**Title:** **The G-protein coupled estrogen receptor in breast tumors positively associates with ERα, and constitutes a clinically significant genomic target of estrogen in breast cancer cells.**

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**Abstract**

**Background:** GPER is a seven transmembrane G-protein-coupled estrogen receptor that mediates rapid estrogen actions. The rising popularity of GPER in the field of breast cancer research stems from the large volumes of data that have revealed- a) its association with clinicopathological variables, b) its role in epidermal growth factor (EGF)- like effects of estrogen, c) its potential as a therapeutic target or a prognostic marker, and d) tamoxifen agonism and endocrine resistance. GPER cross-talks with ERα. However, the literature portrays ambivalence in the nature of their association. Besides, the significance of their association in mammary epithelial cells, or breast tumors, is not clear. Despite the known regulation of GPER by hormones, the mechanism of estrogen-mediated regulation of GPER in breast cancer cells is not completely understood. The current investigation examines the relationship between GPER, and ERα in breast tumors, to understand the mechanistic basis, and to gauge its clinical significance. Given either negative or positive association between GPER and ERα expression in breast cancer, the current investigation also examines the association between GPER upCpGi methylation and ERα expression in breast tumors.

**Methods:** We mined The Cancer Genome Atlas (TCGA)-BRCA data to examine the relationship between GPER and ERα expression. GPER mRNA, and protein expression were analyzed in ERα-positive or -negative breast tumors using western blotting, or RT-qPCR. The Kaplan-Meier Plotter (KM) was employed for survival analysis. The effect of E2, or propylpyrazoletriol (PPT, an ERα agonist) stimulation on GPER expression was studied in MCF-7 and T47D cells, with or without tamoxifen or ERα knockdown. ERα-binding to the GPER locus was explored by analysing ChIP-seq data (ERP000380), in silico prediction of estrogen response elements, and chromatin immunoprecipitation (ChIP) assay. We have also examined the methylation of the GPER upCpGi in ERα-positive and -negative breast tumors using targeted bisulfite sequencing.

**Results:** Clinical data revealed significant positive association between GPER and ERα expression in breast tumors. The median GPER expression in ERα-positive tumors was significantly higher than ERα-negative tumors. High GPER expression was significantly associated with longer overall survival (OS) of patients with ERα-positive tumors. *In vivo* experiments showed a positive effect of E2 on GPER expression. E2 induced GPER expression in MCF-7 and T47D cells; an effect mimicked by PPT. Tamoxifen or ERα-knockdown blocked the induction of GPER. Estrogen-mediated induction was associated with increased ERα occupancy in the upstream region of GPER. Furthermore, treatment with 17β-estradiol or PPT significantly reduced the IC50 of the GPER agonist (G1)-mediated loss of MCF-7 or T47D cell viability. The targeted bisulfite sequencing of the tumor samples revealed that the DMR in the upCpGi of ERα-negative samples were hyper-methylated compared to that in the ERα-positive samples, which were hypo-methylated.

**Conclusion:** In conclusion, GPER is positively associated with ERα in breast tumors, and induced by estrogen-ERα signalling axis. Estrogen-mediated induction of GPER makes the cells more responsive to GPER ligands. GPER expression is negatively associated with methylation in upCpGi is an indirect evidence that ERα and GPER expression are positively associated in breast tumors. More in-depth studies are warranted to establish the significance of GPER-ERα co-expression, and their interplay in breast tumor development, progression, and treatment.

**Keywords:** GPER, ERα, TCGA, TAM, PPT