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Research article

Direct DNA binding by BRCA1 on β-hCG promoter and its clinical implications

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ABSTRACT

Objective: The role of β-hCG in breast cancer is largely unknown, this study aims to analyse the gene expression and clinical implications of β-hCG and its isoforms in various cancers focussing particularly in Breast Invasive Carcinoma (BRCA). A mechanistic approach deciphering the transcriptional regulation of β-hCG by BRCA1 was also explored.

Methods: Data from various comprehensive gene expression platforms like UALCAN, GEPIA2, GENT2, TIMER2, LinkedOmics, and STRING were used to analyse the expression of β-hCG and its clinical implications; Immunohistochemistry and ELISA for β-hCG expression analysis from human breast cancer patients; Electrophoretic mobility shift assay (EMSA) to analyse the direct binding of BRCA1 on β-hCG; Immunoblotting and Luciferase assay to understand the regulation of β-hCG by p53 were performed.

Results: Results from UALCAN and GENT2 gene expression cancer database revealed that TNBC subtypes and high-grade metaplastic carcinoma shows elevated expression of β-hCG and infiltration of various immune cells were also identified in BRCA by TIMER2. It was observed that most of the isoforms of β-hCG (CGB) are upregulated in breast cancers irrespective of hormonal status when BRCA1 gene is mutated according to TIMER2. Similar results were observed with Lymphoid neoplasm diffuse large B-cell lymphoma (LGG) and DLBC (Brain lower grade glioma) when BRCA1 is mutated. These results correlate with our earlier reports indicating expression of β-hCG in BRCA1 defective condition. We have also identified direct binding of BRCA1 on β-hCG promoter.

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Conclusion: All these findings demonstrate the importance of β-hCG as a potential target in BRCA1-deficient carcinomas.

1. Introduction

The mammary gland undergoes several intricate transformational changes during a woman's life; hormones play a major role in keeping physiological homeostasis, the deregulation of which may cause tumorigenesis in these tissues. During pregnancy, elevated levels of various hormones like human Chorionic Gonadotropin (hCG), estrogen, etc. help in the development and differentiation of breasts. Early full-term pregnancy as well as multiparity are associated with decreased risk of breast cancer [1,2]. On the contrary, in women who are carriers of BRCA1 and BRCA2 mutations, multiple pregnancies may increase the risk of development of breast cancers [3]. Also, it has been reported that BRCA1 mutation carriers have considerably higher serum levels of β-hCG than non-mutant carriers [4]. Though BRCA1 represses ER-α transcription in both ligand-dependent and independent manner, most of the BRCA1 defective cancers are ER-α negative [5,6].

Human Chorionic Gonadotropin (hCG) consists of an α subunit (CGA) and a β subunit (CGB) [7]. The α subunit of hCG is common to Thyroid Stimulating Hormone (TSH), Follicle Stimulating Hormone (FSH), and Luteinizing Hormone (LH). CGA is encoded by a single copy gene located on chromosome 6. There are six isoforms of β-hCG subunit which are encoded by six non-allelic genes on chromosome 19 [8]. The α and β polypeptides of hCG are synthesised as distinct mRNA transcripts and assembly of these peptides occur in the endoplasmic reticulum (ER) during the post-translational process [9–11]. Besides the abundant expression of hCG in Gestational Trophoblastic Disease (GTD) and germ cell tumors, β form of hCG has been extensively reported in many non-trophoblastic tumors. Several *in-vitro* studies have demonstrated the tumorigenic potential of β-hCG. In prostate cancer cell lines, β-hCG induced cell migration and invasion [12]. In bladder cancer cell lines, silencing of β-hCG expression led to a significant reduction in growth rate, and in colorectal cancer cell lines, knockout of β-hCG reduced cell migration and invasion [13–15].

β-hCG plays a pivotal role in the regulation of metastasis in epithelial ovarian cancer by triggering the ERK/MMP-2 signaling pathway [16]. In epithelial ovarian cancer, the CGB5 gene promotes tumor growth of OVCAR-3 cells and vasculogenic mimicry formation by tumor xenografts via luteinizing hormone receptor (LHCGR) signal transduction pathway [17]. Earlier we had reported that β-hCG could induce tumor progression in BRCA1 defective breast cancers through the TGFβRII pathway and BRCA1 expression is found to be reduced due to its gene promoter hypermethylation in GTD where hCG expression is high [18–20]. This study systematically evaluates the correlation of various isoforms of β-hCG particularly CGB5 and CGB7 with clinicopathological features and prognosis in patients with BRCA by comprehensive analysis of the data extracted from The Cancer Genome Atlas (TCGA). *In-vitro* studies showed that β-hCG expression is correlated to specific BRCA1 mutation and BRCA1 has been shown to have direct binding with β-hCG promoter. Our results suggest that β-hCG has a significant predictive role in BRCA1 deficient breast cancers and that it requires further investigation in a larger cohort of patient samples.

2. Materials and methods

Various bioinformatic tools were used to assess the functional significance of β-hCG expression status in BRCA defective breast cancers. The clinical significance of the BRCA1 mutations in the tissue microarray was obtained from the ClinVar database.

2.1. Gene expression analysis of β-hCG isoforms from various databases

UALCAN web portal (The **U**niversity of **AL**abama at Birmingham **CAN**cer data analysis Portal) (http://ualcan.path.uab.edu/index.html) was used to analyse the expression profile of different isoforms of CGB (CGB5 and CGB7) since it has already been reported that CGB5 and CGB7 are most prevalent forms of mRNA seen in breast cancer, among major subclasses and histological types in BRCA that utilizes the data deposited in TCGA. Transcripts per million (TPM) were used to depict the gene expression [21]. **GEPIA2** (Gene Expression Profiling Interactive analysis: http://gepia2.cancer-pku.cn/) is a server that allows users to analyse the differential expression of genes in samples from TCGA and GTEx data sets. Here, we employed this tool to analyse the expression of different CGB isoforms in tumor and normal samples across different cancer types and also stage plots of CGB isoforms (CGB5 and CGB7) in BRCA were derived from GEPIA2. CGB isoform expression ($\log_2$(TPM + 1) was compared across various tumor types with match TCGA normal data and results were demonstrated as heat map [22]. Expression of CGB isoforms in different grades, histology, and major subtypes of breast cancer was analysed using **GENT2** (Gene Expression database of Normal and Tumor tissues http://gent2.appex.kr/gent2/) [23]. **TIMER2** (http://timer.cistrome.org/) was used to analyse the correlation of BRCA1 mutation with CGB expression across various cancer types [24]. Here, we have employed **LinkedOmics** (http://linkedomics.org/login.php) portal, which utilizes multiomics HiseqRNA data from TCGA, to analyse correlated genes with different CGB isoforms using the LinkFinder module. The LinkInterpreter module helps in analysing the different pathways associated with these genes. The LinkInterpreter module was used to generate a bar plot depicting the pathways associated with positively and negatively correlated genes of CGB5. Pearson test was used to analyse the correlation of genes with CGB5 [25]. We have used the String protein interaction network platform (https://string-db.org/) to analyse the interaction of BRCA1 with the positively and negatively correlated genes obtained from the LinkFinder module with the CGB5 gene. The correlation analysis of CGB5 and the mutated genes detected by NGS was analysed by **cBioPortal** (https://www.

cbioportal.org/). Three data sets were mainly used for analysis: Breast Invasive Carcinoma TCGA Provisional (1108 patients), Breast Invasive Carcinoma TCGA Cell 2015, Breast Cancer Metabric Nature 2012 & Nat commun 2016 (2509 patient samples) [26].

2.2. Cell culture

Triple-negative breast cancer cell line MDA-MB-231 was obtained from ATCC. Monocyte cell line THP-1 and HEK293T cell lines were obtained from RGCB central cell repository. HCC1937 breast cancer cell line was a kind gift from Dr. Grant McArthur, Peter MacCallum Cancer Centre, VIC, Australia. p53-overexpressed MDA-MB-231 cell line was generated using lentiviral plasmid pRRLNeo-pEF1a-p53ash-mCerulean (Addgene#69579). Lipofectamine (#L3000008, Invitrogen, USA) was used to generate lentiviral particles in the HEK293T cell line. Details of the plasmids are given in the Supplementary Table 1. Transduction of lentiviral particles to the MDA-MB-231 cell line was carried out using polybrene 8 μg/mL (#TR-1003-G, Sigma, USA). Selection of stable clones was done using 200 μg/ml of G418 (#10131-035, Gibco-BRL, Grand Island, NY, USA). Transfection for luciferase assay in HEK293T cell line was performed using Lipofectamine (#L3000008, Invitrogen, USA). THP-1 and HCC1937 cell lines were cultured in RPMI-1640 (#P03-7310, Pan Biotech, Germany) and MDA-MB-231 DMEM (#12100046 Gibco-BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS) (#10270106 Gibco-BRL, Grand Island, NY, USA), 100 U/ml Pen strep cocktail (# 15240062, Invitrogen, USA) in a 5 % CO_2 incubator at 37 °C. Cell lines cells used in the study were STR profiled for authentication.

2.3. Human tissue samples

A total of eleven formalin-fixed paraffin-embedded (FFPE) breast cancer tissues were collected from the Department of Pathology, Government Medical College, Thiruvananthapuram, Kerala, India. These tissues are procured from the patients who are treatment naïve and undergoing primary surgery for breast cancer at the department of surgery, Government Medical College, Thiruvananthapuram, Kerala, India. The exclusion criteria are Patients who are positive for Hepatitis C/HIV/HBsAg and Patients who have undergone any of the treatment modalities for cancer. Immunohistochemistry slides were analysed independently by two board certified pathologists. The study was approved by the Institutional Human Ethics Committee, at both Government Medical College, Thiruvananthapuram, Kerala (HEC.No. 07/13/2019/MCT) and Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala (IHEC No: IHEC/1/2019/13).

2.4. Tissue microarray

Human tissue microarray (#TMAFC1418, US Biolab, Rockville, United States**)** was used for Immunohistochemistry (IHC) analysis. The array consisted of 25 samples (11 cases of Breast Infiltrating duct carcinoma, 13 cases of Ovary cancer, and normal tissues of breast, liver, kidney, and ovary) with a total of 80 cores. The tissue array demographics and clinical significance has been detailed in Supplementary Table 2.

2.5. Immunohistochemical analysis (IHC)

The IHC analysis for FFPE tissues and Tissue microarray (TMA) was analysed by IHC PathnSitu's polyexcel HRP/DAB detection system (#PEH002, PathnSitu, USA) photographed using the compound microscope (U-LH100HG, Olympus, Tokyo, Japan). Quick Score for Immunohistochemical analysis was calculated as previously described elsewhere [27,28]. Two independent, highly experienced pathologists manually assessed and scored the immunohistochemically stained sections. The scoring criteria have been tabulated in Supplementary Table 3.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The serum hCG levels of serum samples collected from breast cancer patients were analysed by ELISA (#ab100533 & #ab178633 Abcam, MA, USA) as per the manufacturer's protocol.

2.7. Mutational analysis for BRCA1

To confirm the mutation status in tissue samples that tested positive for β-hCG (4 samples), next-generation sequencing was done using the hereditary cancer panel, which screens 84 genes. Briefly, genomic DNA was isolated from the FFPE samples and subjected to NGS library preparation. The targeted genes of interest were enriched using the custom-made inherited cancer panel. Sequencing of the libraries was performed on Illumina Novaseq 6000 platform on the 150x2 paired end chemistry for an average of 53.4x coverage. The raw data was demultiplexed, adapter trimmed, and subjected to QC analysis. Only those reads having Phred Score greater than 30 were considered. The filtered reads were aligned to the reference genome GRCh37 and more than 90 % of the reads mapped to the reference genome.

2.8. DNA constructs and protein preparation

His Tag BRCA1(region 758-1057aa) bacterial expression vector was obtained from Addgene (#35085) and one of the fragments of

BRCA1 (504-802aa) was PCR amplified from a wild type BRCA1 containing vector pYFP-BRCA1 and subcloned into pET28b expression vector using *Nde*I (#R0111S, NEB, MA, USA) and *Hin*dIII (#R0111S, NEB, MA, USA) restriction enzymes. The sequences of the expression clones were verified by sequencing. These fragments were expressed in BL21DE3 and grown at 37 °C induced with 1 mM IPTG for 3 h at 30 °C before harvesting. Cell pellets sonicated in Sonication buffer (50 Mm NaH_2PO4, 300 mM NaCl, 5 Mm imidazole, 0.1 % Triton X-100, PMSF, Protease Inhibitor cocktail) clarified by centrifugation at 11000 rpm for 60 min at 4 °C. The His-Tagged proteins were purified by Ni-NTA column, snap frozen in multiple aliquots, and stored at −80 °C until use.

2.9. Western blotting

Immunoblotting of the His Tagged BRCA1 protein, MDA-MB-231and MD-MB-231-p53 overexpressed protein lysates was carried out using the standard wet transfer protocol and proteins were detected with respective antibodies against the overexpressed proteins. The details of the primary and secondary antibodies used have been listed in Supplementary Table 4. The blots were developed using the substrate solution (#T7103A, Western BLoT Hyper HRP Substrate, TAKARA, USA).

2.10. Electrophoretic mobility shift assay

DNA was isolated from the human breast cancer cell line HCC1937, β-hCG promoter region was PCR amplified using specific primers (Forward primer 5′-AAGCCCCTGCCGGGCATAC-3′ and Reverse primer: 5′-TGGATCGCGGGGAAGG-3′) (Supplementary Table 5). Gel purified β-hCG promoter DNA amplicon fragment was quantified and labelled using γP32 using polynucleotide kinase enzyme (#M0201S, NEB, MA, USA).

Purified protein and labelled DNA binding were performed in a 20 μl reaction buffer containing 20 mM Tris (pH 8.0), 50 mM NaCl, 4mM $MgCl_2$, 0.1 % Triton X-100, 10 % glycerol and incubated for 30 min at room temperature. After the samples were incubated, they were run in pre-electrophoresed 5 % polyacrylamide gel in 0.5 X TBE for 2 h at 4 °C. The gels were vacuum dried and imaged in a phosphoimager (Typhoon FLA 9500, GE Health Care).

2.11. Immunofluorescence

Cells were grown in 24 well plates over the coverslip and fixed with acetone: methanol (1:1) at −20 °C for 15 min. Cells were then blocked with 3 % BSA in PBST and incubated overnight with primary antibodies. After overnight incubation, unbound antibodies were washed with 1X PBS. Further fluorescent conjugated secondary antibodies were incubated at room temperature for 1 h. DAPI (#10236276001, Roche, Germany) was used as a counter stain for nucleic acid staining followed by mounting and observed under a confocal microscope.

2.12. Statistical analysis

The difference in gene expression among the major subclasses and histology types in BRCA was calculated using a student's t-test. Survival analysis of patients with ER-negative cancer subtype based on CGB5 expression was done by Kaplan -Meier curve with p values from log-rank test. Wilcoxon test was used to analyse the statistical significance between BRCA1 mutation and CGB expression, p-value <0.05 was considered significant. Pearson and Spearman's tests were used for correlation analysis of the LinkedOmics data set. The correlation between different immune cell types and CGB7 were analysed by Spearman's test and $p < 0.05$ was considered statistically significant. *In-vitro* experiments were carried out in triplicates and error bars are expressed as mean ± SD. ANOVA (One-way or two-way) and non-parametric student *t*-test was used to determine the difference between two or more experimental groups. Statistical analysis was performed using the GraphPad Prism (Version 8.0) and statistical significance is indicated as (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

3. Results

3.1. Expression of β-hCG isoforms in major subclass and histology types of breast invasive carcinoma

β-hCG subunit is encoded by six genes CGB1, CGB2, CGB3, CGB5, CGB7 and CGB8. The allelic forms of CGB3 and CGB7 are CGB9 and CGB6. CGB3 is now referred to as CGB, it is located close to the LHβ gene (CGB4). Previous studies from our lab have shown enriched expression of CGB5 and CGB7 in BRCA1 defective breast cancer both *in-vitro* and *in-vivo*. Hence, we analysed the expression of CGB5 and CGB7 in major subclasses (Fig. 1 A and C) and also among different tumor histology subtypes (Fig. 1B and D) in the UALCAN cancer database. Among the major subclasses with TNBC (Triple negative breast cancer) subtypes, the CGB5 transcript was overexpressed in TNBC-(UNS) unspecified (Fig. 1A). When compared to Normal and HER2+, the TNBC subtypes exhibited higher expression of CGB5 (Normal Vs HER2+ $p = 0.0005$), (Normal Vs TNBC-UNS (Unspecified) $p = 0.02$), (HER2+ Vs TNBC-BL1 (Basal Like 1) $p = 0.01$), (HER2+ Vs TNBC-MSL (Mesenchymal stem cell like) $p = 0.04$), HER2+ Vs TNBC-M (Mesenchymal) ($p = 0.02$), HER2+ Vs TNBC-UNS $p = 0.01$) and among TNBC subtypes TNBC-UNS exhibited higher expression of CGB5 (TNBC-BL1 vs TNBC-UNS $p = 0.03$), TNBC- IM (Immunomodulatory) Vs TNBC-UNS $p = 0.03$), TNBC-M vs TNBC-UNS $p = 0.04$). In all other isoforms, CGB1, CGB2, CGB, and CGB8, TNBC-BL2 showed a high expression (Data not shown) when compared to normal and other subtypes except for CGB7 where a high expression was observed in normal samples when compared to other subtypes (Fig. 1C). A high expression of CGB5 was

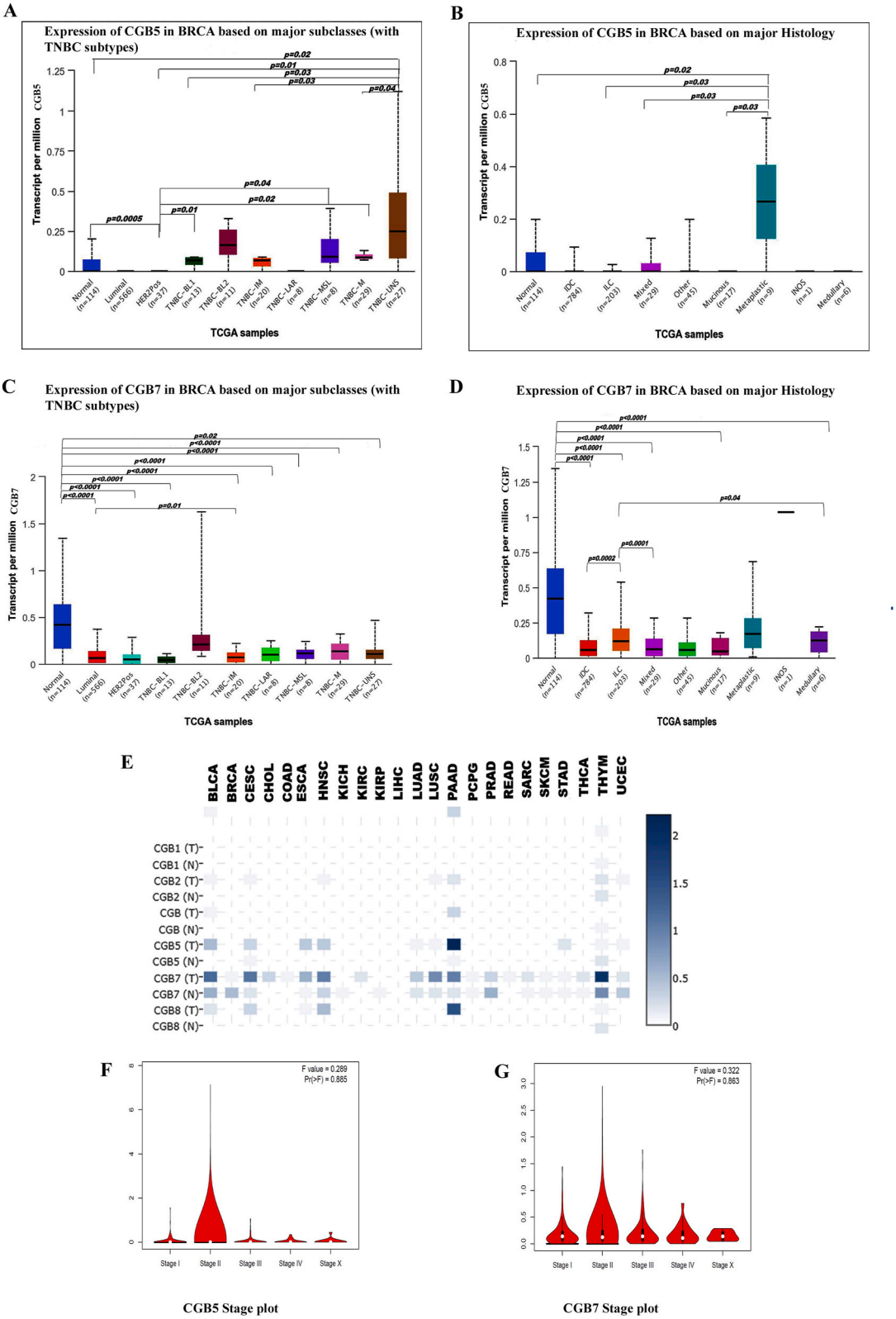

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Fig. 1. Expression analysis of CGB5 and CGB7 in Breast Invasive carcinoma using UALCAN: (A and C) Expression of CGB5 & CGB7 in the major subclass of breast cancer with TNBC subtypes (B and D) Expression of CGB5 & CGB7 in major histological subtypes of BRCA. **Expression analysis using GEPIA2:** (E) Heat map showing the expression of, CGB1, CGB2, CGB, CGB5, CGB7 and CGB8 isoforms in TCGA Normal and tumor (F and G) Expression of CGB5 and CGB7 in different stages of breast cancer.

observed in metaplastic carcinoma than in other histological subtypes (Fig. 1B). We analysed the TCGA expression data of different isoforms of β-hCG (CGB1, CGB2, CGB, CGB5, CGB7, CGB8) among tumors and matched normal in different cancer types using GEPIA2 (Fig. 1E). High expression of CGB5 and CGB7 transcripts was observed in PAAD (Pancreatic adenocarcinoma) and THYM (Thymoma) respectively. Low to moderate expression of different isoforms of β-hCG transcripts were observed in the rest of the cancers and normal samples analysed. The expression of CGB5 (Fig. 1F) and CGB7 (Fig. 1G) in different stages of BRCA was plotted using GEPIA2.

Using the GENT2 platform, we analysed the expression of CGB5 in different Grades (Fig. 2 A) and histology of breast cancer (Fig. 2 B). CGB5 was found to be overexpressed in grade 3 and grade 2 when compared to grade 1 (Fig. 2 A). A high expression of CGB5 was observed in poorly differentiated samples (Fig. 2 B) as well as in ER-negative (Fig. 2C), PR-negative (Fig. 2 D), and Her2-negative cancers (Fig. 2 E). BRCA1 mutated cancers are usually high grade poorly differentiated tumors which are mainly ER, PR, and Her2 negative, this could be a reason for the upregulation of CGB5 in these samples [29]. Also, ER-negative breast cancers positive for CGB5 expression showed poor prognosis when compared to ER-positive breast cancers (Fig. 2F).

Breast Invasive Carcinoma patients with higher expression of CGB, CGB5, and CGB8 are associated with poor prognosis.

Gene outcome of CGB, CGB5, and CGB8 was analysed in different tumor types using TIMER2. The overall survival related to CGB, CGB5 (Fig. 3A and B), and CGB8 expression was also analysed which showed poor prognosis in BRCA ($p < 0.05$*) (Fig. 3D). BRCA-basal type with higher expression of CGB5 showed a worse prognosis ($p < 0.01$ **) (Fig. 3C). TIMER2 employs a Cox proportional hazard model to analyse the outcome of gene expression. CGB, CGB5, and CGB8 showed an increased risk with Z score ($p < 0.05$, $Z > 0$) in BRCA but only CGB5 showed an increased risk in BRCA-basal type. Only isoforms of CGB with statistically significant increased risk are represented.

3.2. Correlation of β-hCG and BRCA1 in other cancer types

Earlier we reported a high expression of β-hCG in BRCA1 mutated breast cancer *in-vitro* and *in-vivo* [19]. Here, we analysed the differential expression of β-hCG isoforms in correlation to the BRCA1 mutation status in different types of cancers. The heat map of β-hCG isoform expression in the presence of BRCA1 mutation is represented in (Fig. 3E). The heat map depicts the log2 fold changes of differential expression of β-hCG isoforms in each cancer type. BRCA and its subtype BRCA-Her2 both had significant levels of CGB expression (Fig. 3F and G), but BRCA-Basal and BRCA-LumA had higher levels of CGB2 and CGB5, respectively (Fig. 3I and J), CGB5 expression was also observed in BRCA (Fig. 3 K). Additionally, CGB2 expression was seen in DLBC (Lymphoid neoplasm diffuse large B-cell lymphoma) (Fig. 3H). Furthermore, LGG (Brain lower grade glioma) showed a higher expression of CGB7 (Log2 fold change: higher level in mutants $p < 0.05$, $\log2FC > 0$) (Fig. 3 L). CGB1 and CGB8 did not show any significant correlation with BRCA1 mutation in any of the tumor types ($p > 0.05$) (Data not shown).

3.3. The link between CGB isoforms and other genes in BRCA

The different isoforms of β-hCG (CGB, CGB1, CGB2, CGB5, CGB7, and CGB8) was analysed to see the correlation with other genes using RNAseq data from LinkedOmics. Positively and negatively correlated genes of CGB5 are represented (Fig. 4 A). Data of other isoforms of β-hCG analysis are not shown. Red dots indicate positively correlated genes and green dots indicate negatively correlated genes. Gene enrichment analysis using the GSEA tool and GO analysis for biological process elucidated that positively correlated genes are mainly involved in the collagen metabolic process, extracellular structure organization, cell surface receptor signaling pathway involved in heart development, hormone metabolic process, and others which are represented in (Fig. 4 B), whereas negatively regulated genes are involved in the regulation of DNA metabolic process, DNA templated transcription elongation, ncRNA transcription, gene silencing, etc. Heatmaps were used to show the top 50 significantly upregulated and downregulated genes (Fig. 4C and D). We have also performed a string analysis of positively and negatively correlated genes of the CGB5 isoform which depicted them to be associated with the BRCA1 gene.

3.4. Infiltration of immune cells and its link with β-hCG in breast invasive carcinoma

Infiltration of immune cells upon β-hCG expression was analysed in BRCA using the TIMER2 platform which also links data from other sources like CIBESORT, XCELL, QUANTISEQ, MCP-COUNTER, TIDE, and EPIC. Given that β-hCG is an immune suppressor we thought of analysing its immunological association with breast cancer. The association between CGB7 and different immune cell types was analysed, and tumor purity was negatively associated with CGB7 expression indicating a high expression in BRCA ($p < 0.0001$).

Cancer-associated fibroblast showed positive correlation (0.179, $p < 0.0001$), Macrophage M2 showed negative correlation (−0.148, $p < 0.0001$), T-cell $CD4^+$ cells showed negative correlation (−0.249, $p < 0.0001$), T cell $CD8^+$ cells showed negative correlation (−0.108, $p < 0.001$), Myeloid-derived suppressor cells (MDSC) showed positive correlation (0.102, $p < 0.001$), T cell regulatory (Tregs) showed negative correlation (−0.151, $p < 0.0001$) (Fig. 4 E).

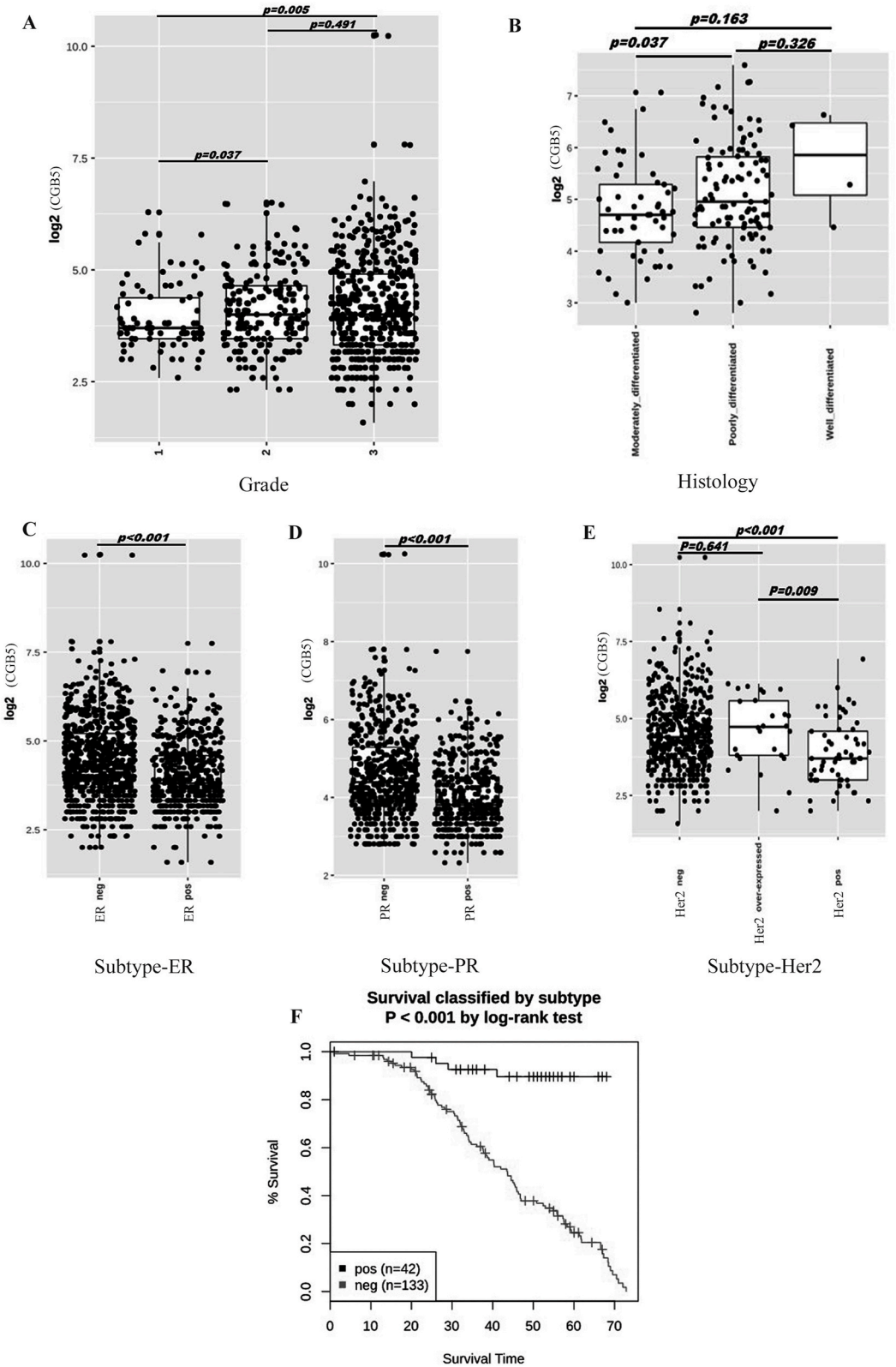


Fig. 2. Expression analysis of CGB5 using GENT2: (A) Expression of CGB5 in different grades of breast cancer (B) Expression of CGB5 in different histology types (C, D and E) Expression of CGB5 in ER-negative, PR-negative and Her2-negative breast cancer subtype (F) CGB5 positive ER-negative patients showing poor survival analysed by GENT2.

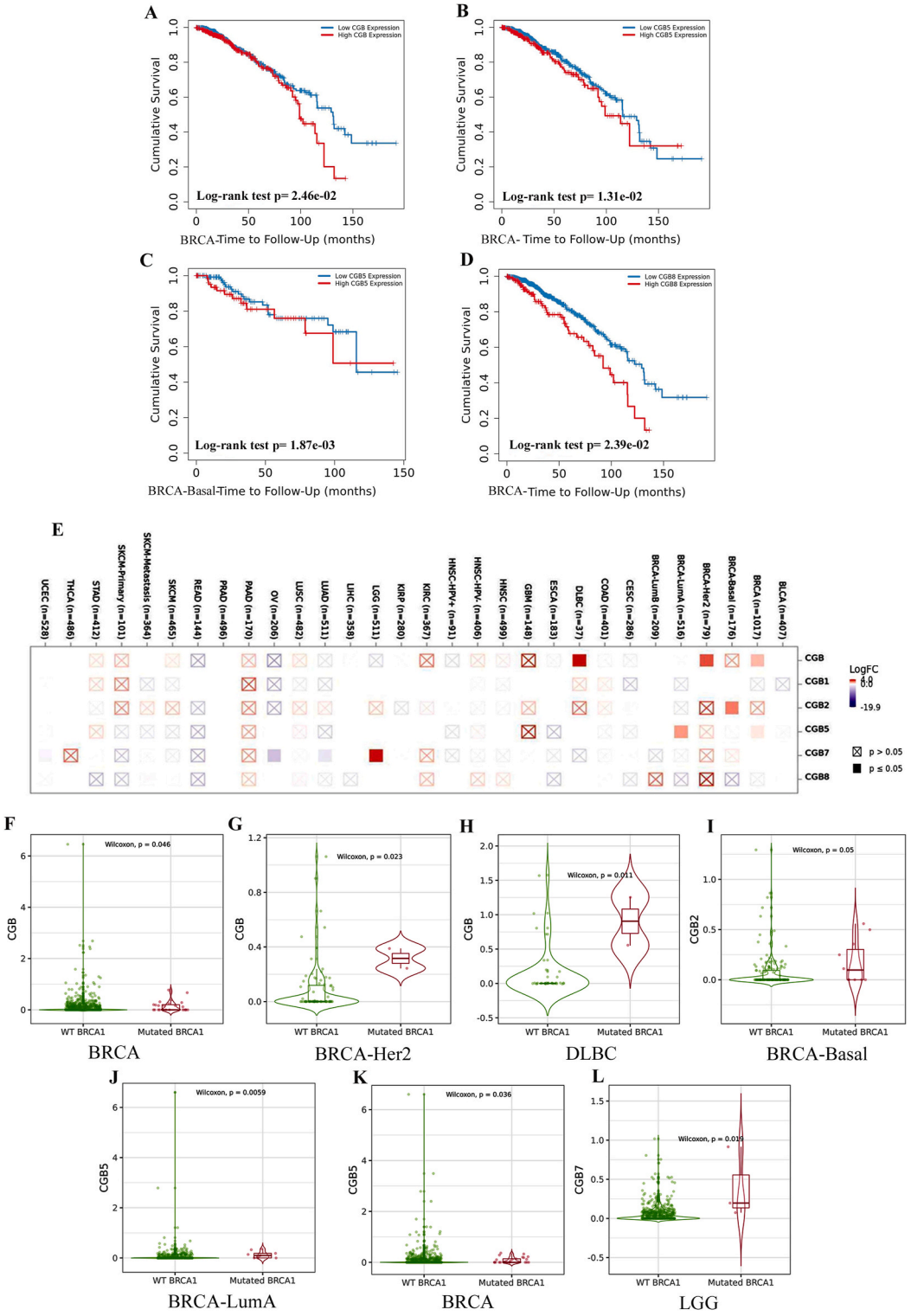


Fig. 3. Gene outcome of β-hCG isoforms in Breast Invasive Carcinoma (TIMER2): (A) Cumulative survival of CGB in breast invasive carcinoma, BRCA n = 1100, log-ranktest p = 2.46e-02 * (B) Cumulative survival of CGB5 in breast invasive carcinoma n = 1100, log-ranktest p = 1.31e-02 * (C) Cumulative survival of CGB5 in BRCA-basal n = 191, log-ranktest p = 1.87e-03 ** (D) Cumulative survival of CGB8 in BRCA n = 1100, log-ranktest p = 2.39e-02 * **Expression of β-hCG in correlation to BRCA1 mutation analysed by TIMER2:** (E) Heat map showing the differential expression of CGB isoforms across various tumor types with red box indicating higher expression in mutants (higher level in mutants ($p < 0.05$,log2FC > 0) (F, G and H) Differential CGB expression level in BRCA (n = 1017), BRCA-Her2 (n = 79) & Lymphoid neoplasm diffuse large B-cell lymphoma DLBC (n =

37) (I) Differential CGB2 expression level in BRCA-Basal (n = 176) (J and K) Differential CGB5 expression level in BRCA-LumA (n = 516) & BRCA (n = 1017) (L) Differential CGB7 expression level in LGG (n = 511). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Expression of β-hCG in breast cancer tissues and serum samples

Immunohistochemical analysis showed β-hCG expression in 4 out of the 11 breast cancer tissues analysed. β-hCG expression was observed to be cytoplasmic (Fig. 5A). In tissue microarray, β-hCG expression was observed in 4 out of 7 BRCA1 mutated breast cancer tissues and 2 out of 4 BRCA1 wild type tissues (Fig. 5C representative images shown), and among ovarian cancer tissues β-hCG expression was observed in 4 out of 6 BRCA1 mutated tissues and 3 out of 7 BRCA1 wild type tissues (Fig. 5D representative images shown). The β-hCG H-score based on the intensity and percentage of positive cells was calculated which showed a significant difference in β-hCG H-score between BRCA1 mutated samples than BRCA1 wild type samples ($p < 0.05$) in both breast cancer and ovarian cancer samples (Fig. 5E and F). The clinical significance of each mutation in breast cancer and ovarian cancer tissues was examined by ClinVar (Supplementary Table 6) and it was observed that not all BRCA1 mutations will lead to the expression of β-hCG. However, serum levels of β-hCG were low in all the breast cancer patient samples (Fig. 5 G).

3.6. BRCA1 mutation analysis in breast cancer tissues

Next generation sequencing using a hereditary cancer panel that screened 84 genes revealed that the tissue samples possessed mutations in several genes, whilst one of the tissues also possessed a BRCA1 mutation (Supplementary Table 7). TCGA data set analysis of mutated genes detected by NGS did not show any significant correlation with β-hCG other than BRCA2 and BARD1 (Supplementary Table 8).

3.7. BRCA1 directly binds to the promoter of β-hCG

We have previously reported that BRCA1 could inversely regulate the expression of β-hCG [30]. To identify the direct binding of BRCA1 to β-hCG promoter, the fragment of BRCA1 which was previously known to bind DNA, amino acids (504–802) and (758–1057) [31], were expressed in E.coli and further purified using His tag (Supplementary Figs. S1A and B). We performed EMSA using these fragments and found out that the fragment (504–802) alone exhibited direct binding with the promoter of β-hCG (Fig. 6B).

3.8. Role of p53 in the regulation of β-hCG expression

We have also identified the role of p53 in the regulation of β-hCG. Luciferase activity of both the isoforms of β-hCG was induced upon transfection of wildtype p53 (Fig. 6D and E) in HEK293T cells (Supplementary Fig. S1C). Western blot analysis of β-hCG upon stable overexpression of p53 in the MDA-MB-231 cell line showed overexpression of β-hCG and the expression of BRCA1 was downregulated by the negative feedback mechanism (Fig. 6F and G).

3.9. Macrophages accumulate hCG

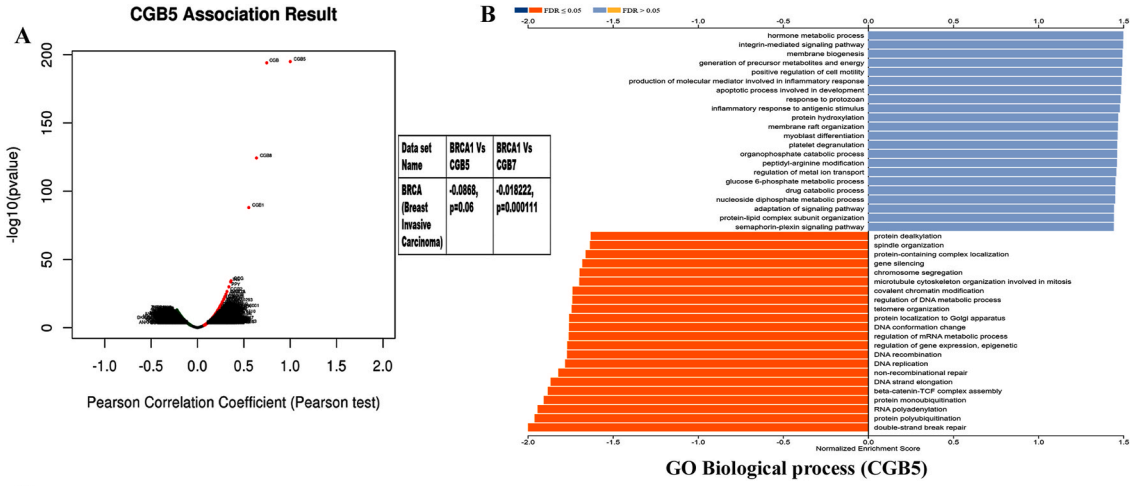
Immunohistochemical analysis of breast cancer tissues showed positive β-hCG staining in macrophages. Further, to analyse human macrophage accumulation of β-hCG, we exogenously supplemented β-hCG in increasing concentrations (60IU–500IU) to phorbol 12-myristate 13-acetate (PMA) treated THP1 cells which are differentiated macrophages. Immunofluorescence analysis was done to analyse the uptake of β-hCG and enhanced fluorescence was observed for THP1 cells treated with 250 and 500IU of β-hCG for 30 min (Fig. 6H and I). Exogenous supplementation of β-hCG for 24 h resulted in diminished fluorescence which could be attributed to the degradation of β-hCG by macrophages. A significant uptake of β-hCG was observed in cells treated with 250 and 500IU of β-hCG compared to the control.

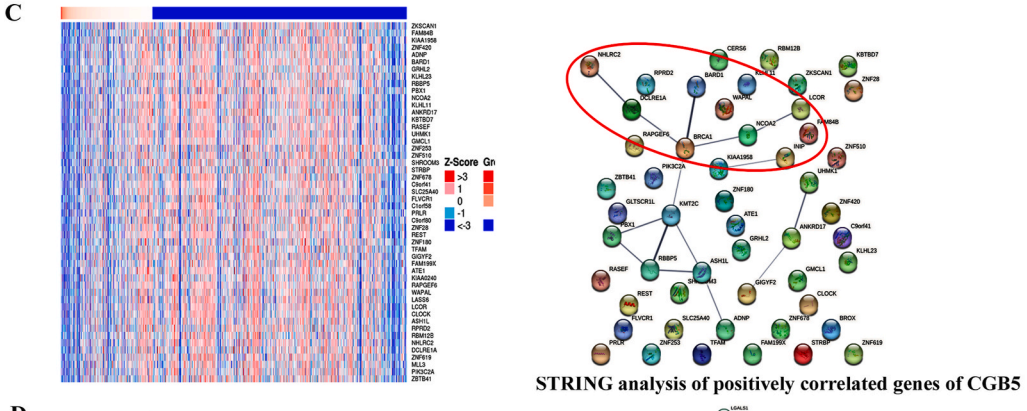
4. Discussion

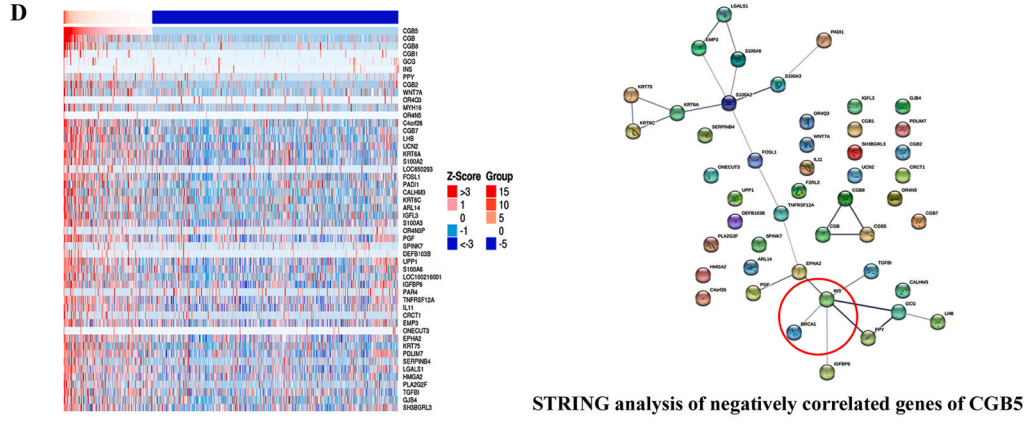
We have already demonstrated from our laboratory that β-hCG expression is associated with BRCA1 status both *in vitro* in BRCA1 mutated cell lines and *in vivo* in BRCA1 conditional knockout mouse models. β-hCG induces migration, and invasion and shows high metastatic potential in BRCA1 defective breast cancer. Tumor progression by β-hCG in BRCA1 defective breast cancer is achieved by TGF-βRII signaling [19].

Considering the findings of this work, we first examined the expression of β-hCG (CGB) and its isoforms in breast cancer and their correlation with the BRCA1 gene. For this, we utilized different extensive platforms to analyse the expression of β-hCG in various tumor types and its correlation with the BRCA1 gene. The lower expression of β-hCG in the BRCA data set could be due to the lower percentage of BRCA1-mutated breast cancers. Lack of information regarding BRCA1 mutation status in data sets such as UALCAN, GENT2, and GEPIA2 makes it challenging to analyse the correlation between β-hCG and mutation status of BRCA1, however, analysis with TIMER2 provided inclusiveness of BRCA1 mutated patients.

A high expression of CGB5 was observed in the TNBC subtype when compared to normal and other subtypes in breast cancer.







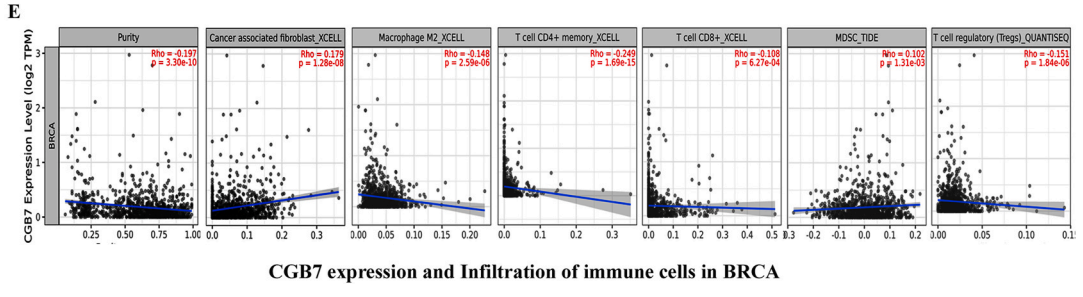


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Fig. 4. Co-expressed genes of CGB5 in Breast Invasive Carcinoma (BRCA) using LinkedOmics: (A) Co-expressed genes with CGB5, Pearson test was used for correlation analysis (red dots indicate positive correlation and green dots indicate negative correlation (B) Bar Plot showing the significantly enriched genes of GO (biological process) of CGB5 in BRCA (C) Heat map showing the 50 positively regulated genes with CGB5 and STRING analysis showing the interaction of BRCA1 with positively regulated genes (D) Heat map showing the 50 negatively regulated genes with CGB5 and STRING analysis showing the interaction of BRCA1 with negatively regulated genes. **Association between tumor infiltrating immune cells and CGB7 in Breast Invasive Carcinoma (BRCA) in TIMER2:** (E) Cancer associated fibroblast (CAF) and Myeloid derived suppressor cells (MDSC) showed positive correlation and Macrophage M2, T-cell $CD4^+$, T cell $CD8^+$, T cell regulatory (Tregs) showed negative correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Among the histological subtypes, a high expression was observed in metaplastic carcinoma. This could be due to the majority of TNBC cancers being observed with BRCA1 mutation and metaplastic breast cancer is also associated with BRCA1 mutation. Triple negative breast cancers are more likely to be associated with BRCA1 mutation [32] which could be the reason for the upregulation of β-hCG isoforms in TNBCs. Furthermore, BRCA1 mutated breast cancer belongs to the TNBC-BL1 and TNBC-BL2 subtypes which also showed a higher expression of CGB5 and CGB8. Moreover, TNBC-M and TNBC-MSL subtypes are enriched with TGFβRII and Wnt/β-catenin signaling [33]. It has been previously reported the overexpression of β-hCG in BRCA1 defective breast cancer and also β-hCG binds to the TGFβRII receptor and mediates SMAD signaling to promote tumorigenesis in BRCA1 defective breast cancer [18,19]. Canonical Wnt/β-catenin signaling induces slug-mediated repression of BRCA1 [34] which could be one of the reasons for the overexpression of CGB5 and CGB8 in TNBC-M and TNBC-MSL subtypes. More than 90 percent of Metaplastic carcinoma are associated with TNBC and two-thirds of metaplastic breast carcinoma are associated with BRCA1 promoter hypermethylation which also leads to lower expression of BRCA1 [35]. BRCA1 mutated breast cancer patients also display metaplastic carcinomas, particularly chondroid metaplastic carcinomas [36]. β-hCG genes could have a significant impact on triple negative breast cancers regardless of their low expression. In stage II breast cancer, there was notable upregulation of β-hCG expression. Additionally, high levels of CGB5 expression were detected in breast tumors characterized by grade 3 and poor differentiation. CGB5 expression analysis in the GENT2 platform among the subtype classification, also showed that CGB5 expression was high in ER, PR, and HER2-negative tumors and survival analysis also showed poor prognosis in ER-negative samples with CGB5 expression. Survival analysis of CGB, CGB5, and CGB8 showed a worse prognosis with increased risk ($p < 0.05$) in BRCA by TIMER2. CGB5 showed a poor prognosis in the BRCA-basal type.

Next, we analysed the expression of β-hCG isoforms with mutation status of BRCA1 in various tumors. β-hCG was found to be significantly upregulated in BRCA, DLBC, and LGG with BRCA1 mutation. Most of the isoforms of CGB, CGB2, and CGB5 are upregulated in BRCA, BRCA-basal, BRCA-Her2, and BRCA-LumA irrespective of hormonal status when the BRCA1 gene is mutated. Similarly, when BRCA1 mutated cancers were analysed, CGB7 and CGB8 were upregulated in LGG, and CGB (CGB3) was expressed in DLBC. These results correlate with our earlier reports of high expression of β-hCG in BRCA1 defective condition. However, the CGB isoforms were more expressed in BRCA when compared to other tumor types. This highlights the importance of targeting β-hCG in BRCA1 mutated conditions and the potential of β-hCG to emerge as a marker in BRCA1 mutated breast cancers.

The analysis of the positively and negatively correlated genes with β-hCG isoforms by LinkedOmics database showed that although BRCA1 was not one of the top 50 negatively correlated genes, CGB7 was more significantly negatively correlated ($p = 0.0001$), and CGB5 with less significance ($p = 0.06$). BARD1, RMI1, NBN, and DCLRE1A which are among the top 50 negatively correlated genes are closely linked with BRCA1 and help in maintaining genomic integrity. Most of the pathways that β-hCG negatively regulates in BRCA are involved in maintaining genomic integrity, such as DNA replication and recombination, as well as the regulation of DNA metabolic processes, which indicates the tumorigenic potential β-hCG in breast cancer. One of the important genes negatively regulated by β-hCG is BARD1 which forms a stable heterodimer with BRCA1. This association helps BRCA1 with nuclear localization and also helps in sequestering BRCA1 to the DNA damage foci [37]. According to the reports from our earlier studies BRCA1 transcriptionally represses the expression of β-hCG, which underpins the analysis results from LinkedOmics which showed a negative expression of BARD1 with β-hCG. As a result of paucity of BARD1 expression which leads to impeding the transport of BRCA1 to the nucleus and thereby inhibiting the BRCA1-mediated transcriptional regulation could be a reason for the upregulation of β-hCG. Similarly, other genes like DCLRE1A are also linked with BRCA1 and help in maintaining genomic instability. Among the positively regulated genes of β-hCG, INS has a direct association with BRCA1. INS or Insulin like growth factors have an important role in breast tumorigenesis and BRCA1 targets the insulin-IGF1 axis. BRCA1 suppresses the IGF-I-R (Insulin like growth factor I receptor) [38].

As β-hCG is an immune suppressor, we analysed the infiltration of various immune cells upon expression of CGB7 in BRCA, and TcellCD4+, TcellCD8+, Tregs, and Macrophage M2 showed a significant negative correlation whereas cancer-associated fibroblast, MDSC showed significant positive infiltration. Previously from our laboratory, we have reported the reduction in CD4+/CD8+ ratio in tumors followed by differentiation of activated $CD4^+$ cells to CD4+/CD25+/FOXP3+ regulatory T-cells in BRCA1 deficient β-hCG overexpressed condition [39].

Further on, we analysed the β-hCG expression in breast cancer tissues and human tissue microarray BRCA1 mutated samples wherein 36 and 57 % of the samples showed cytoplasmic β-hCG expression respectively. Among ovarian cancers, 66 % of BRCA1 mutated tissues showed β-hCG expression. Clinical significance was not observed for all BRCA1 mutations. Only pathogenic variations in exon 11 or frameshift deletions or insertion, resulting in premature translational termination, before exon 11 results in the expression of β-hCG. Except for the ovarian cancer mutation (185del AG) in exon 2, positive expression of β-hCG was not seen, which could be attributed to a certain hypomorphic activity of the BRCA1 gene. Expression of β-hCG was observed in BRCA1 wild type tissue also which could be possibly due to the BRCA1 promoter hypermethylation in these tissues. Our previous studies have already reported the inverse correlation of BRCA1 promoter hypermethylation and β-hCG expression in GTD tissues [40].

Our EMSA results confirmed the direct interaction between BRCA1 and β-hCG wherein the amino acid fragment (504–802) of

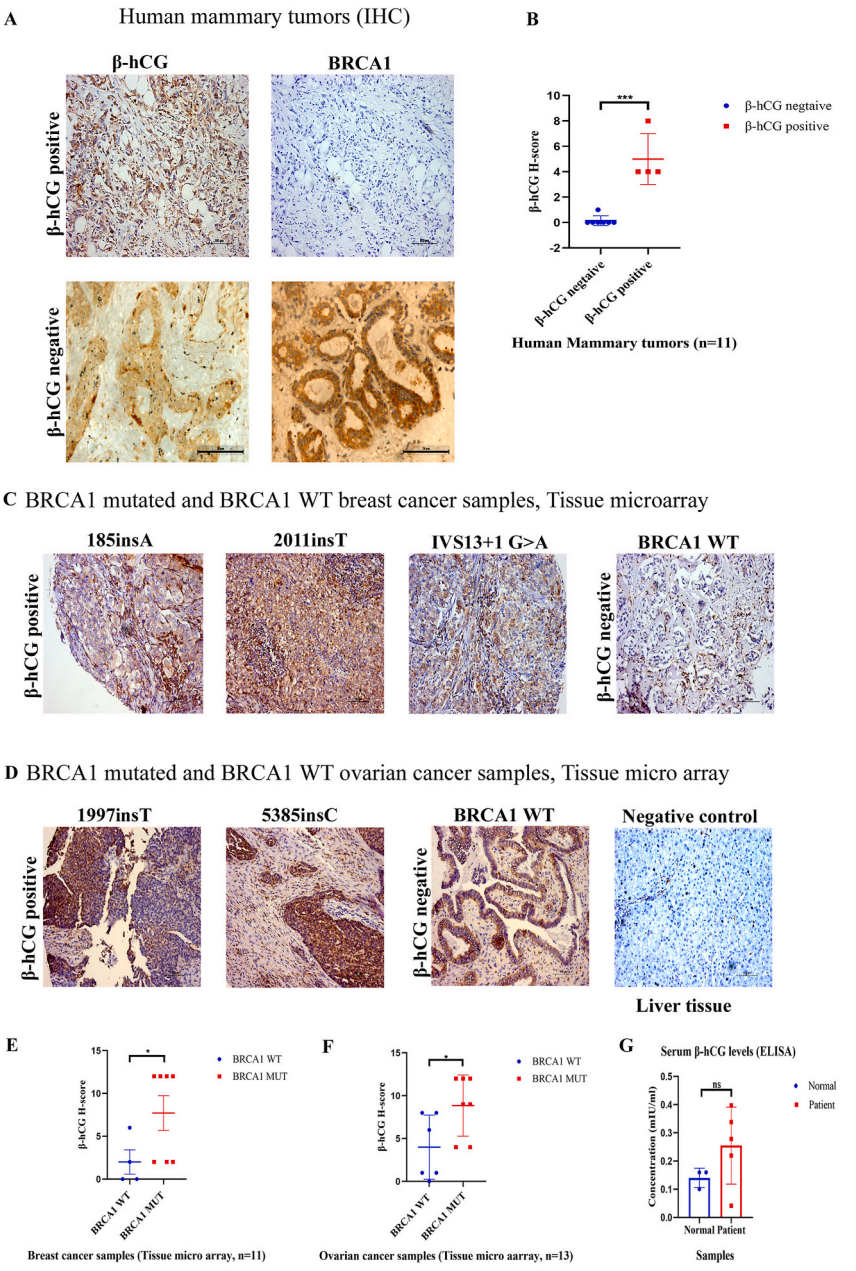

Fig. 5. Expression of β-hCG in human mammary and ovarian carcinomas by Immunohistochemistry: (A) Expression of β-hCG in breast cancer samples (n = 11) (B) β-hCG H-Score analysed between β-hCG positive and negative samples. **Tissue microarray analysis:** (C) β-hCG positive expression in BRCA1 mutated and BRCA1 wild type breast cancer tissues showing no expression (D) BRCA1 mutated ovarian cancer tissues stained positive for β-hCG and BRCA1 wild type ovarian cancer tissues showing no expression, also liver tissue which served as negative control (E and F) β-hCG H-Score analysed between BRCA1 wild type and BRCA1 mutated samples (G) Serum levels of β-hCG in breast cancer patients. (IHC images are 10x and 40× magnification with scale bar 100 μm and 20 μm, statistical significance: $p \leq 0.05$ *, $p \leq 0.001$***, $p > 0.05$ ns)

BRCA1 showed direct binding with the β-hCG promoter. The fragment (504–802) belongs to exon 11 of BRCA1, a mutation that results in overexpression of β-hCG since the mutation in this region could abolish the inverse regulation of BRCA1 over β-hCG.

Tissue samples that showed positivity for β-hCG by IHC were subsequently analysed by NGS to identify BRCA1 mutation. As indicated in Supplementary Table 8, only genes exhibiting a Spearman correlation with a p value of less than 0.01 have been taken into consideration. Since only one out of 4 tissue samples showed BRCA1 mutation, we further analysed whether the mutated genes detected by NGS (Supplementary Table 7) in the remaining samples had any correlation with β-hCG by using TCGA data set analysis from cBioportal (Supplementary Table 8). TCGA data set analysis showed significant negative correlation between the BRCA1, BRCA2, and BARD1 with β-hCG, and a positive correlation with SMARCB1, any of these genes are mutated in the 4 tissue samples that showed

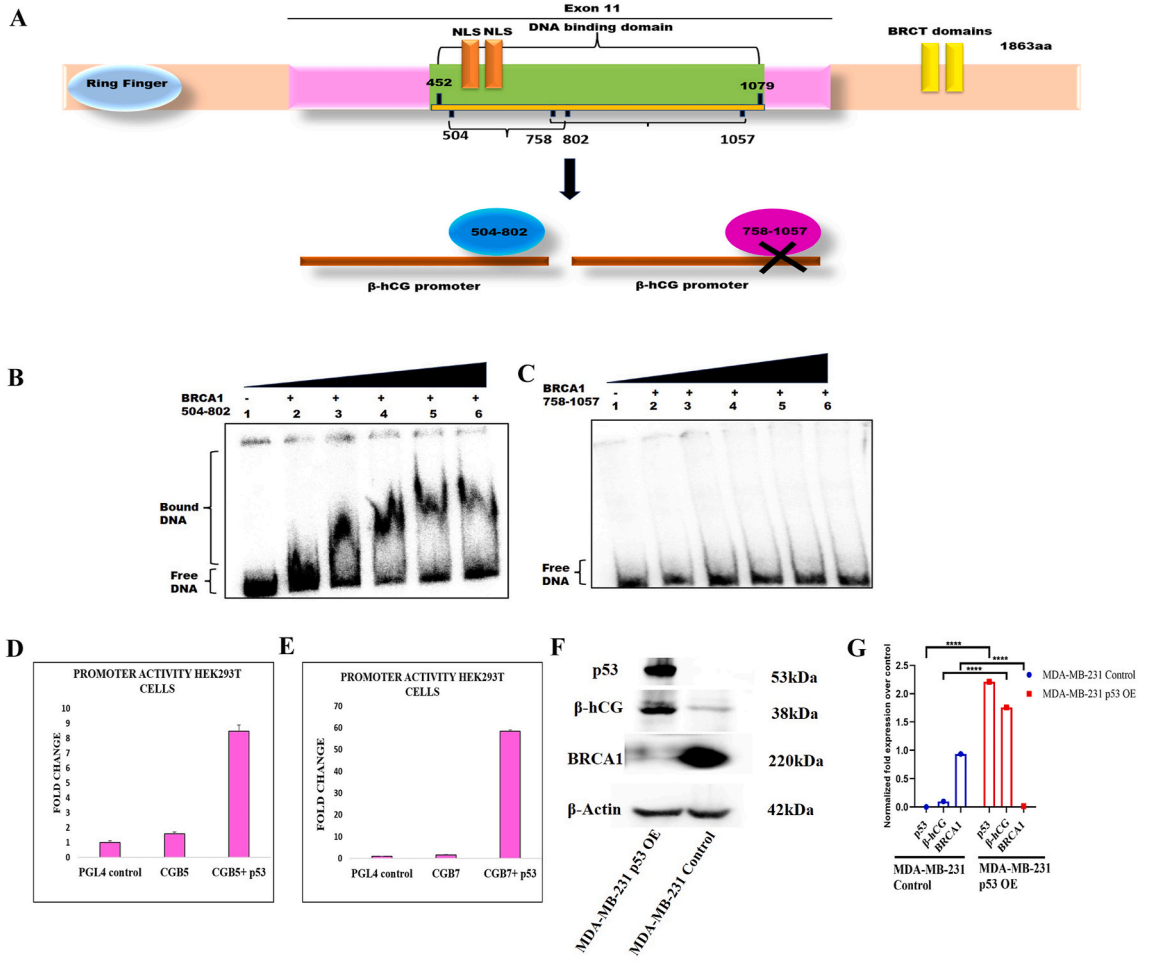

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Fig. 6. Direct binding of BRCA1 to β-hCG promoter:(A) Schematic representation of BRCA1 gene domain used for cloning and purification (B) EMSA Showing the binding of BRCA1 domain (504–802) on β-hCG promoter in increasing concentrations; Lane 1: 0 μM, Lane 2: 5 μM, Lane 3: 10 μM, Lane 4: 15 μM, Lane 5:20 μM, Lane 6: 25 μM (C) EMSA Showing no binding of BRCA1 domain (758–1057) on β-hCG promoter in increasing concentrations; Lane 1: 0 μM, Lane 2: 5 μM, Lane 3: 10 μM, Lane 4: 15 μM, Lane 5:20 μM, Lane 6: 25 μM. **Regulation of p53 by β-hCG:** (D and E) Relative luciferase activity of CGB5 and CGB7 upon overexpression of p53 in HEK293T cells (F and G) Western blot analysis of p53, β-hCG and BRCA1 upon stable overexpression of p53 in MDA-MB-231 cell line. **β-hCG uptake by macrophages:** (H and I) Immunofluorescence analysis of β-hCG uptake by THP-1 cells (60x, scale bar 20 μm, statistical significance: $p \leq 0.05$ *, $P \leq 0.01$**, $p > 0.05$ ns) Uncropped/non-adjusted immunoblots, Coomassie stained gel and autoradiogram are provided in the Supplementary Fig. S2 and Supplementary Fig. S3.

expression of β-hCG. The BRCA1 gene is closely associated with BRCA2 and BARD1, all of which are important in DNA damage repair. BRCA1 and BRCA2 are involved in the HR pathway, BRCA1 mainly helps in signal mediation during DNA damage whereas BRCA2 are repair initiators also known as effectors [41]. BRCA1-BARD1 heterodimer plays an important role in DNA repair, this heterodimer formation also helps in the retention of BRCA1 in the nucleus masking its nuclear export signal (NES) [42]. SMARCB1 which showed a strong positive correlation with β-hCG in TCGA analysis is also linked with the BRCA1/2 pathway genes [43]. SMARCB1 seemed to have a negative correlation with BRCA1 (Spearman correlation- −0.17; p value $9.39e^{-7}$). SMARCB1 mutation that we have seen in the tissue is in intron 4 which is a heterozygous splice region intron variant, and does not directly change the encoded amino acid sequence of the SMARCB1 protein (ClinVar Variation ID 825362). These results confirm the negative correlation between BRCA1 and β-hCG.

We have also demonstrated from our laboratory previously that β-hCG reprogrammes macrophages from M1 antitumorigenic to M2 pro-tumorigenic phenotype causing immune suppression and promoting tumorigenesis in BRCA1 deficient tumors [39]. Therefore β-hCG uptake by macrophages could be attributed to the differentiation of M1 macrophage to M2 macrophage.

We have also showed the regulation of β-hCG by p53 in breast cancer cell lines. Transfection of wild type p53 in the MDA-MB-231 cell line resulted in overexpression of β-hCG and downregulation of BRCA1 through a negative feedback mechanism. Also, by Luciferase assay it has been shown that p53 induces β-hCG. Earlier reports show the induction of β-hCG by p53 and its role in human reproduction [44], here we report the role of p53 in the induction of β-hCG in breast cancer cell line and downregulation of BRCA1 and further its role in tumorigenesis has yet to be validated. We have used p53 antibody which is a recombinant fusion protein containing a sequence corresponding to amino acids 1–393 of human p53 (NP_000537.3, ABclonal) that detects wild type but not mutant p53 protein in MDA-MB-231 cells. Therefore, we did not get expression of p53 in MDA-MB-231 control cells which contain R280K mutant p53 [45,46]. All these findings demonstrate the importance of β-hCG as a potential target in BRCA1-deficient carcinomas. Owing to the limited availability of BRCA1 mutation databases, a significant portion of the correlation between BRCA1 mutation was not analysed. Only information regarding available β-hCG isoforms was included. The reduced expression of β-hCG could be attributable to the fact that only 5–10 % of all breast cancers are hereditary with BRCA1 mutation, which limits the expression of β-hCG in BRCA. The reliance on databases with limited BRCA1 mutation data could constrain the robustness of the correlation analyses, underscoring the need for more comprehensive datasets to validate these findings further. However, the importance of β-hCG in BRCA1 deficient conditions cannot be neglected.

Ethics statement

The research study was subject to a comprehensive review and adhered to the principles outlined in the Declaration of Helsinki. The research protocol used in this study received approval from both the Institutional Human Ethics Committee (IHEC) of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, India with approval number IHEC/1/2019/13 and the Human Ethics Committee (HEC) at the Government Medical College, Thiruvananthapuram, Kerala, India with approval number HEC.No.07/13/2019/MCT. Written informed consent for study participation was acquired from all participants. Furthermore, all participants willingly provided consent for the publication of their case details and images, with measures in place to safeguard their anonymity. The study was carried out in strict adherence to all pertinent regulations and guidelines by Indian Council for Medical Research (ICMR).

Ethics approval

The study was approved by the Institutional Human Ethics Committee, at both Government Medical College, Thiruvananthapuram, Kerala (HEC.No. 07/13/2019/MCT) and Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala (IHEC No: IHEC/1/2019/13).

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

CRediT authorship contribution statement

Neethu Krishnan: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Methodology, Formal analysis, Data curation. **Neetha R L:** Writing – review & editing, Methodology. **Arathy V. Warrier:** Writing – review & editing, Methodology. **Induprabha Yadev:** Validation, Resources. **Jaimie Anandan:** Resources, Methodology. **Sankar Sundaram:** Writing – review & editing, Validation, Methodology. **Arathi Rajan:** Methodology. **Prianka Kumari:** Writing – review & editing. **Shreya Sara Ittycheria:** Writing – review & editing. **Manasa V.G:** Writing – review & editing. **Serbin Mohammed:** Resources. **Preethamol S:** Resources. **Rakesh Sathish Nair:** Writing – review & editing. **Priya Srinivas:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37064.

References

[1] P.F. Slepicka, S.L. Cyrill, C.O. Santos, Pregnancy and breast cancer : pathways to understand risk and prevention, Trends Mol. Med. 25 (2019) 866–881.
[2] J. Russo, R. Moral, G.A. Balogh, D. Mailo, I.H. Russo, The protective role of pregnancy in breast cancer, Breast Cancer Res. 7 (3) (2005) 131–142, https://doi.org/10.1186/bcr1029.
[3] H. Jernstrom, Pregnancy and risk of early breast cancer in carriers of BRCA1 and BRCA2, Lancet 354 (9193) (1999 Nov 27) 1846–1850, https://doi.org/10.1016/s0140-6736(99)04336-6.
[4] H. Deng, X. Wang, J. Wang, J.X. Day, B. Liu, Comprehensive analysis of serum tumor markers and BRCA1/2 germline mutations in Chinese ovarian cancer patient, Mol Genet Genomic Med 7 (6) (2019 Jun) e672, https://doi.org/10.1002/mgg3.672.
[5] Saijun Fan l, Yong Xian Ma, Chenguang Wang, Ren-Qi Yuan, Qinghui Meng, Ji-An Wang, Michael Erdos, Itzhak D. Goldberg, Webb Paul, Peter J. Kushner, Richard G. Pestell, Eliot M. Rosen, p300 Modulates the BRCA1 inhibition of estrogen receptor activity, Cancer Res. 62 (1) (2002) 141–151. Jan 1.
[6] A.M. Hosey, et al., Molecular basis for estrogen receptor α deficiency in BRCA1-linked breast cancer, J. Natl. Cancer Inst. 99 (2007) 1683–1694.
[7] J.L. Jameson, A.N. Hollenberg, Regulation of chorionic gonadotropin gene expression, Endocr. Rev. 14 (1993) 203–221.
[8] T. Otanis, F. Otanis, M. Krychs, D. Chaplin, I. Boimes, Identification of a promoter region in the CGP gene cluster, J. Biol. Chem. 263 (15) (1988 May 25) 7322–7329.
[9] M. Kanda, et al., Genetic fusion of an alpha-subunit gene to the follicle-stimulating hormone and chorionic gonadotropin- subunit genes : production of a bifunctional protein, Mol Endoclino 13 (11) (1999 Nov) 1873–1881, https://doi.org/10.1210/mend.13.11.0372.
[10] D.L. Blithe, Structure and Function of the Gonadotropin Free a Molecule, vols. 156–157, 1994.
[11] B.P. Peters, et al., A kinetic comparison of the processing and secretion of the alpha beta dimer and the uncombined alpha and beta subunits of chorionic gonadotropin synthesized by human choriocarcinoma cells, J. Biol. Chem. 259 (1984) 15123–15130.
[12] J. Yang, et al., Vaccination with the repeat β-hCG C-terminal peptide carried by heat shock protein-65 (HSP65) for inducing antitumor effects, Tumor Biol. 33 (2012) 1777–1784.
[13] Z. Li, C. Li, L. Du, Y. Zhou, W. Wu, Human chorionic gonadotropin β induces migration and invasion via activating ERK1/2 and MMP-2 in human prostate cancer DU145 cells, PLoS One 8 (2) (2013) e54592, https://doi.org/10.1371/journal.pone.0054592.
[14] B. Burczynska, et al., Stable knockdown of hCGβ mRNA expression in bladder cancer cells results in significant growth inhibition, Anticancer Res. 33 (2013) 3611–3614.
[15] J. Li, et al., B subunit of human chorionic gonadotropin promotes tumor invasion and predicts poor prognosis of early-stage colorectal cancer, Cell. Physiol. Biochem. 45 (2018) 237–249.
[16] W. Wu, et al., β-hCG promotes epithelial ovarian cancer metastasis through ERK/MMP2 signaling pathway, Cell Cycle 18 (2019) 46–59.
[17] S. Gao, et al., Effects of HCG on human epithelial ovarian cancer vasculogenic mimicry formation in vivo, Oncol. Lett. 12 (2016) 459–466.
[18] Satheesh Kumar Sengodan, Sreelatha K. Hemalatha, Revathy Nadhan, Thara Somanathan, Arun Peter Mathew, Arkadiusz Chil, Janusz Kopczynski, Rakesh Sathish Nair, P.S. Jerald Mahesh Kumar, β-hCG induced mutant BRCA1 ignites drug resistance in susceptible breast tissue, Carcinogenesis 40 (11) (2019 Nov) 1415–1426, https://doi.org/10.1093/carcin/bgz070.
[19] S.K. Sengodan, et al., BRCA1 regulation on β-hCG: a mechanism for tumorigenicity in BRCA1 defective breast cancer, Oncogenesis 6 (2017).
[20] J.V. Revathy, Satheesh kumar sengodan, K. Sreelatha, N. Hemalatha, BRCA1 promoter hypermethylation in human placenta: a hidden link with β-hCG expression, Carcinogenesis 41 (5) (2020 Jul 10) 611–624, https://doi.org/10.1093/carcin/bgz117.
[21] D.S. Chandrashekar, et al., UALCAN: an update to the integrated cancer data analysis platform, Neoplasia 25 (2022) 18–27.

[22] Z. Tang, B. Kang, C. Li, T. Chen, Z. Zhang, GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis, Nucleic Acids Res. 47 (2019) W556–W560.
[23] S.J. Park, B.H. Yoon, S.K. Kim, S.Y. Kim, GENT2: an updated gene expression database for normal and tumor tissues, BMC Med. Genom. 12 (2019).
[24] T. Li, et al., TIMER2.0 for analysis of tumor-infiltrating immune cells, Nucleic Acids Res. 48 (2020) W509–W514.
[25] S.V. Vasaikar, P. Straub, J. Wang, B. Zhang, LinkedOmics: analyzing multi-omics data within and across 32 cancer types, Nucleic Acids Res. 46 (2018) D956–D963.
[26] J. Gao, et al., Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal, Sci. Signal. 6 (2013).
[27] K.P. Padmavathy, et al., Clinical evaluation of proline, glutamic acid, and leucine-rich protein 1 expression in astrocytomas and correlations with the proliferation marker ki-67, J. Mol. Neurosci. 71 (4) (2021) 724–733, https://doi.org/10.1007/s12031-020-01690-w. Apr.
[28] D. Kaemmerer, et al., Comparing of IRS and Her2 as immunohistochemical scoring schemes in gastroenteropancreatic neuroendocrine tumors, Int. J. Clin. Exp. Pathol. 5 (3) (2012) 187–194.
[29] E. Honrado, J. Benítez, J. Palacios, The Pathology of hereditary breast cancer, Hered. Cancer Clin. Pract. 2 (3) (2004) 131–138, https://doi.org/10.1186/1897-4287-2-3-131.
[30] S.K. Sengodan, et al., BRCA1 Regulation on β -hCG : a Mechanism for Tumorigenicity in BRCA1 Defective Breast Cancer, 2017, https://doi.org/10.1038/oncsis.2017.75.
[31] T.T. Paull, D. Cortez, B. Bowers, S.J. Elledge, M. Gellert, Direct DNA binding by Brca1, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 6086–6091.
[32] H. Chen, J. Wu, Z. Zhang, Y. Tang, X. Li, Association between BRCA status and triple-negative breast cancer, Front. Pharmacol. 9 (2018 Aug 21) 909, https://doi.org/10.3389/fphar.2018.00909, eCollection 2018.
[33] B.D. Lehmann, et al., Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies, J. Clin. Invest. 121 (7) (2011 Jul) 2750–2767, https://doi.org/10.1172/JCI45014.
[34] Z. Wu, et al., Canonical Wnt signaling regulates Slug activity and links epithelial – mesenchymal transition with epigenetic Breast Cancer 1 , Early Onset (BRCA1) repression, Proc. Natl. Acad. Sci. U.S.A. 109 (41) (2012 Oct 9) 16654–16659, https://doi.org/10.1073/pnas.1205822109. Epub 2012 Sep. 24.
[35] B. Weigelt, C. Eberle, C.F. Cowell, C.K.Y. Ng, J.S. Reis-filho, More than a Special Type, Nature Publishing Group, 2014, https://doi.org/10.1038/nrc3637.
[36] M. Yamashita, et al., Metaplastic carcinoma of the breast and BRCA1 germline mutation : a case report and review, Hered. Cancer Clin. Pract. 19 (1) (2021 Jan 6) 3, https://doi.org/10.1186/s13053-020-00162-x.
[37] B.R. Henderson, The BRCA1 breast cancer suppressor : regulation of transport , dynamics , and function at multiple subcellular locations, Sci. Tech. Rep. 2012 (2012) 796808.
[38] H. Werner, BRCA1 : an endocrine and metabolic regulator, Front. Endocrinol. 13 (2022 Mar 31) 844575, https://doi.org/10.3389/fendo.2022.844575. eCollection2022.
[39] G.R. Varghese, et al., βhCG mediates immune suppression through upregulation of CD11b+Gr1+ myeloid derived suppressor cells, CD206+ M2 macrophages, and CD4+FOXP3+ regulatory T-cells in BRCA1 deficient breast cancers, Immunology (2023), https://doi.org/10.1111/imm.13673.
[40] R. Nadhan, et al., BRCA1 promoter hypermethylation in human placenta: a hidden link with β-hCG expression, Carcinogenesis 41 (2020) 611–624.
[41] Roy, et al., BRCA1 and BRCA2: different roles in a common pathway of genome protection, Nat. Rev. Cancer 12 (1) (2011 Dec 23) 68–78, https://doi.org/10.1038/nrc3181.
[42] Finger, et al., New concepts on BARD1: regulator of BRCA pathways and beyond, Int. J. Biochem. Cell Biol. 72 (2016 Mar) 1–17, https://doi.org/10.1016/j.biocel.2015.12.008.
[43] Laco, et al., SMARCB1/INI1-deficient sinonasal carcinoma shows methylation of RASSF1 gene: a clinicopathological, immunohistochemical and molecular genetic study of a recently described entity, Pathol. Res. Pract. 213 (2) (2017) 133–142, https://doi.org/10.1016/j.prp.2016.10.012. Feb.
[44] S. Sohr, K. Engeland, Cell Cycle the tumor suppressor p53 induces expression of the pregnancy-supporting human chorionic gonadotropin (hCG) CGB7 gene, Cell Cycle 10 (2011) 3758–3767.
[45] Hui, et al., Mutant p53 in MDA-MB-231 breast cancer cells is stabilized by elevated phospholipase D activity and contributes to survival signals generated by phospholipase, D. Oncogene 25 (55) (2006) 7305–7310, https://doi.org/10.1038/sj.onc.1209735. Nov 23.
[46] Hui, et al., Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53, Mol. Cell Biol. 24 (13) (2004 Jul) 5677–5686, https://doi.org/10.1128/MCB.24.13.5677-5686.2004.