (p=0.09; Fig. 5B). No association was identified between the

absolute value of α+/β+ or α+/β- *hTERT* expression and *BRCA1* levels (data not shown). α+/β+ *hTERT* was expressed at a higher percentage in samples with low *BRCA1* expression compared with samples with high *BRCA1* expression (Fig. 5C). A high percentage of α-/β+ *hTERT* expression was identified in samples with high *BRCA1* expression. Similarly 6/7 low *BRCA1* expressing tissue samples did not express α-/β+ *hTERT* (p=0.003; Fig. 5D). The relative expression of of α-/β- *hTERT* was positively correlated with high *BRCA1* expression and not detected in tissues expressing low *BRCA1* (p=0.04; Fig. 5E). A reverse trend was identified in the correlation of the percentage of α+/β+ *hTERT* with BRCA1 expression, which did not reach statistical significance (p=0.1; Fig. 5F). No significant association between the percentage of α+/β- *hTERT* and BRCA1 expression was found (data not shown).

3.6 Correlation of hTERT expression with clinical prognostic factors

We did not identify any correlations between total *hTERT* and *hTERT* splice variants with tumor stage, grade and histology in either EOC tissues or primary cultures (data not shown). Furthermore, we did not observe any changes in survival time between positive and negative total *hTERT* in EOC tissues or primary cultures (data not shown). Interestingly, analysis of survival data after grouping positive samples into low and high hTERT levels based on the mean expression showed that patients with no or low total *hTERT* had comparable survival times, and that they had significantly longer survival times than patients with high *hTERT* expression in primary EOC cultures (q=0.04; Fig. 6A). A similar trend was observed in the EOC tissue although

this did not reach statistical significance (q=0.1). After excluding patients who were negative for total *hTERT* expression, we observed an association between total *hTERT* expression and survival; patients with high total *hTERT* expression had shorter survival times than patients with low total hTERT expression in primary EOC cultures (*q*=0.02; Fig. 6B). A similar trend was observed in the EOC tissue although this did not reach statistical significance (q=0.1). Generally, *hTERT* splicing did not show significant changes with survival. However, patients who expressed α+/β- *hTERT* in primary EOC culture samples showed a longer survival period compared to those that did not (*q*=0.0003; Fig 6C).

4. Discussion

The aim of this study was to evaluate the expression of *hTERT* splice variants in EOC tissues and primary EOC cultures. We assessed the expression of 4 exon α and β *hTERT* variants (α+/β+, α-/β+, α+/β- and α-/β-), previously identified in melanoma, thyroid and cervical cancer [25,8,26]. Other possible *hTERT* variants, such as the γ- deletion or exon 2 deletions [27], were not examined here. We then correlated *hTERT* splice variant expression with expression of *MCPH1/BRIT1* and *BRCA1*, which are *hTERT* negative regulators in EOC. To our knowledge this is the first study that evaluates correlations between the expression levels of the *hTERT* regulators MCPH1 and BRCA1, hTERT variant expression and telomerase activity.

We initially characterised telomerase activity and the expression of 4 *hTERT* splice variants in ovarian cancer cell lines. Similarly to a recent study by Khosravi and colleagues, we found that the cell lines expressed a mixed

pattern of all 4 *hTERT* variants [28]. There was a higher level of expression for α-/β+ *hTERT* compared to the α+/β-, and α-/β- *hTERT* variants. Similarly, a lung adenocarcinoma cell line study demonstrated greater expression of the α-/β+ *hTERT* variant compared to α+/β- and α-/β+ *hTERT* variants [29]. In contrast, the α-/β+ *hTERT* variant accounted for only 1% of the total *hTERT* in a panel of cell lines originating from different organs [24]. Such contradictory findings may indicate that the α-/β+ and α+/β- *hTERT* variants have little impact on overall telomerase activity in a variety of cell lines.

No correlation was identified between the expression levels of the different *hTERT* splice variants and telomerase activity in the ovarian cancer cell lines. Potentially other mechanisms may contribute to the regulation of telomerase such as post transcriptional factors like phosphorylation or the presence of telomerase inhibitors or activators such as protein phosphatase 2A or Akt kinase respectively [30-31]. Another possible explanation for these findings may be that the regulation of telomerase activity by hTERT splice variants is tumour-specific and that there could be additional splice variants present in ovarian cancer cell lines that were not investigated in this study [32].

Our study indicates that *MCPH1/BRIT1* functions as a negative regulator of total *hTERT* activity in EOC tissues and primary cultures, in samples expressing high or low total *hTERT*. In terms of *hTERT* variants, α+/β+ *hTERT* and *MCPH1/BRIT1* expression were found to be associated; *MCPH1/BRIT1* expression was diminished in EOC tissue samples that expressed high α+/β+ *hTERT*. For *BRCA1* expression, no major difference in the samples showing low or high total *hTERT* in either EOC tissues or primary

cultures was observed. The correlation of *BRCA1* with *hTERT* alternate splice variants only revealed a trend between *BRCA1* and α+/β+ *hTERT* expression in EOC tissues. In samples with no *hTERT* activity we observed no negative regulation of *BRCA1* and *MCPH1/BRIT1*. When samples were grouped into positive and negative total *hTERT* activity, those with negative activity demonstrated low levels of *MCPH1/BRIT1* expression in EOC tissues (p=0.01) and primary cultures (p=0.10). Similarly, samples with negative total *hTERT* demonstrated low levels of *BRCA1* expression in primary culture compared to positive total *hTERT* samples (*p*=0.04). Taken together, these findings indicate that *BRCA1* and *MCPH1/BRIT1* have adverse regulatory effects on the expression of *hTERT* only when samples are positive for *hTERT* expression.

Previously, BRCA1 siRNA knockdown in T47D, MCF-7 and DU-145 cell lines was found to increase hTERT mRNA expression and telomerase activity [33]. Taking that study together with our findings, we propose that *MCPH1/BRIT1* and *BRCA1* might act as tumor suppressors via the negative regulation of *hTERT*. Furthermore, our findings indicate that decreased *MCPH1/BRIT1* expression may increase the levels and/or the activity of telomerase.

The most commonly identified dominant negative regulator of telomerase activity in both EOC tissue samples and primary cultures was the α+/β- *hTERT* splice variant, consistent with previous studies that identified a negative correlation between telomerase activity and relative α+/β- *hTERT* expression in melanoma and lung cancer [25,29]. In our study, relative and absolute expression of α+/β- *hTERT* were not associated with *BRCA1* or

MCPH1/BRIT1 in EOC tissue or primary cultures. Conversely, there was a positive correlation of α-/β+ *hTERT* and α-/β- *hTERT* with both *MCPH1/BRIT1* and *BRCA1* in their absolute, as well as their relative, levels of *hTERT* expression. A α-/β+ *hTERT* plasmid construct confirmed this correlation in cell lines. A previous study had also shown decreased telomerase activity following transfection with an α-/β+ *hTERT* plasmid construct [21].

The value of *hTERT* expression as a prognostic biomarker remains controversial. An association between *hTERT* and tumor stage has been reported in bladder cancer [34] and tumour grade in breast cancer [35]. In contrast *hTERT* was not a prognostic marker in lung cancer [29] or gastric cancer [36]. Furthermore, the role of *hTERT* mRNA splicing in the regulation of telomerase activity remains unclear. The utility of *hTERT* gene products in clinical diagnostics therefore remains uncertain.

hTERT has been considered an important predictor of survival. Shorter survival was observed in *hTERT*-positive patients with non-small cell lung cancer [37] and pediatric intracranial ependymoma [38]. In our samples, a simple positive and negative *hTERT* expression classification did not correlate with survival. However, an association with survival was identified by classing total *hTERT* as either high or low in primary EOC primary cultures and a trend in the the tissue samples. Patients with a low total *hTERT* expression had a longer life span. Potentially the higher sensitivity of the method used in our study could account for the conflict with results from previous studies. It is possible that we could detect a lower level of total *hTERT* expression using the TaqMan qRT-PCR assay compared to the immunohistochemistry [37] or *in situ* hybridization [38] used in earlier studies. Further research on a larger

sample size would be required for this potentially important observation to be confirmed.

In conclusion, the negative association between *MCPH1/BRIT1* and the functional form of *hTERT* (α+/β+) supports its role as a negative regulator of telomerase activity in primary EOC samples.

Conflict of interest

The authors declare that they have no conflict of interest.

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Authors' roles

R.A. J.B. E.E.M and S.M.B designed the study. R.A. and S.M.B. drafted the manuscript and prepared the figures. R.A. and A.B-R performed the experiments. S.C.B. and S.A.B developed the Telomerase and hTERT splice variant real-time assays. R.H. and N.G. provided tissue samples and clinical data. S.C.B, J.B, E.E.M. and S.A.B. assisted in drafting the manuscript and figures. All authors read and critically revised the manuscript and provided their final approval before submission.

Figure legends

Fig. 1. *hTERT* splice variant expression in ovarian cancer cell lines

(A) Absolute (fold change) and (B) relative (percentage) *hTERT* splice variant expression in the ovarian cancer cell lines OVCA433, SKOV-3, TR175 and 1847. Expression of α-/β+ h*TERT* was higher than the expression of α+/β- and α-/β- h*TERT*. Bars represent the average expression of *hTER*T splice variants in the cell lines.

Fig. 2. mRNA expressions of total *hTERT, hTERT* splice variants, *MCPH1/BRIT1 and BRCA1* in EOC tissues and primary cultures

For all graphs the mean value, is depicted by a red dotted line and was used to separate the samples into high and low expression (A) Total *hTERT* expression in EOC tissues (n=15). Benign samples are highlighted in the blue box, and one normal ovary tissue sample is shown in red. (B) Total *hTERT* expression in the primary EOC culture samples. (C) Detection of *hTERT* variants in EOC tissues. (D) Detection of *hTERT* variants in primary EOC cultures. (E) *MCPH1/BRIT1* expression in EOC tissues ranged from a 0.011- to 1.83-fold change (Relative to fetal brain standard curve). (F) *MCPH1/BRIT1* expression in primary EOC cultures ranged from a 0.003- to 5.26-fold change. (G) *BRCA1* expression in EOC tissues ranged from a 0.363- to 37.3-fold change (Relative to normal ovary standard curve). (H) *BRCA1* expression in primary EOC cultures ranged from a 0.01- to 17.38-fold change.

Fig. 3. Correlation of total *hTERT* with *MCPH1/BRIT1* and *BRCA1* expression in EOC tissues and primary cultures

(A) In EOC tissues, a significant negative correlation between *MCPH1/BRIT1* and total *hTERT* was identified in positive total *hTERT* samples *(p*=0.05). Samples with negative total *hTERT* exhibited low *MCPH1/BRIT1* when compared to samples which expressed total *hTERT (p*=0.01). (B) In EOC cultures, *MCPH1/BRIT1* and total *hTERT* showed same pattern of association, such that sample with low total *hTERT* demonstrated high levels of *MCPH1/BRIT1* in total *hTERT* positive samples *(p*=0.03). Samples with negative total *hTERT* showed low levels of *MCPH1/BRIT1* when compared with samples with positive total *hTERT*, however, this did not reach statistical significant *(p*=0.10). (C) In EOC tissue samples, no association was identified between *BRCA1* and total *hTERT.* (D) In EOC culture samples high *BRCA1* expression was identified in patients exhibiting positive total *hTERT* expression, when compared to samples that did not expressed total *hTERT* expression (*p*=0.04). However, no difference in *BRCA1* expression was identified between low and high total *hTERT* expression (*p*=0.83) (MW).

Fig. 4. *MCPH1/BRIT1* and *hTERT* splice variant expression in EOC tissues and primary cultures

MCPH1/BRIT1 was correlated with absolute (A-C) and relative expression (D-G) of *hTERT* variants. (A) Samples with low *MCPH1/BRIT1* expression showed higher α+/β+ *hTERT* compared to samples with high *MCPH1/BRIT1 (p*=0.04). Both α-/β+ (B) and α-/β- *hTERT (C)* were positively correlated with *MCPH1/BRIT1 (p*=0.02). (D) *MCPH1/BRIT1* expression decreased in samples exclusively expressing α+/β+ or α+/β+ and α+/β- *hTERT*. While, high *MCPH1/BRIT1* expression samples demonstrated the expression of α-/β+

and/or α-/β- *hTERT*. (E) A high percentage of α+/β+ *hTERT,* ranging from 39% to 100%, with a mean of 71%, was identified in low *MCPH1/BRIT1* expression samples compared with the mean of 23% in high *MCPH1/BRIT1* expression samples (p=0.01). (F) No α-/β+ *hTERT* expression was detected in low *MCPH1/BRIT1* samples, in comparison to the elevated mean of 30% (p=0.02) for α-/β+ in high *MCPH1/BRIT1* expression samples. (G) No α-/β- *hTERT* expression was detected in low *MCPH1/BRIT1* samples, in comparison to the elevated mean of 27% (p=0.02) for α-/β- *hTERT* in high *MCPH1/BRIT1* expression samples. (H) Demonstrated *MCPH1/BRIT1* expression in cell lines transfected with α-/β+ *hTERT* construct. *MCPH1/BRIT1* expression increased in 3/5 cell lines transfected with α-/β+ *hTERT* compared to their matched untransfected control cells (HEK 293, SKOV-3 and 1847). 2/5 cell lines (TR175 and OVCA433) did not showed the positive correlation between *MCPH1/BRIT1* and α-/β+ *hTERT* expression.

Fig. 5. *BRCA1* and *hTERT* splice variant expression in EOC tissues

BRCA1 was correlated with absolute (A-B) and relative expression of *hTERT* variants (C-F). (A) *BRCA1* expression was positively correlated with α-/β+ *hTERT* expression *(p*=0.003). (B) The expression of the α-/β- *hTERT* also had a weak positive correlation with *BRCA1* expression *(p*=0.09). (C) Correlation between *BRCA1* expression and each *hTERT* isoform related to other isoforms. Expression of α-/β+ and α-/β- *hTERT* was more frequent in samples expressing high *BRCA1*. (D-E) Percentage of α-/β+ and α-/β- hTERT expression was strongly correlated with *BRCA1* expression (p=0.003 and p= 0.04 respectively). (F) A weak negative association was detected between low

α+/β+ *hTERT* expression and high *BRCA1* expression, and high α+/β+ *hTERT* with with low *BRCA1* expression (p=0.1) (MW).

Fig. 6. Patient Kaplan-Meir survival plots of *hTERT* expression in EOC tissues and primary cultures

Grouping positive total *hTERT* expression samples into low and high categories based on the mean expression showed that patients with low and negative total *hTERT* have comparable survival times and high total *hTERT* patients have a shorter survival time compared to patients with no or low total *hTERT* expression in (A) primary EOC cultures (q=0.04). Further analysis after excluding patients who did not expressed total *hTERT* revealed that patients with low total *hTERT* have longer survival time compared to patients with high total *hTERT* in (B) primary EOC cultures (q=0.02). (C) Splice variant analysis in primary EOC cultures idenfied increased survival times in patients with low α+/β- expression compared to patients with high expression (q=0.0004).

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Abbreviations

BRIT1 - BRCT-repeat inhibitor of *hTERT* expression

cDNA - complementary DNA

DTT - dithiothreitol

EOC - Epithelial ovarian cancer

hTERT – human Telomerase reverse transcriptase

MW - Mann-Whitney

OCT - Optimal cutting temperature

qRT- PCR – Quantitative reverse transcriptase polymerase chain reaction

Highlights

- High total *hTERT* expression was associated with low *MCPH1/BRIT1* expression.

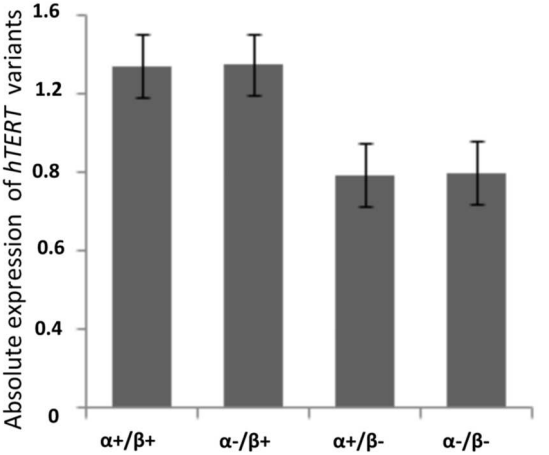
- A strong positive association between *MCPH1/BRIT1* and both α-/β+ and α-/β- hTERT.

- A negative association between *MCPH1/BRIT1* and the functional form of *hTERT* α+/β+.

- A positive association between *BRCA1* and α-/β+ *hTERT* and α-/β- *hTERT* expression.

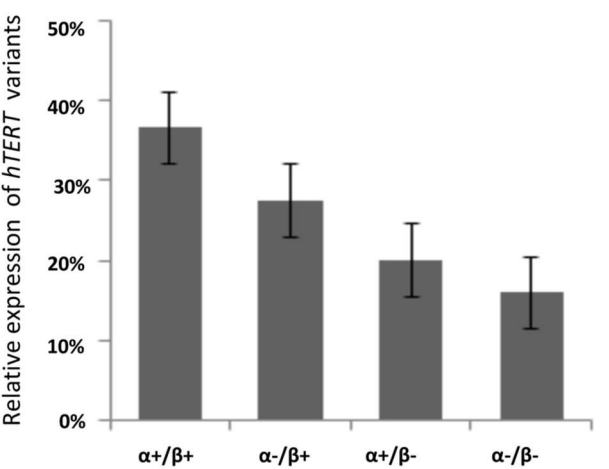
- These findings support a regulatory effect of *MCPH1/BRIT1* and *BRCA1* on telomerase activity.

A



hTERT splicing expression in EOC cell lines

B



hTERT splicing expression in EOC cell lines

Figure 1

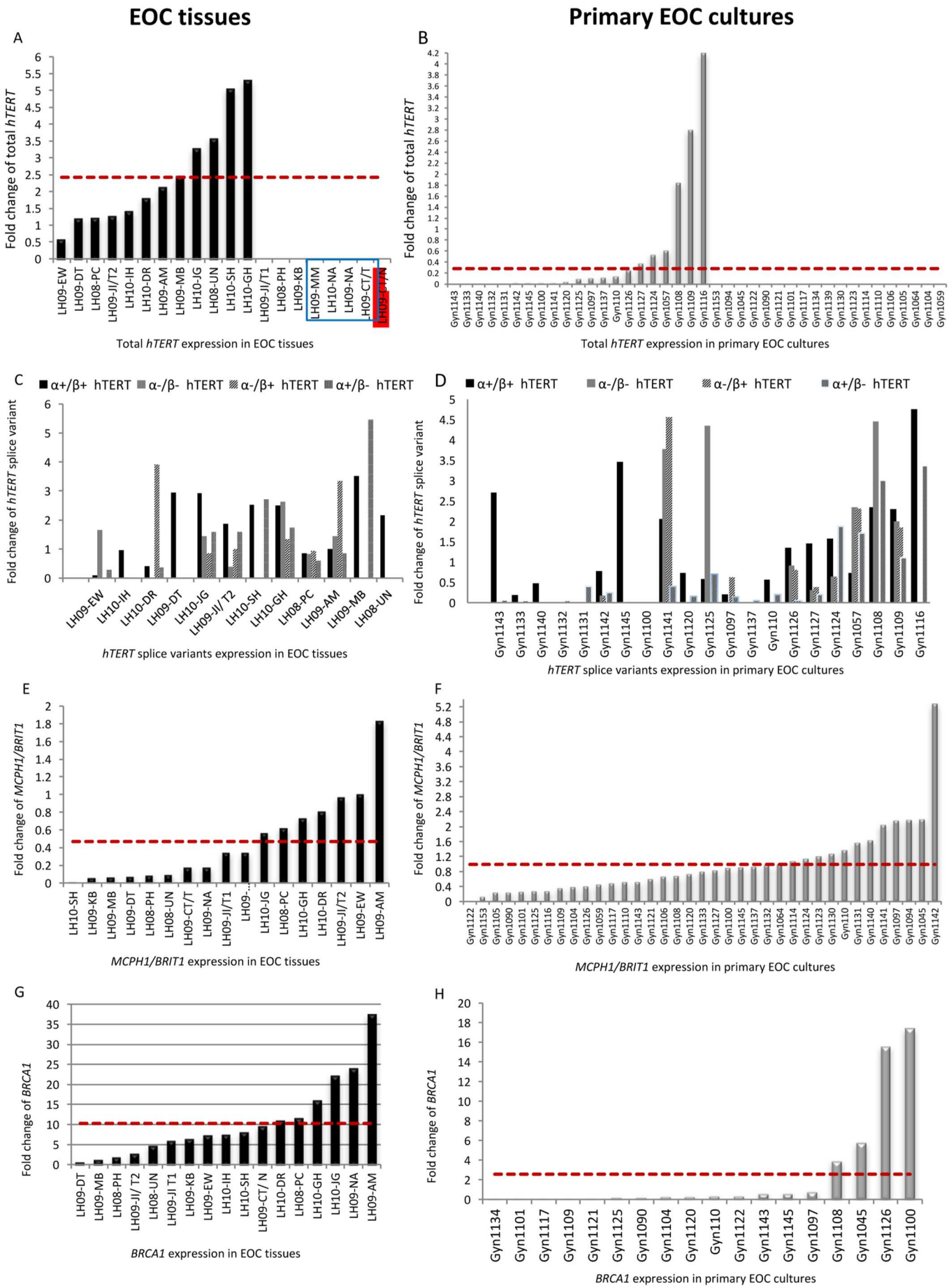

EOC tissues
Primary EOC cultures
A
Fold change of total hTERT
Total hTERT expression in EOC tissues
B
Fold change of total hTERT
Total hTERT expression in primary EOC cultures
C
α+/β+ hTERT
α-/β- hTERT
α-/β+ hTERT
α+/β- hTERT
Fold change of hTERT splice variant
hTERT splice variants expression in EOC tissues
D
α+/β+ hTERT
α-/β- hTERT
α-/β+ hTERT
α+/β- hTERT
Fold change of hTERT splice variant
hTERT splice variants expression in primary EOC cultures
E
Fold change of MCPH1/BRIT1
MCPH1/BRIT1 expression in EOC tissues
F
Fold change of MCPH1/BRIT1
MCPH1/BRIT1 expression in primary EOC cultures
G
Fold change of BRCA1
BRCA1 expression in EOC tissues
H
Fold change of BRCA1
BRCA1 expression in primary EOC cultures

Figure 2

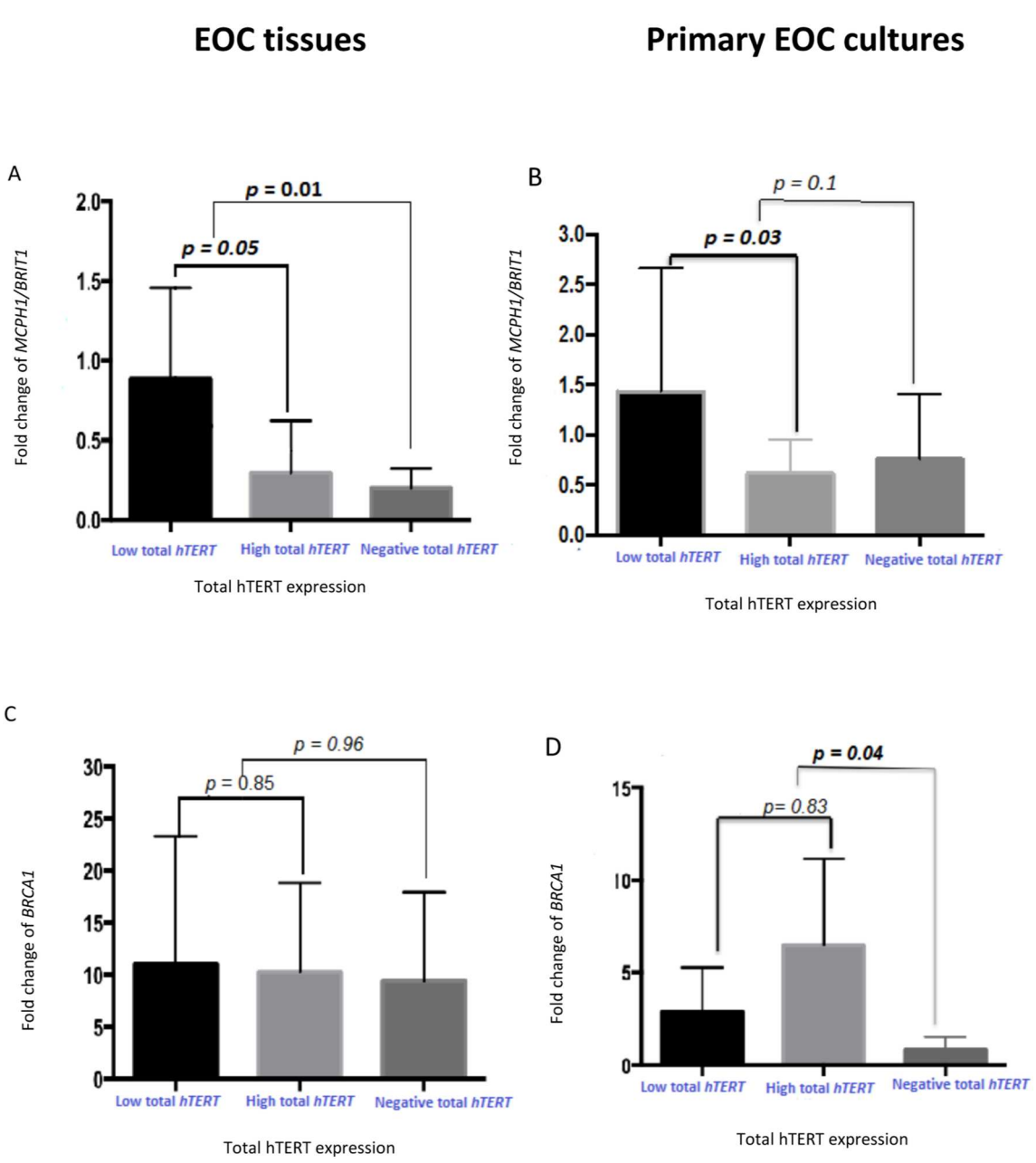

EOC tissues
Primary EOC cultures
A
p = 0.01
p = 0.05
Fold change of MCPH1/BRIT1
2.0
1.5
1.0
0.5
0.0
Low total hTERT
High total hTERT
Negative total hTERT
Total hTERT expression
B
p = 0.1
p = 0.03
Fold change of MCPH1/BRIT1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Low total hTERT
High total hTERT
Negative total hTERT
Total hTERT expression
C
p = 0.96
p = 0.85
Fold change of BRCA1
30
25
20
15
10
5
0
Low total hTERT
High total hTERT
Negative total hTERT
Total hTERT expression
D
p = 0.04
p= 0.83
Fold change of BRCA1
15
10
5
0
Low total hTERT
High total hTERT
Negative total hTERT
Total hTERT expression

Figure 3

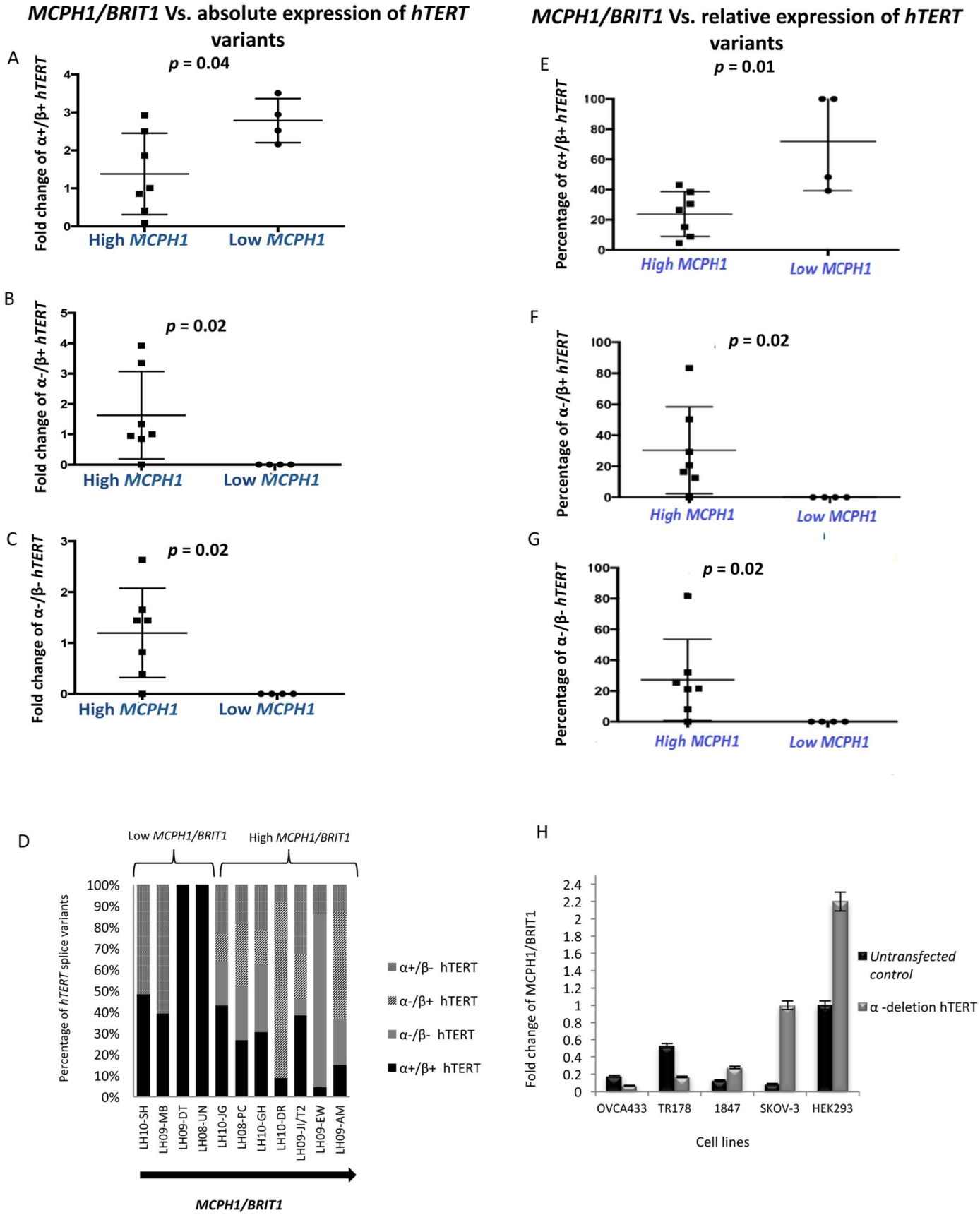

MCPH1/BRIT1 Vs. absolute expression of hTERT variants
A
p = 0.04
Fold change of α+/β+ hTERT
High MCPH1
Low MCPH1
B
p = 0.02
Fold change of α-/β+ hTERT
C
p = 0.02
Fold change of α-/β- hTERT
MCPH1/BRIT1 Vs. relative expression of hTERT variants
E
p = 0.01
Percentage of α+/β+ hTERT
F
p = 0.02
Percentage of α-/β+ hTERT
G
p = 0.02
Percentage of α-/β- hTERT
D
Low MCPH1/BRIT1
High MCPH1/BRIT1
Percentage of hTERT splice variants
α+/β- hTERT
α-/β+ hTERT
α-/β- hTERT
α+/β+ hTERT
LH10-SH
LH09-MB
LH09-DT
LH08-UN
LH10-JG
LH08-PC
LH10-GH
LH10-DR
LH09-JI/T2
LH09-EW
LH09-AM
MCPH1/BRIT1
H
Fold change of MCPH1/BRIT1
Untransfected control
α -deletion hTERT
OVCA433
TR178
1847
SKOV-3
HEK293
Cell lines

Figure 4

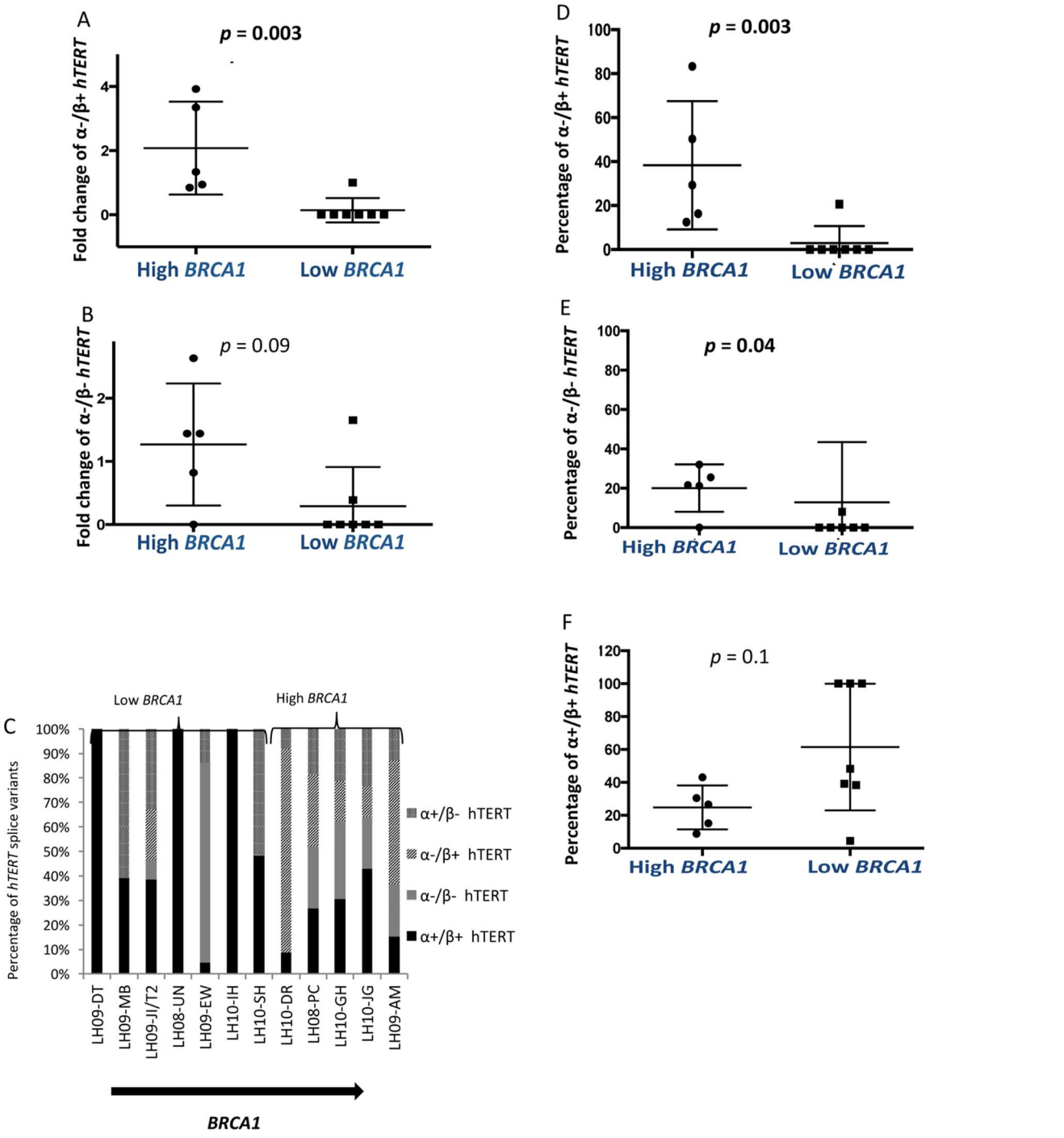

BRAC1 Vs. absolute expression of hTERT variants
BRCA1 Vs. relative expression of hTERT variants
A
p = 0.003
Fold change of α-/β+ hTERT
High BRCA1
Low BRCA1
B
p = 0.09
Fold change of α-/β- hTERT
High BRCA1
Low BRCA1
C
Low BRCA1
High BRCA1
Percentage of hTERT splice variants
α+/β- hTERT
α-/β+ hTERT
α-/β- hTERT
α+/β+ hTERT
LH09-DT
LH09-MB
LH09-JI/T2
LH08-UN
LH09-EW
LH10-IH
LH10-SH
LH10-DR
LH08-PC
LH10-GH
LH10-JG
LH09-AM
BRCA1
D
p = 0.003
Percentage of α-/β+ hTERT
High BRCA1
Low BRCA1
E
p = 0.04
Percentage of α-/β- hTERT
High BRCA1
Low BRCA1
F
p = 0.1
Percentage of α+/β+ hTERT
High BRCA1
Low BRCA1

Figure 5

Kaplan-Meir survival plots

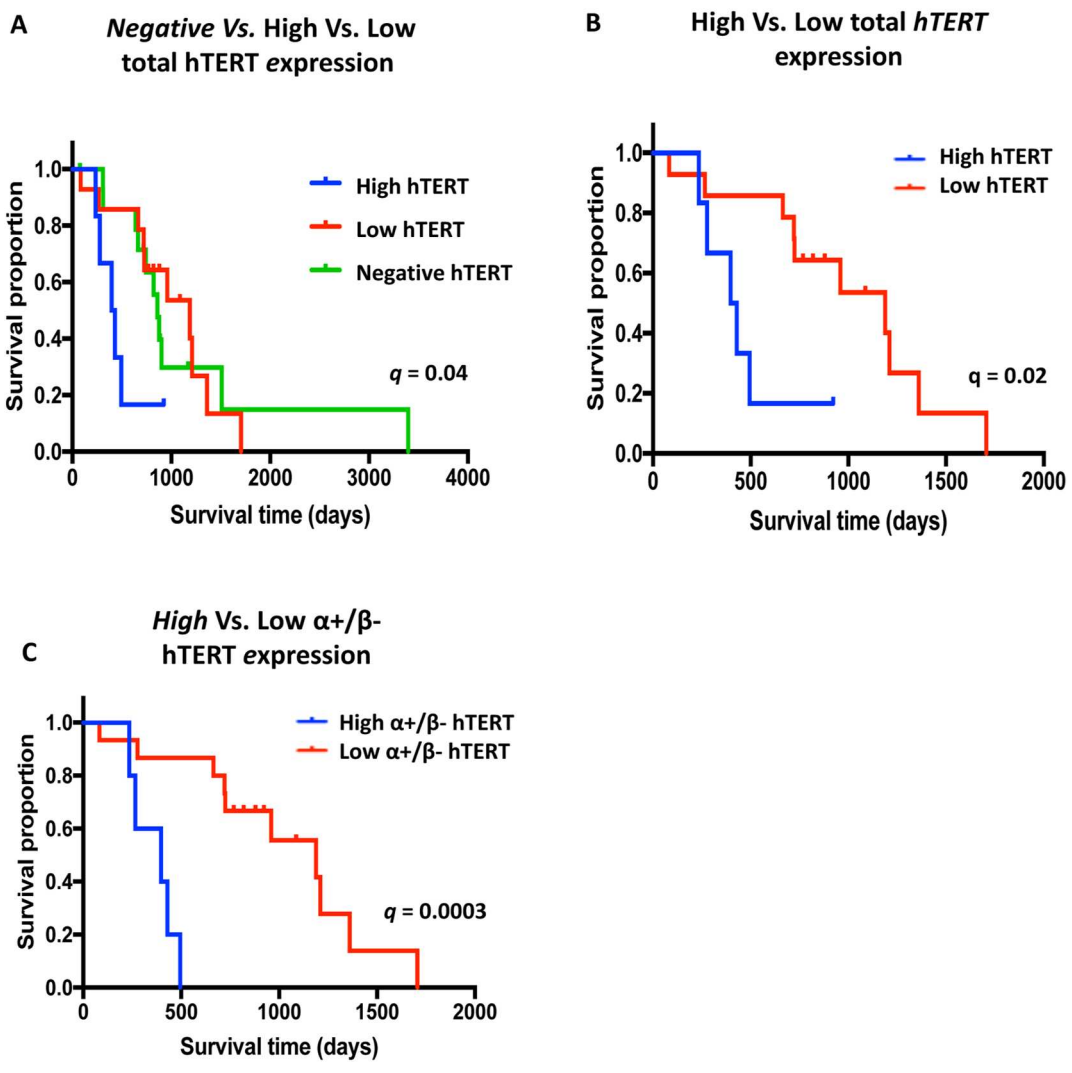


Figure 6