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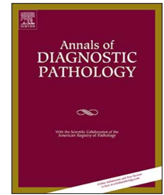

Methylation-associated silencing of *SFRP1* gene in high-grade serous ovarian carcinomas

Vedran Kardum[a], Valentina Karin[b], Mislav Glibo[b], Anita Skrtic[c], Tamara Nikuseva Martic[b], Nermina Ibisevic[d], Faruk Skenderi[d], Semir Vranic[d,e,*], Ljiljana Serman[b]

[a] *Department of Obstetrics and Gynecology, University Hospital Merkur, Zagreb, Croatia*
[b] *Department of Biology, School of Medicine, University of Zagreb, Zagreb, Croatia*
[c] *Department of Pathology, School of Medicine, University of Zagreb, Croatia*
[d] *Department of Pathology, Clinical Center, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*
[e] *School of Medicine, University of Sarajevo, Sarajevo, Bosnia and Herzegovina*

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ABSTRACT

Wnt is a highly conserved signaling pathway responsible for tissue regeneration, maintenance and differentiation of stem cells in adults. Its aberrant activation through reduced expression of Wnt signaling pathway inhibitors, such as proteins from the SFRP family, is commonly seen in many tumors. In the present study we explored SFRP1 protein expression using immunohistochemistry in 11 low-grade serous ovarian carcinomas (LGSC), 42 high-grade serous ovarian carcinomas (HGSC), and 5 normal ovarian tissues (controls). *SFRP1* gene methylation was analyzed by methylation-specific PCR in 8 LGSCs, 13 HGSCs and control samples. *SFRP1* gene was unmethylated and SFRP1 protein expression was strong in normal ovaries (n = 5). Although *SFRP1* gene was unmethylated in almost all of the LGSC cases (7/8, 88%), SFRP1 protein expression was significantly lower than in normal ovaries ($p < 0.05$). Seven out of 13 HGSCs (54%) showed *SFRP1* gene hypermethylation and protein expression level was also significantly lower than in normal ovaries ($p < 0.001$). Our preliminary data show loss of SFRP1 protein expression caused by the SFRP1 promoter hypermethylation in a subset of HGSCs. SFRP1 protein expression was also lost in LGSCs but different regulatory mechanisms may be involved. Further studies should elucidate the clinical and therapeutic relevance of the observed molecular alterations.

1. Introduction

Ovarian cancer is the sixth most common cancer among women in Europe, with incidence of 9.4 and mortality of 5.2 per 100,000 women (GLOBOCAN 2012). Incidence increases by age with peak between age 60 and 70 (median 63) when > 70% of patients have been diagnosed with advanced stage of the disease [1].

Ovarian epithelial carcinoma is the most common type of ovarian cancer, representing approximately 85% of ovarian cancers and, based on histopathology, immunohistochemistry and molecular genetic analysis, at least 5 main types are currently distinguished: high-grade serous carcinoma (HGSC), representing about 70% of all ovarian cancers; endometroid carcinoma, about 10%; clear-cell carcinoma, 10% of cases; mucinous carcinoma, 3% of cases; and low-grade serous carcinoma (LGSC), < 5% [2,3].

LGSC may develop *de novo* or following a diagnosis of serous tumor of low malignant potential [4]. LGSCs are genetically stable and harbor *KRAS* (19%), *BRAF* (5%–38%) or *ERBB2* mutations (6%) [2]. They are characterized by young age at diagnosis (mean age 45–57), relative chemoresistance and relative prolonged survival compared with HGSCs [5]. In contrast, HGSCs affect older women (mean age 55–65 years, median 64 years), are typically aggressive tumors and account for the vast majority of deaths due to ovarian cancer (10 year survival 30% - 40%) [6]. HGSCs may occur from *de novo* mutation but also arise from the epithelial mucosal lining of the fallopian tube fimbria or from endosalpingiotic deposits on the ovarian or peritoneal surfaces [7,8]. HGSCs are genetically highly unstable, with mutations frequently affecting *BRCA1*, *BRCA2* and *TP53* genes [2,9].

Wnt signaling pathway has been shown to be tighly related to cancer development and progresssion [10]. Wnt signaling pathway acts through two main mechanisms: the “canonical” or Wnt/β-catenin pathway and several “non-canonical” pathways that are β-catenin independent [11-13]. Both pathways are activated by binding of extracellular Wnt ligand to the frizzled (Fz) family membrane receptor, which then activates the family of dishevelled cytoplasmic proteins (DVL). At the level of DVL, the Wnt signal branches into at least three

* Corresponding author at: Department of Pathology, Clinical Center and School of Medicine, University of Sarajevo, Bolnicka 25, BA-71000 Sarajevo, Bosnia and Herzegovina.
E-mail address: semir.vranic@bosnianpathology.org (S. Vranic).

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major cascades, canonical, and less known Planar Cell polarity and Wnt/Ca^{2+}. In canonical or β-catenin dependent pathway, DVL family proteins (DVL1, DVL2 and DVL3) protect β-catenin from degradation by inhibiting the degradation complex. This results in the citoplasmic accumulation of β-catenin, which then translocates into the nucleus and induces the transcription of Wnt-responsive genes. Aberant acctivation of Wnt/β-catenin signaling is linked to variety of human diseases, including cancers [14-16].

The Wnt pathway is regulated by the Wnt antagonists, such as secreted frizzled-related proteins (SFRPs), a family of 5 secreted glycoproteins which directly bind Wnts and block the Wnt signaling pathway. SFRPs are extracellular soluble proteins with a cysteine rich domain also present in Fz receptor and wingless-type (Wnt) proteins [12,13,17,18].

SFRPs demonstrates tumor suppressor activity which means that they represent negative regulators of cell growth [12-14]. Increased expression of the *SFRP* gene in cancer cells indicates a better prognosis, while reduced expression, most often due to their promoter hypermethylation, contributes to the progression and invasiveness of the tumor. Thus, decreased expression of SFRP1 and SFRP3 proteins is present in ovarian carcinomas, kidney, prostate and colon cancer [19-22] and in a number of other malignancies.

The aim of this study was to investigate the SFRP1 protein expression, *SFRP1* gene methylation status, and their relationship, in HGSCs and LGSCs.

2. Materials and methods

2.1. Tissue samples

Fifty-eight formalin-fixed paraffin embedded (FFPE) samples of normal and malignant ovarian tissues were used for the study. Representative slides were reviewed by board certified pathologists (S.V., A.S.) to confirm the diagnosis (low- and high-grade serous ovarian carcinomas) and select appropriate materials of both normal and malignant tissues for immunohistochemical and molecular analyses. The diagnoses of ovarian carcinomas were in concordance with the most recent WHO classification of the tumors of the female reproductive organs [8]. In selected cases, additional immunohistochemical work up (CK7, CK20, CDX-2, WT-1, Napsin-A, CA-125, vimentin, p53) was employed to render the correct diagnosis. For the study purposes 42 cases of HGSCs were subcategorized according to the predominant morphologic pattern: papillary, solid, micropapillary, intracystic, transitional cell-like, and cribriform patterns. The study was approved by the Ethical Committees, School of Medicine, University of Zagreb and University Hospital Merkur.

2.2. Immunohistochemistry (IHC)

Immunohistochemical staining was performed using biotin-avidin-streptavidin horseradish peroxidase method (DAKO #K0679) on 4 μm thick paraffin embedded sections that were placed on silanized glass slides (DakoCytomation, Denmark). Tissue sections were deparaffinized in xylene (4 ×, 10 min) and rehydrated in a graded ethanol series, 100% and 96% ethanol (1 ×, 5 min), 70% ethanol (1 ×, 2 min) and water (30 s). Antigen retrieval was performed by heating the sections in microwave oven 2 × for 10 min at 600 W in Dako Target Retrieval Solution (Dako Corporation, USA). The endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 10 min. Sections were incubated with primary antibody SFRP1 (rabbit polyclonal anti-human; Clone: sc-13,939, Santa Cruz Biotechnology, USA, dilution 1:200) overnight at 4 °C. Dako REAL Envision detection system (DakoCytomation, Carpinteria, USA) was utilized for visualization as suggested by the manufacturer and the sections were counterstained with hematoxylin. Normal ovarian tissues were used as positive control as adjacent normal ovarian tissue (stroma and blood vessels) overexpressed SFRP1 protein in > 50% of the cells with ≥ 1 + intensity. Negative controls were treated in the same way with the omission of incubation with primary antibodies.

Ovarian serous carcinomas and normal tissues stained for SFRP1 protein were interpreted by two pathologists independently as follows: score 0 (no staining), score 1 (< 10% tumor cells), score 2 (10–50% of tumor cells), and score 3 (> 50% of tumor cells) as previously suggested [23]. SFRP1 protein expression was scored in both membranous/cytoplasmic and nuclear pattern of staining as previously reported [19,21]. In case of discordant interpretation, the pathologists would review cases together to obtain a full concordance. Immunohistochemical results were interpreted blindly in regards to the *SFRP1* methylation status.

2.3. Methylation-specific PCR (MSP)

FFPE samples of normal ovarian tissues (n = 5), LGSCs (n = 8) and HGSCs (n = 13) were randomly selected for MSP. DNA was isolated from 2 × 10 μm FFPE tissue sections as previously described (Vrsalovic et al., 2004) and treated with bisulfite using the MethylEdge Bisulfite Conversion System (Promega, Madison, Wisconsin, USA) according to manufacturer's instruction. Bisulfite-treated DNA was then used for methylation-specific PCR reaction (MSP). Sequences of the primers for MSP of the *SFRP1* promoter region were synthesized according to Guo et al. [24]: methylated primers, F: 5′-TGTAGTTTTCGGAGTTAGTGTCGCGC-3′, R:5′-CCTACGATCGAAAACGACGCGAACG-3′ (126 bp); unmethylated primers, F: 5′- GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT-3′, R:5′-CTCAACCTACAATCAAAAACAACACAAACA-3′ (135 bp). All PCRs were performed using TaKaRa EpiTaq HS (for bisulfite-treated DNA) (TaKaRa Bio, USA): 1XEpiTaq PCR Buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 20 pmol of each primer (Sigma-Aldrich, USA), 50 ng of DNA, and 1.5 units of TaKaRa EpiTaq HS DNA Polymerase in a 50 μl final reaction volume. PCR cycling conditions were as following: initial denaturation at 95 °C for 30 s, followed by 35 cycles consisting of three steps: 95 °C for 30 s, the respective annealing temperature for 30 s, 72 °C for 30 s, followed by a final extension at 72 °C for 7 min. For the amplification of methylated *SFRP1* promoter region the annealing temperature was 65.5 °C, while for unmethylated *SFRP1* promoter region was 63.1 °C. PCR products were separated on 2% agarose gel stained with GelStar nucleic acid stain (Lonza Rockland, Inc. Rockland, USA) and visualized on a UV transilluminator. Methylated Human Control (Promega, Madison, Wisconsin, USA) was used as positive control for methylated reaction, human white blood cell DNA was used as positive control for unmethylated reaction, and nuclease-free water was used as negative control. DNA was isolated from white blood cells according to the simple salting out procedure [25].

2.4. Statistical analysis

The expression of SFRP1 protein across the tumors and benign ovary tissue samples was statistically analyzed using GraphPad Prism 5/01 program (GraphPad Software, Inc., San Diego, CA, USA). Distribution of the data was assessed by Kolmogorov-Smirnov test and Shapiro-Wilk W-test.

The difference in expression of SFRP1 proteins among ovarian tumors samples in comparison with normal ovarian tissue was assessed by Wilcoxon signed rank test. The difference in expression of SFRP1 proteins among ovarian tumors samples was assessed by Mann-Whitney test. $P < 0.05$ was considered statistically significant.

3. Results

Out of 58 FFPE samples used in the study, 42 were HGSC, 11 were LGSC and 5 control (normal) ovarian tissue samples.

Average age of patients with HGSC was 60 years (range, 37–81 years) and those with LGSC was 64 years (range, 48–86 years).

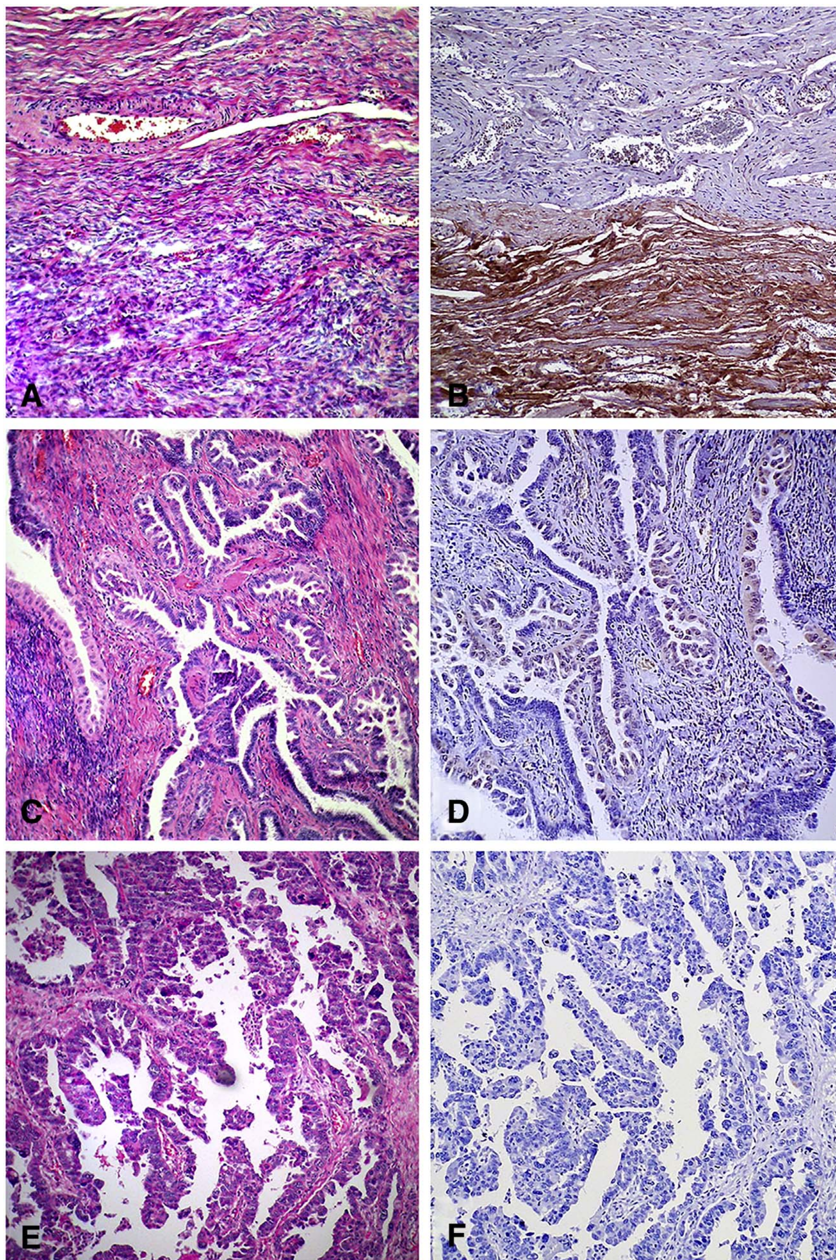

Fig. 1. Characteristic immunohistochemical staining of SFRP1 protein expression in normal ovarian tissue showing retained protein expression (B), LGSC showing diffuse protein expression (D) and HGSC showing complete loss of the protein expression (F). Hematoxylin and eosin staining of normal ovarian tissue (A), LGSC (C) and HGSC (E). LGSC, low.grade serous ovarian cancer; HGSC, high-grade serous ovarian cancer.

3.1. SFRP1 protein status in normal ovary and cancer tissues

All normal ovarian tissue (stroma and blood vessels) samples (n = 5) as well as normal ovarian tissues adjacent to the malignant tissues showed no *SFRP1* gene methylation and retained SFRP1 protein expression in > 50% of the cells (Fig. 1B; Fig. 2).

When compared to normal tissues, both LGSCs and HGSCs exhibited significantly lower SFRP1 protein expression (median: 1, range 0–3, p = 0.0211, Z - 2.449, Wilcoxon signed rank test; and median: 0, range 0–3, $p < 0.0001$, Z - 3.811, Wilcoxon signed rank test, respectively). However, between the LGSCs and HGSCs, SFRP1 protein expression was not significantly different (p = 0.1154, Mann-Whitney test).

Four out of 11 LGSCs (37%), had no expression of SFRP1 protein; 3 tumors (27%) had SFRP1 protein expression in < 10% of tumor cells; one LGSC case (9%) had protein expression in 10–50% of cells; and 3 LGSCs (27%) showed diffuse protein expression in > 50% of cells (Fig. 1D). Similarly to healthy ovaries, 7/8 (87.5%) LGSCs exhibited no *SFRP1* gene hypermethylation while only one LGSC (12.5%) had only a weakly detectable methylation (Fig. 2).

In contrast, 26 out of 42 HGSCs (62%) had a complete absence of SFRP1 protein expression (Fig. 1E); 5 cases (12%) had protein expression in < 10% of cells and remaining 9 HGSCs (21%) had protein expression in 10–50% of tumor cells. Only 2 HGSCs (5%) showed diffuse SFRP1 protein expression in > 50% of neoplastic cells. Seven out of 13 randomly selected HGSCs (54%) exhibited *SFRP1* gene methylation (Fig. 2).

We observed no significant differences in SFRP1 protein expression between different morphologic patterns of HGSC (papillary, solid, micropapillary, intracystic, transitional cell-like, cribriform) (p = 0.58).

4. Discussion

In the present study we explored the status of SFRP1 protein/gene in a cohort of low- and high-grade serous ovarian carcinomas (LGSC and HGSC) in comparison with the normal ovarian tissues. SFRP1 protein, a member of the SFRP family of Wnt signaling pathway inhibitors, has a tumor suppressor activity in majority of tumor types analyzed thus far, and is the only member of the SFRP family whose expression is

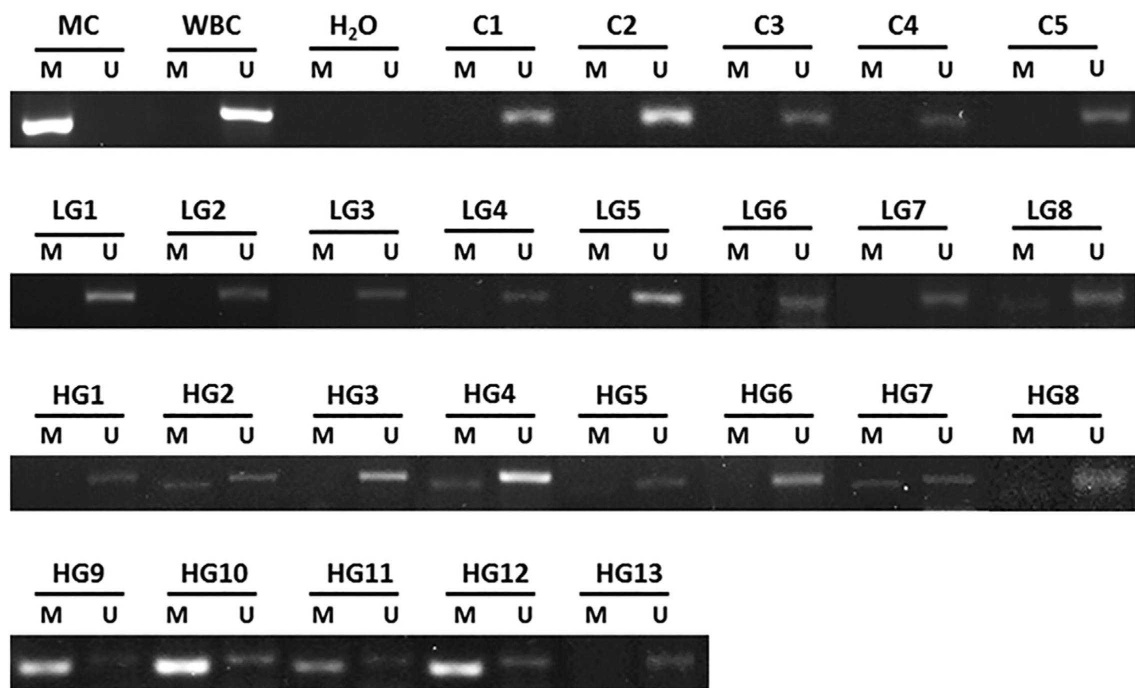

Fig. 2. A representative example of methylation-specific PCR analysis for *SFRP1* promoter in normal ovarian tissues (controls, C1-C5), low-grade serous ovarian carcinomas (LGSC, LG1-LG8) and high-grade serous ovarian carcinomas (HGSC, HG1-HG13). The presence of a visible PCR product in lanes marked U indicates the presence of unmethylated promoters; the presence of a product in lanes marked M indicates the presence of methylated promoters. Methylated human control (MC) was used as positive control for methylated reaction, human white blood cell DNA (WBC) was used as positive control for unmethylated reaction, and water was used as a negative control.

consistently reduced in numerous cancers [26], such as in prostate, colon, kidney, lung cancer, intrahepatic cholangiocarcinoma, hepatocellular carcinoma, testicular germ cell and ovarian malignant tumors [19,20,22,27-29]. Expression of *SFRP1* gene is controlled, among other main mechanisms, by DNA methylation, which is increased in many tumors and tumor cell lines, as recent reports indicate [30]. Our results show that healthy ovarian tissues retain SFRP1 protein expression with unaltered *SFRP1* gene DNA methylation status. In contrast, > 50% of tested HGSC harbored hypermethylation of *SFRP1* gene, followed by the loss of SFRP1 protein. These results are in line with previous data on the ovarian carcinoma cell lines [31]. Takada et al. also showed that the addition of DNA demethylation agent 5-aza-2′-deoxycytidine resulted in promoter demethylation and consequent re-expression of SFRP1 [31]. The remaining HGSC did not show *SFRP1* gene methylation, which could be partially explained by existence of additional epigenetic mechanisms of gene regulation. In case of *SFRP1*, this may be histone H3 lysine 27 trimethylation (H3K27me3), a post-translational histone modification, which is associated with transcriptional repression [32]. This mechanism could also explain the lower SFRP1 protein levels in LGSCs and HGSCs compared to the normal tissues. *SFRP1* gene was almost unmethylated in all LGSCs, with only 1 out 8 analyzed tumors displaying low level of *SFRP1* gene methylation. Lower gene expression along with absence of methylation in this group of LGSCs could also be explained by mutations that may continuously accumulate in tumors, especially considering the currently accepted hypothesis that LGSCs more commonly develop from precancerous lesions, in contrast to HGSCs whose appearance is typically *de novo* [5]. Also, the lack of statistically significant difference in SFRP1 protein expression between LGSCs and HGSCs may be due to the small number of tested cases and this represents one of the key limitations of our study. We also point out here other mechanisms that may contribute to the *SFRP1* gene (and consequently SFRP1 protein) silencing such as various miRNAs as recently shown in a case of ovarian cancer; thus a study of Wu et al. [33] showed that miR-27a plays a vital role in promoting the cancer stem cell-like phenotype in ovarian cancer cells acting against different components of Wnt signaling pathway including SFRP1 protein. Similar mechanisms have also been observed in other cancer subtypes such as pancreatic, gastric, breast, and oral squamous cell carcinoma [28,33-35].

In conclusion, the SFRP1 protein is downregulated in a subset of HGSCs, and that downregulation is caused by *SFRP1* promoter hypermethylation. Additionally, SFRP1 protein expression was also lost in LGSCs, but different regulatory mechanisms may be involved. Further studies should elucidate the clinical and therapeutic relevance of the observed molecular alterations.

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Conflict of interest

The authors report no conflict of interests.

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