Original Research

Transmembrane G protein–coupled receptor 30 (GPR30) gene polymorphisms in Korean Women with Ovarian Cancer: An experimental study

Ji Young Park[1], Gun Oh Chong[2], Yoon Hee Lee[2], Hyun Jung Lee[2], Chul Min Park[3],

Eun Hye Lee[1], Dae Gy Hong[2]

[1]*Department of Pathology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea*
[2]*Department of Obstetrics and Gynecology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea*
[3]*Department of Obstetrics and Gynecology, School of Medicine, Jeju National University, Jeju, Republic of Korea*

Summary

Purpose: To compare genetic distribution of fourteen (14) G protein–coupled receptor 30 (*GPR30*) single-nucleotide polymorphisms (SNPs) in patients with ovarian cancer or benign gynecological disease. M*aterials and Methods:* We examined 117 Korean females with postoperative specimens available for DNA extraction. We used predesigned PCR/Sanger or Sequencing Primer and TaqMan® SNP Genotyping Assays for SNP genotyping of the G*PR30* gene and performed immunohistochemical staining for assessing GPR30 protein expression. We analyzed linkage disequilibrium (LD) and evaluated differences in genotype and allele frequency between the two groups. *Res*ults: We observed no differences in genotype distribution and allele frequency between the two groups. Two LD blocks were determined in both groups. All specimens of both groups revealed intensive staining with a high percentage of positive cells exhibiting GPR30 expression. Conclusions: This study could not demonstrate differences in the genetic distribution of fourteen GPR30 gene polymorphisms in Korean women with ovarian cancer and disease-free controls.

Key words: Ovarian cancer; G protein–coupled receptor 30; Single-nucleotide polymorphism; Korean.

Introduction

Ovarian cancer is a very challenging cancer in terms of determining early diagnosis and treatment. The intra-abdominal location of the ovary and the preponderance of advanced disease at initial presentation make characterization of changes in the ovarian surface epithelium (OSE) consistent with intraepithelial neoplasia difficult to judge [1]. Thus, little information is available about the very early molecular and genetic events associated with ovarian carcinogenesis.

Although causes of ovarian cancer are not entirely elucidated, several risk factors associated with changes in sex hormones during a woman's lifetime have been reported. For example, studies have demonstrated that estrogen, one of the primary sex hormones, affects ovarian cell proliferation and have suggested that women who receive hormone replacement therapy (HRT) during menopause with estrogen for 5 or more years are more likely to develop ovarian cancer because of the exposure of the OSE to estrogen [2]. Both estrogen receptor (ER) and progesterone receptor (PR) are critical in promoting the effects of estrogen and progesterone on proliferation and apoptosis in ovarian cancer cells [3].

Estrogens, mainly estradiol (E2), exert their classically slow biological effects through the nuclear alpha (ERα) and beta (ERβ) receptor isoforms [4, 5]. Either ERs at the plasma membrane or transmembrane G protein–coupled receptor 30 (GPR30) mediate rapid non-genomic responses to estrogen [6].

A recent study reported that GPR30 binds to estradiol with an affinity approximately 10 times higher than that of ERα [7]. GPR30 is localized in the cell membrane as well as in the intracellular membranes [6,8]. In gynecological diseases, several studies demonstrated a significant correlation between GPR30 expression and disease. For example, Long et al. suggested that abnormal expression of GPR30 might be involved in malignant transformation, invasion, and metastasis of endometrioid ovarian cancer [9]. Additionally, another report indicated GPR30 was preferentially expressed in high-risk epithelial ovarian cancer and correlates with lower survival rates [10].

We hypothesized that, because cumulative estrogen exposure could contribute to the development of ovarian cancer, GPR30 gene single-nucleotide polymorphisms (SNPs) could affect susceptibility to ovarian cancer development. Hence, this study aimed to examine the prevalence of fourteen GPR30 gene SNPs in Korean women with ovarian cancer.

Table 1. — *The characteristics of 14 SNPs selected in this study*

SNPs	Gene region	Genome position[a]	Transcript change	Protein change
rs3808350	2 kb upstream	1086257	c.-1434A > G	
rs3808351	5'-UTR	1087023	c.-668G > A	
rs3802141	5'-UTR, Exon 3	1091720	c.-9T > C	
rs117290655	Exon 3	1091742	c.14C > T	Ser5Phe
rs34497267	Exon 3	1091758	c.30G > A	Val10=
rs11544331	Exon 3	1091775	c.47C > T	Pro16Leu
rs61735235	Exon 3	1092039	c.311C > G	Ala104Gly
rs61747089	Exon 3	1092111	c.383T > A	Val128Asp
rs4266553	3'-UTR	1093035	c.*179C > G	
rs1133041	3'-UTR	1093055	c.*199C > T	
rs12702047	3'-UTR	1093084	c.*228G > A	
rs10235056	3'-UTR	1093112	c.*256G > A	
rs76649173	3'-UTR	1093189-1093190	c.*333_*334delTC	
rs3808353	3'-UTR	1093336	c.*480G > A	

The SNP numbers are reference SNP ID in National Center for Biotechnology Information. [a]*Derived from the current versions of the reference human genome assembly (GRCh38).*
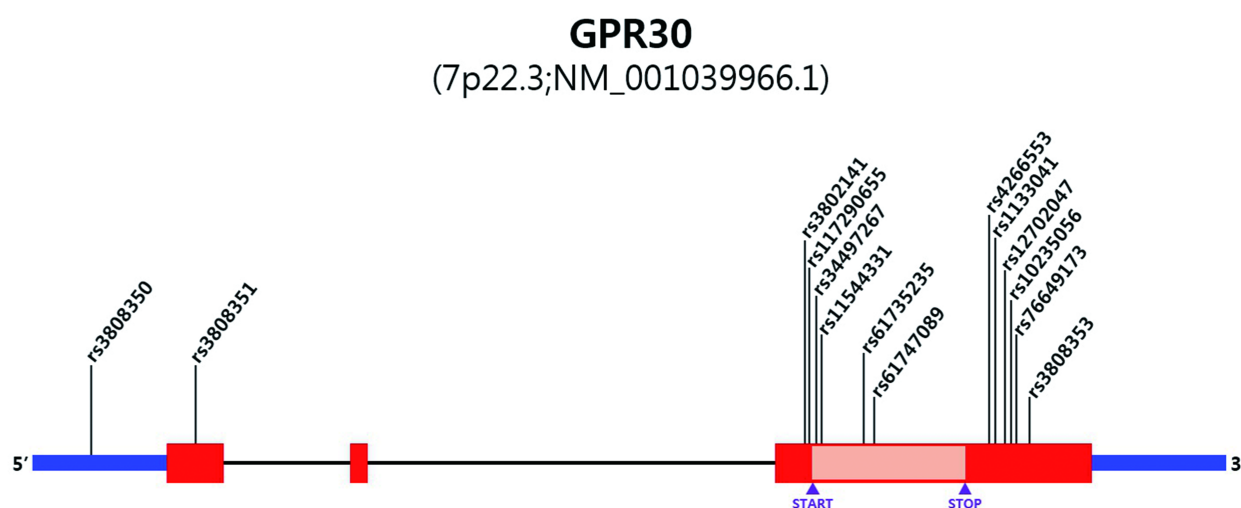

Figure 1. — Localization of selected fourteen (14) SNPs in the GPR30 gene region, including 1 kb upstream and downstream sequence (Chr7:1,085,807-1,094,815, GRCh38.p7). SNP numbers were assigned according to NCBI dbSNP. Red boxes = CDS (coding sequence); blue line = 5′-UTR or 3′-UTR; black line = intron; START and STOP indicate the translational start and stop codons, respectively.

Materials and Methods

Study Design and Population

We reviewed medical records of patients and compared the distribution of the genotype and allele frequencies of fourteen (14) GPR30 SNPs in patients with and without ovarian cancer.

We examined 117 Korean females who underwent surgical intervention for ovarian cancer or benign uterine diseases. The ovarian cancer group in this study comprised 48 females. The final pathological diagnosis was ovarian cancer with or without a gynecological benign disease such as benign uterine or ovarian diseases. The remaining 69 patients were diagnosed with benign uterine diseases without gynecological and/or other malignancies. Female patients with benign disease included 35 patients with uterine adenomyosis and 34 patients with uterine leiomyoma.

In this study, we targeted nine SNPs in the GPR30 gene by the Variation Viewer (https://www.ncbi.nlm.nih.gov/variation/view/) with minor allele frequencies (MAFs) $\geq$ 0.05. In addition, we included three SNPs with MAF between 0.01 and 0.05, and two missense variants (rs117290655, rs61735235, rs61747089) located in exon 3 that result in amino acid alterations within the GPR30 protein (Table 1; Figure1). Furthermore, we used Haploview software (Ver 4.2; http://www.broadinstitute.org/haploview/haploview) to perform linkage disequilibrium (LD) and constructed LD patterns using an algorithm designed by Gabriel et al. with
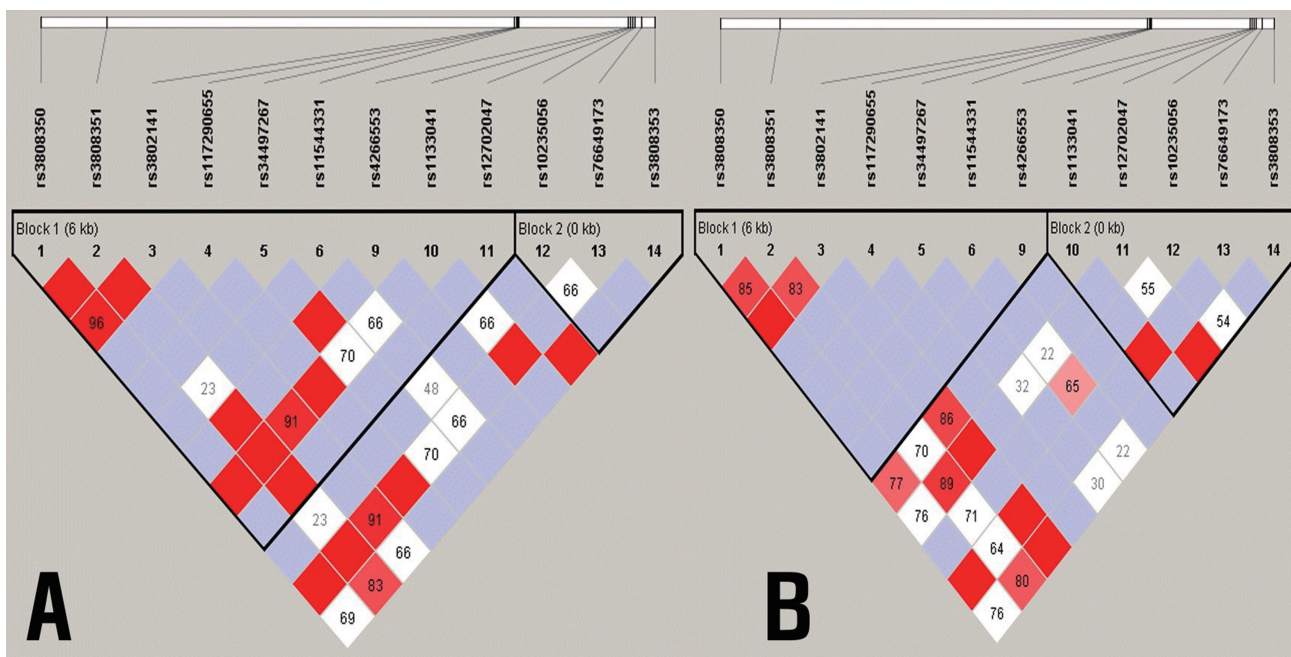
Figure 2. — Panel A = Benign (cancer-free) group; Panel B = Ovarian cancer group. The LD structure of the region of GPR30 according to Haploview (solid spine of LD; D′ > 0.8). Markers with LD [D′ < 1 and log of the odds (LOD) > 2] are shown in red through pink with color intensity decreasing with decreasing D′ value. Regions of low LD and low LOD scores (D′ < 1 and LOD < 2) are shown in white. The highlighted numbers indicate the D′.

Table 2. — *Clinical and pathological distribution of ovarian cancer*

	n = 48 (100%)
Stage, *n* (%)	
Ia	2 (4.17)
IC	1 (2.08)
IIA	5 (10.42)
IIB	3 (6.25)
IIC	2 (4.17)
IIIB	2 (4.17)
IIIC	31 (64.58)
IVB	2 (4.17)
Histology, *n* (%)	
Serous	32 (66.67)
Mucinous	3 (6.25)
Endometrioid	9 (18.75)
Clear cell	2 (4.17)
Others	2 (4.17)
Tumor grade (FIGO)	
1	4 (8.33)
2	3 (6.25)
3	41 (85.42)
Lymph node, *n* (%)	
Metastasis (+)	29 (60.41)
Metastasis (−)	17 (35.42)
Not sampled	2 (4.17)

MAF $\geq$ 1% [11].

DNA Extraction and Genotyping

We extracted genomic DNA from postoperative uterine tissue with the Qiagen QIAamp Fast DNA Tissue Kit (Qiagen, Valencia, CA) and determined DNA concentrations using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). In addition, we performed polymerase chain reaction (PCR) in 50 μL volumes using 100 ng of genomic DNA, 25 μL of 2× Qiagen Multiplex PCR Master Mix, 10 pmol of each primer (Qiagen) using a SimpliAmpT^M Thermal Cycler (Life Technologies, Carlsbad, CA). For twelve GPR30 SNPs, predesigned PCR/Sanger Sequencing primer pairs (Thermo Fisher Scientific, Waltham, MA) were used. The cycling parameters in this study were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 90 sec, 72 °C for 40 sec, and a final extension of 72 °C for 10 min. PCR products were purified using the GeneAll Expin Kit (GeneAll Biotechnology, Seoul, Republic of Korea). Sanger sequencing was conducted using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA) and an ABI PRISM 3730 automated sequencer (Applied Biosystems). Sequencing results were prepared using reference sequences (GPR30(GPER1)/NM_001039966/NP_001035055/ENST00000397092.5) and the alignment program BLAST V2.5.0 (NCBI) and the portion of variation that occurred was determined. In addition, rs3808350 and rs3808351 were genotyped using TagMan®-^{b}ased genotyping assay

Table 3. — *Distribution of GPR30 genotypes and alleles in ovary cancer and benign diseases*

	Ovary cancer, $n = 48$, (%)	Benign, $n = 69$, (%)	p
s3808350			
AA/GG/GA	11(22.9)/18(37.5)/19(39.6)	12(17.4)/25(36.2)/32(46.2)	0.68
A/G	41(42.7)/55(57.3)	54(39.1)/84(60.9)	0.33
rs3808351			
GG/AA/GA	32(66.7)/5(10.4)/11(22.9)	46(66.7)/1(1.4)/22(31.9)	0.07
G/A	75(78.1)/21(21.9)	114(82.6)/24(17.4)	0.24
rs3802141			
CC/TT/CT	21(43.8)/9(18.8)/18(37.5)	29(42.0)/12(17.4)/28(40.6)	0.94
C/T	60(62.5)/36(37.5)	86(62.3)/52(37.7)	0.54
rs117290655			
CC/TT/CT	46(95.8)/0(0)/2(4.2)	61(88.4)/1(1.4)/7(10.1)	0.33
C/T	94(97.9)/2(2.1)	129(93.5)/9(6.5)	0.1
rs34497267			
GG/AA/GA	46(95.8)/0(0)/2(4.2)	65(94.2)/0(0)/4(5.8)	0.52
G/A	94(97.9)/2(2.1)	134(97.1)/4(2.9)	0.52
rs11544331			
CC/TT/CT	46(95.8)/1(2.1)/1(2.1)	67(97.1)/0(0)/2(2.9)	0.46
C/T	93(96.9)/3(3.1)	136(98.6)/2(1.4)	0.33
rs4266553			
CC/GG/CG	47(97.9)/0(0)/1(2.1)	64(92.8)/0(0)/5(7.2)	0.39
C/G	95(99.0)/1(1.0)	133(96.4)/5(3.6)	0.21
rs1133041			
CC/TT/CT	25(52.1)/4(8.3)/19(39.6)	35(50.7)/8(11.6)/26(37.7)	0.84
C/T	69(71.9)/27(28.1)	96(69.6)/42(30.4)	0.4
rs12702047			
GG/AA/CA	37(77.1)/2(4.2)/9(18.8)	56(81.2)/1(1.4)/12(17.4)	0.63
G/A	83(86.5)/13(13.5)	124(89.9)/14(10.1)	0.27
rs10235056			
GG/AA/GA	45(93.8)/0(0)/3(6.3)	67(97.1)/0(0)/2(2.9)	0.33
G/A	93(96.9)/3(3.1)	136(98.6)/2(1.4)	0.33
rs76649173			
(TC/TC)/(-/-)/(-/TC)	25 (52.1)/2(4.2)/21(43.8)	35(50.7)/8(11.6)/26(37.7)	0.35
(TC/TC)/(-/-)	71(74.0)/25(26.0)	96(69.6)/42(30.4)	0.28
rs3808353			
GG/AA/GA	36 (75.0)/2(4.2)/10(20.8)	54(78.3)/1(1.4)/14(20.3)	0.65
G/A	82(85.4)/14(14.6)	122(88.4)/16(11.6)	0.31

on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). using predesigned TaqMan® S^{N}P genotyping assays. 10 μL PCR reactions were comprised 10 ng of genomic DNA, 5.0 μL of 2× TaqMan Universal PCR Master Mix No UNG, and 0.5 μL of 20× SNP genotyping assay (probes and primers). We performed genotyping per the manufacturer's theromocycling recommendations (60°C for 1 min, 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 60 s).

Immunohistochemistry

We performed immunohistochemical analysis using the LAB-SA (labeled with Avidin–Biotin) immunoenzymatic antigen detection system (Lab Vision/Neo Markers, Fremont, CA). For GPR30, rabbit polyclonal anti-GPCR (ab12563; Abcam, Cambridge, UK) diluted 1 : 50 was used. We scored the immunohistochemical results using a semiquantitative scoring system and compared expression of GPR30 by scoring the percentage of positive cells and intensity in glandular cells of postoperative tissues in both groups.

Statistical Analysis

Continuous variables were calculated and presented as a mean (+/- standard deviation), categorical variables are presented as proportions. We used the independent sample t-test to compare continuous variables between the cancer and disease-free groups. The difference in genotype distributions between patients with and without ovarian cancer were assessed by a logistic regression model. Fisher's exact test used to analyze allele frequency. We tested the Hardy–Weinberg equilibrium using the χ^2 test. All frequency and
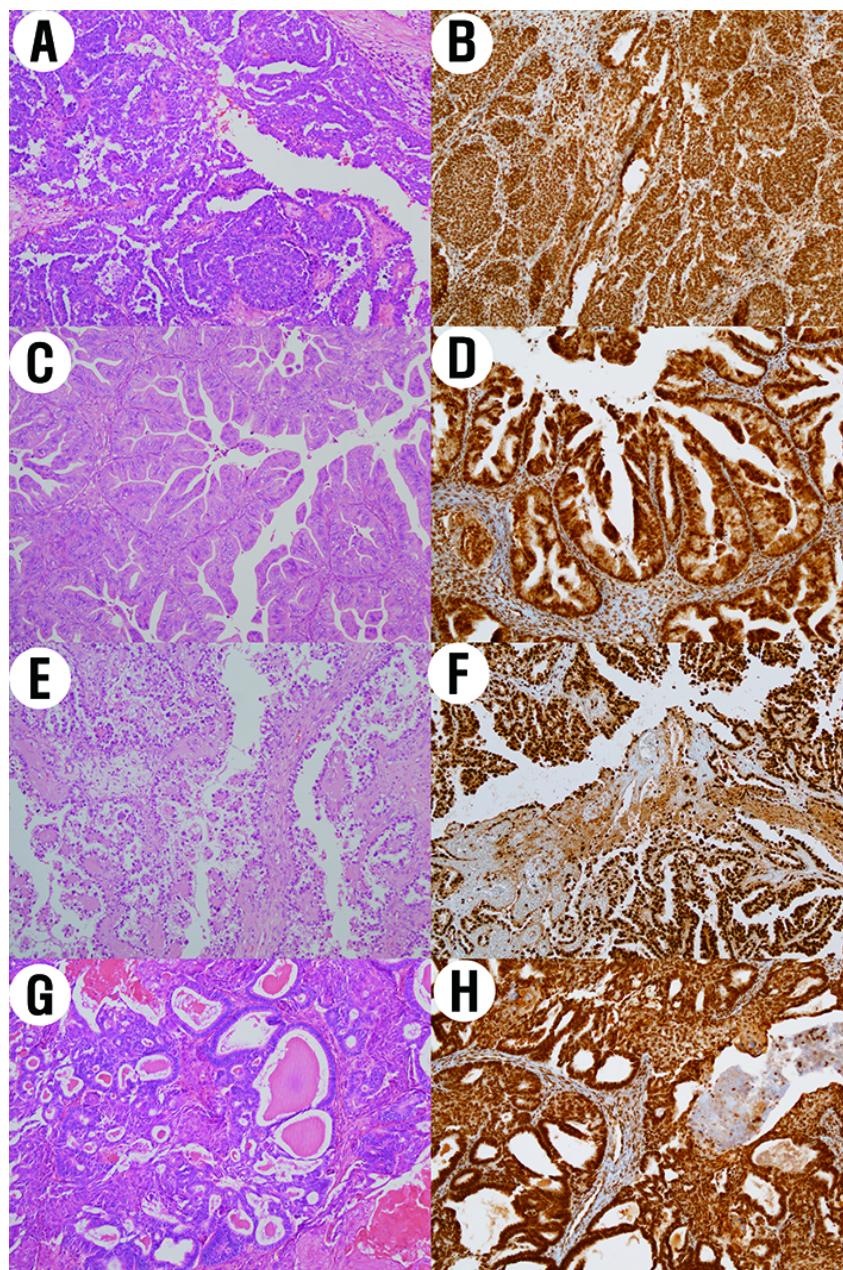
Figure 3. — Hematoxylin–eosin (H&E) and GPR30 immunohistochemical (IHC) staining in high-grade serous, mucinous, clear cell, and endometrioid ovarian cancer. Panels A, C, E, and G display H&E staining. Panels B, D, F, and H display IHC staining ($\times$ 100). Note diffuse nuclear staining of GPR30. Panels A and B, high-grade serous ovarian carcinoma. Panels C and D, mucinous ovarian carcinoma. Panels E and F, clear cell ovarian carcinoma. Panels G and H, endometrioid ovarian carcinoma.

statistical calculations were performed using SPSS ver.2.0 (IBM Corporation, Armonk, NY). We considered a two-sided $p < 0.05$ as statistically significant.

Results

Table 2 summarizes the characteristics of the patient cohort with ovarian cancer. The χ^2 test revealed that all observed genotype frequencies were at the Hardy–Weinberg equilibrium ($p > 0.05$). Two SNPs (rs62735235 and rs61747089) did not represent minor genotype and het-

erozygous genotypes. Furthermore, twelve SNPs did not demonstrate a different distribution of genotype between both groups, and differing allele frequencies were also not detected (Table 3).

We found that LD patterns presented two LD blocks in both groups (Figure 2). Twelve SNPs comprised two blocks in each group and two SNPs (rs62735235 and rs61747089) did not.

Immunohistochemical analysis of the 117 patients included in our study confirmed the expression of the GPR30

protein in both groups. Immunohistochemistry presented diffuse nuclear staining of GPR30 (Figure 3). While intracytoplasmic or membrane staining was observed in some cases, immunohistochemistry could not establish a difference in expression between both groups. In all specimens of both groups, glandular cells were stained significantly and exhibited a high percentage of cells with positive GPR30 expression.

Discussion

To our knowledge, this is the first study to investigate the effect of GPR30 SNPs on ovarian cancer in Korean women. Although we hypothesized that GPR30 SNPs could affect the susceptibility to ovarian cancer development because cumulative estrogen exposure contributes to ovarian carcinogenesis [12], this study could not establish any correlation between fourteen GPR30 SNPs and ovarian cancer. Despite identifying no SNP that exhibited a statistical difference in the distribution of genotype or allele frequency between patients with and without ovarian cancer, However, in this study, rs3808351 (distribution of GPR30 genotype, p = 0.07) demonstrated the potential to exhibit a statistical genotypic difference in a large-scale study.

Few studies have reported the role of GPR30 SNPs in other disease types, but several previous studies were aimed at revealing a correlation between GPR30 SNPs and estrogen-sensitive diseases. For example, a previous study conducted on breast cancer patients reported that the A allele of SNP rs3808351, which is located in the 5'-untranslated region, was significantly less frequent in patients with a large tumor or G3 grading, thereby suggesting that this SNP exerts a protective effects on breast cancer development [13]. In addition, a higher relative risk of gynecomastia has been reported in patients with the GG genotype of rs3808350 and the AA genotype of rs3808351 [14]. Moreover, these investigators reported that the G allele of rs3808350 and the A allele of rs3808351 frequently occurred in patients with gynecomastia [14]. Another study suggested that the presence of the GG genotype of the GPR30 rs3808351 polymorphism and the G allele of the GPR30 rs3808351 polymorphism affected the characteristics and development of leiomyoma in the Turkish population [15].

Several studies have established a correlation between GPR30 protein expression and the development or progression of breast, endometrial, and ovarian cancer [16-18]. Regarding endometrial cancer, GPR30 overexpression reportedly occurred more frequently in tumors with deep myometrial invasion, high-grade biologically aggressive histological subtypes, advanced stage, and was associated with lower patient survival rates [17]. In ovarian cancer, Long et al. suggested that abnormal expression of GPR30 could be involved in malignant transformation, invasion, and metastasis of endometrioid ovarian cancer [9]. However, another study reported that GPR30 expression, both at the mRNA and protein level, was detectable in all tissue samples of benign, borderline, and malignant ovarian tumors [18]. Moreover, data obtained indicated that neither GPR30 mRNA nor protein differed between benign and malignant tumors. Further, GPR30 positivity demonstrated no association with clinical parameters including survival rates [18]. These findings are consistent with the results of the current study. Specifically, our findings revealed that in all specimens examined, glandular cells exhibited a high percentage of positive staining and no statistical difference in staining was observed between ovarian cancer and cancer-free patients.

Some early studies of GPR30 suggested a correlation between GPR30 expression and the development and progression of ovarian cancer [10, 19]. However, to date, the relationship between ovarian cancer and GPR30 remains controversial [18]. Our present study did not demonstrate any differences at the protein and gene levels in ovarian cancer and non-cancer controls. In addition, the carcinogenic effect of estrogen in the ovary has not been rigorously established. Data pertaining to the impact of estrogens on ovarian cancer risk are primarily derived from a case-control series investigating the impact of oral contraceptives or HRT on ovarian cancer risk. These studies suggest that estrogens may, perhaps, counteract the cancer-preventive effect of progestins [20].

Considering the high relevance between GPR30 and estrogen-sensitive gynecological malignancies such as breast and endometrial cancer, the correlation between GPR30 and ovarian cancer remains unclear. This view may be attributed to the fact that ovarian cancer is not typically considered estrogen-sensitive, but estrogen may still affect ovarian cancer progression. Although GPR30 is involved in rapid estrogen signaling, a potential role for this mechanism in the development of ovarian cancer remains elucive.

This study has both strengths and limitations. Its strengths are as follows: First, this is the first study to investigate the role of GPR30 gene polymorphism in ovarian cancer. Second, the study cohort in this study was strictly divided into two groups through operation field findings and pathological review of specimens. However, its limitations are its retrospective nature and small sample size. These limitations may not provide sufficient data to elucidate minor genetic impacts on the development of ovarian cancer.

In conclusion, this study did not demonstrate a genetic impact of GPR30 SNPs on ovarian cancer. Further extensive studies are warranted to elucidate potential impacts of GPR30 on ovarian cancer.

Ethics Approval and Consent to Participate

All subjects gave their informed consent for inclusion before they participate in the study. The study was conducted in accordance with the Declaration of Helsinki. and the protocol was approved by Kyungpook National University Hospital (KNUH) Institutional Review Board (IRB) (approval number : 2016-03-027).

Acknowledgments

Conflict of interest

There is no conflict of interest. The authors have no commercial, proprietary, or financial interests in the products or companies described in this article.

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Corresponding Author:
DAE GY HONG, M.D, Ph.D,
Department of Obstetrics and Gynecology,
Kyungpook National University Medical Center, Daegu (Republic of Korea)
e-mail: chssa0220@hanmail.net