New insights into the role of PTCH1 protein in serous ovarian carcinomas

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Abstract

Background

The Hedgehog (Hh) signaling pathway is essential for normal embryonic development, while its hyperactivation in adult organism is associated with development of various cancers, including ovarian cancer. The role of the Hh signaling pathway in ovarian cancer, as well as in certain histological subtypes of ovarian cancer, is poorly understood. Therefore, we investigated the role of PTCH1 protein and changes in the promoter methylation status of the corresponding gene, in a cohort of low- (LGSC) and high-grade (HGSC) serous ovarian carcinomas and HGSC cell lines (OVCAR5, OVCAR8 and OVSAHO).

Methods

PTCH1 protein expression level was analyzed using immunohistochemistry in tissue samples, and by immunofluorescence and Western blot in cell lines. DNA methylation pattern of *PTCH1* gene were analyzed by methylation-specific PCR (MSP). Mann-Whitney U test was used to compare differences in expression of PTCH1 protein among ovarian tumor samples compared with normal tissue samples, while Spearman's correlation was used to test the association between DNA promoter methylation of the *PTCH1* gene and expression of the corresponding protein.

Results

PTCH1 protein expression level was significantly higher in HGSCs and LGSCs compared with control tissues (healthy ovaries and fallopian tubes). Similarly, cancer cell lines exhibited significantly higher PTCH1 protein expression in comparison with normal fallopian tube non-ciliated epithelium cell line (FNE1). Nuclear localization of the PTCH1 protein in tumor tissue and cultured tumor cells suggests that this protein could play an active tumor promoter role in the nuclei of serous ovarian carcinoma cells. PTCH1 protein fragments of different molecular weights were detected in the cell lines, indicating possible proteolytic cleavage of this protein, resulting in the generation of soluble N-terminal fragments that are translocated to the nucleus. DNA methylation of the *PTCH1* gene promoter was not in line with the expression level of this protein, suggesting that possibly other mechanisms, either epigenetic or posttranslational, regulate *PTCH1* gene expression and protein level in serous ovarian carcinomas.

Conclusions

Our results indicate that PTCH1 protein could play an active tumor promoter role in the pathogenesis of serous ovarian carcinoma.

Background

Ovarian cancer (OC) is the eighth leading cause of cancer-related deaths in women and is the third most common gynecological malignancy [1]. This disease is a major clinical challenge in gynecologic oncology, with the highest mortality rate of all malignancies of the female reproductive system [2]. Most patients have almost no symptoms in the early stage of the disease, while nonspecific symptoms, associated with more frequent benign conditions, occur in the advanced stage of OC that significantly complicates the diagnosis of this disease [2, 3]. The diagnosis is further complicated because there are different histological subtypes of ovarian cancer with different biological and clinical features [4].

Serous ovarian carcinomas are the most common form of ovarian cancer and account for approximately 75% of all ovarian epithelial tumors. High- (HGSC) and low-grade (LGSC) serous ovarian carcinomas represent about 70% and < 5% of all epithelial ovarian cancers, respectively [5]. Although both are serous in histological type, HGSCs and LGSCs are two different entities with different pathogenesis, molecular and genetic changes, origin, and prognosis [5]. The exact origin of the LGSC and HGSC is still unknown. LGSC most likely arises from fallopian tube epithelium (FTE) [6], while the origin of HGSC is probably dual, and it may arise from ovarian surface epithelium (OSE) or FTE [7].

Aberrant activation of several signaling pathways, including the Hedgehog (Hh) signaling pathway, has been observed in ovarian cancer [8–10]. The Hh signaling pathway is an evolutionarily conserved signaling pathway that is essential for the development of a normal embryo [11]. However, in the adult organism, this signaling pathway is inactive in most organs, so its aberrant activation in adulthood is associated with the development of various tumors [11, 12].

The Hh signaling pathway is activated when one of the three Hh ligands, Sonic Hedgehog (SHH), Indian Hedgehog (IHH) or Desert Hedgehog (DHH), binds to 12-pass transmembrane receptor Patched 1 (PTCH1) or Patched 2 (PTCH2) thus suppressing its activity. In the absence of Hh ligands, activated PTCH1 represses Hh signaling [13, 14]. PTCH1 is the main receptor of the Hh signaling pathway. The human *PTCH1* gene encodes a transmembrane glycoprotein of 1447 amino acids (~ 161 kDa) [15]. PTCH1 receptor contains transmembrane domain (TMD), two large extracellular domains (ECDs), ECD1 and ECD2, and three large cytoplasmic domains, N-terminal domain (NTD), middle loop (ML) and C-terminal domain (CTD) [16, 17].

Although PTCH1 is a negative regulator of Hh signaling, this receptor serves as a marker of canonical Hh signaling activation [18]. Since the *PTCH1* gene contains binding sites for GLI transcription factors, its expression is enhanced when Hh signaling is activated, creating a negative feedback loop [19, 20]. If this protein loses its function, either due to gene mutations or epimutations, aberrant activation of the Hh signaling will occur [12]. Inactivating mutations and hypermethylation of the *PTCH1* gene have been observed in various cancers [21–27]. However, numerous studies have shown that PTCH1 protein, otherwise known to act as a tumor suppressor, has increased expression in many different cancers, including breast, prostate, lung, colon, brain tumors, and melanoma [28–30]. A recent study has shown that the PTCH1 receptor can also serve as a transporter that releases chemotherapeutic agents out of the cell and thus contributes to chemotherapy resistance [30]. The increased expression of PTCH1 protein in

tumor tissue can be explained by possible changes in the structure and function of this protein in malignant tissue. These changes could be triggered by mutations in the *PTCH1* gene whereby PTCH1 could lose its original tumor suppressor role and gain a new tumor promoter role [22].

Increased expression of the PTCH protein has also been observed in ovarian cancer, where expression of this protein was increased stepwise in benign, borderline, and malignant neoplasms [31]. PTCH protein expression was associated with increased tumor cell proliferation and was positively correlated with poor survival of patients with ovarian cancer [31, 32]. On the other hand, there are studies with conflicting results where reduced expression of PTCH1 protein has been observed in tumor tissue and ovarian cancer cell lines [33, 34]. Patients with decreased expression of PTCH1 protein were found to have a poorer prognosis than patients with increased expression of this protein [34].

Although the studies mentioned above have shown that PTCH1 could be involved in the molecular pathogenesis of ovarian cancer, its role in ovarian cancer, as well as in certain histological subtypes of ovarian cancer, has not been sufficiently investigated. Therefore, we decided to investigate the role of PTCH1 protein, as well as changes in the promoter methylation status of the corresponding gene, in serous ovarian carcinomas. We found that PTCH1 protein may play an active role in the molecular pathogenesis of both LGSCs and HGSCs. Also, we indicated the importance of nuclear localization of PTCH1 protein in cancer cells, which can be linked with the development and progression of these carcinomas, and showed that of DNA methylation of the *PTCH1* gene promoter is not a potential mechanism involved in the pathogenesis of serous ovarian carcinomas.

Methods

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) samples of 48 serous ovarian carcinomas (low-grade, LGSC, n = 11; and high-grade, HGSC, n = 37), 20 samples of normal ovarian tissue, and 10 samples of normal fallopian tube tissue were used for the study. Representative slides were reviewed by board-certified pathologists (S.V., A.S.) to confirm the diagnosis (LGSCs and HGSCs) and select appropriate normal and malignant tissues for immunohistochemical and molecular analyses. The tissue samples used in the study were a part of the archival collection of cancer tissue samples from the University of Zagreb School of Medicine, assembled in collaboration with University Hospital Merkur (Zagreb), both of which are part of the Scientific Center of Excellence for Reproductive and Regenerative Medicine.

Cell lines

HGSC cell lines OVCAR5, OVCAR8, and OVSAHO, and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (used as normal control) were used in this study. OVCAR5 and OVCAR8 were a kind gift from Dr. Ernst Lengyel, University of Chicago, USA. OVSAHO was obtained from the Japanese Collection of Research Bioresources and FNE1 from the Live Tumor Culture Core (LTCC) at the University of Miami, Sylvester Comprehensive Cancer Center, USA.

Cell culture

OVCAR5, OVCAR8, and OVSAHO cell lines were grown in Dulbecco's modified Eagle's Medium (DMEM) with 4.5 g/L D-glucose and L-glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) along with 10% FBS (Gibco, Thermo Fisher Scientific), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 1% MEM vitamins (Gibco, Thermo Fisher Scientific), 1% MEM nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin-streptomycin (Sigma-Aldrich). FNE1 cells were grown in FOMI media (LTCC, University of Miami, Sylvester Comprehensive Cancer Center, USA) supplemented with 25 ng/mL of cholera toxin (Sigma-Aldrich) in Corning® Primaria™ cell culture dishes (Corning, New York, NY, USA). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Immunohistochemistry (IHC)

Immunohistochemical staining was performed using the biotin-avidin-streptavidin horseradish peroxidase method (DAKO #K0679) on 4µm thick paraffin-embedded sections that were placed on silanized glass slides (DakoCytomation, Denmark) as previously described [35]. The primary antibodies used in this experiment are listed in Table 1. Normal placental tissue was used as a positive control. Negative control was treated in the same way with the omission of incubation with primary antibodies.

Table 1
List and specifications of anti-PTCH1 primary antibodies used in immunohistochemistry, immunofluorescence and western blot.

Antibody	Antigen	Host and clonality	Epitope	Dilution	Manufacturer and cat.no.
Anti-PTCH1a	PTCH1	Rabbit polyclonal	1–50 aa	IHC – 1:500 IF – 1:100 WB – 1:1000	Abcam, Cambridge, UK; ab129341
Anti-PTCH1b	PTCH1	Rabbit polyclonal	1–50 aa	IHC – 1:300 IF – 1:100 WB – 1:1000	Abcam, Cambridge, UK; ab53715
Anti-PTCH1c	PTCH1	Rabbit polyclonal	1–80 aa	IHC – 1:300 IF – 1:100 WB – 1:1000	Aviva Systems Biology, San Diego, CA, USA; OASG05688
Anti-PTCH1d	PTCH1	Mouse monoclonal	122–436 aa	IHC – 1:50 IF – 1:25 WB – 1:500	Novus Biologicals, Centennial, CO, USA; NBP1-47945

aa – amino acid; IHC – immunohistochemistry; IF – immunofluorescence; WB – western blot

Serous ovarian carcinomas and normal tissues stained for PTCH1 protein (using anti-PTCH1a antibody) were interpreted independently by two pathologists (S.V., A.S.) 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 [36]. PTCH1 protein expression was observed in the cytoplasm and in the nucleus of epithelial and stromal cells. In case of discordant interpretation, the pathologists reviewed cases together to obtain a complete concordance. Few samples of HGSC and LGSC tissues were stained for PTCH1 protein using anti-PTCH1b, anti-PTCH1c, and anti-PTCH1d antibodies to confirm subcellular localization of PTCH1 protein.

Immunofluorescence

Cells grown on glass coverslips (Gerhard Menzel, Braunschweig, Germany) were fixed with 4% paraformaldehyde solution (Santa Cruz Biotechnology, Dallas, TX, USA) for 15 min at room temperature (RT). Samples were washed with 1 × DPBS buffer (Gibco, Thermo Fisher Scientific, Waltham, MA, SAD) and permeabilized with buffer containing 0.1% saponin (Sigma-Aldrich) in 1 × DPBS (PMS buffer) for 10 min at RT. After permeabilization, cells were incubated in Blocking buffer (PMS buffer with 5% FBS) for 1 h at RT. Samples were incubated overnight at 4°C with primary antibodies listed in Table 1. Cells were washed and incubated for 1 h at RT with anti-rabbit Alexa Fluor 488 (dilution 1:300; A-21206; Invitrogen, Carlsbad, CA, USA) or anti-mouse Cy3 antibody (dilution 1:300; 715-165-150; Jackson ImmunoResearch, Ely, UK) including Hoechst stain solution (dilution 1:10; NucBlue Live ReadyProbes Reagent; Invitrogen). After incubation with secondary antibodies, samples were washed and mounted using a fluorescence mounting medium (Dako, Carpinteria, CA). Cells were examined using an inverted Leica SP8 X FLIM confocal microscope.

Western blot

Total proteins were isolated from OVCAR5, OVCAR8, OVSAHO and FNE1 cell lines using ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris/HCl – pH 8, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing complete protease inhibitor (Roche, Basel, Switzerland) and 1 mM PMSF (Thermo Fisher Scientific, Waltham, MA, USA) as previously described [37]. Subcellular Protein Fractionation Kit for Cultured Cells Proteins (Thermo Fisher Scientific) was used for segregation and enrichment of proteins from five different cellular compartments (cytoplasmic, membrane, nuclear soluble, chromatin-bound, and cytoskeletal proteins). Proteins were enriched and extracted according to the manufacturer's instructions.

For Western blot analysis, 10% polyacrylamide gel was used to separate 10 µg of total protein samples and 10 µL of each protein fraction. Western blot was performed as previously described [37]. Anti-PTCH1 primary antibodies used in this experiment are listed in Table 1. The membranes were also probed with rabbit polyclonal anti-GAPDH antibody (dilution 1:2000, Cat No. IMG- 5143A, IMGENEX, San Diego, USA) and mouse monoclonal anti-H3K4me2 antibody (dilution 1:2000; clone CMA303, 05-1338; Millipore, Sigma-Aldrich, Burlington, MA, SAD) as controls to verify the efficiency of protein extraction from different cellular compartments, and loading controls. Peroxidase-conjugated secondary antibodies (dilution 1:5000, goat anti-rabbit, Cat No. P0448, Dako; dilution 1:12 500, goat anti-mouse, Cat No. 170–6516, Bio-Rad, Hercules, CA, USA) and SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) were used to visualize reactive bands.

Methylation-specific PCR (MSP)

DNA was isolated from two 10 µm sections of FFPE tissue as previously described [38]. DNA was also extracted from cultured cells using cell lysis buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, 0.5% SDS, 0.1 mg/mL proteinase K; 1 mL per 10^8 cells). Samples were incubated in lysis buffer overnight at 300 rpm and 50°C. An equal volume of ROTI®Phenol/Chloroform/Isoamyl alcohol (Carl Roth, Karlsruhe, Germany) was added to the lysed cell suspensions. Samples were vortexed vigorously and

subsequently centrifuged at 16 000 × g for 5 min at RT. The aqueous phase containing the purified DNA was transferred to a clean tube. DNA was precipitated using ice-cold absolute ethanol. Isolated DNA was treated with bisulfite using the MethylEdge Bisulfite Conversion System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Bisulfite-treated DNA was used for methylation-specific PCR reaction (MSP). Primers for *PTCH1* promoter region were synthesized according to Peng et al. [39]: methylated primers, F: 5'- AATTAAGGAGTTGTTGCGGTC-3', R: 5'- GCTAAACCATTCTATCCCCGTA – 3' (125 bp); unmethylated primers, F: 5'- ATTAAGGAGTTGTTGTGGTTGT – 3', R: 5'- ACTAAACCATTCTATCCCCATA – 3' (124 bp). All PCRs were performed using TaKaRa EpiTaq HS (for bisulfite-treated DNA) (TaKaRa Bio, Kusatsu, Shiga, Japan): 1XEpiTaq PCR Buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 20 pmol of each primer (Sigma-Aldrich), 50 ng of DNA, and 1.5 Units of TaKaRa EpiTaq HS DNA Polymerase in a 50 µl final reaction volume. PCR cycling conditions for both unmethylated and methylated primers were as follows: initial denaturation at 95 ºC for 30 sec, followed by 40 cycles consisting of three steps: 95 ºC for 30 sec, 61 ºC for 30 sec, 72 ºC for 30 sec, followed by a final extension at 72 ºC for 7 min. 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) was used as a positive control for methylated reaction, unmethylated human EpiTect Control DNA (Qiagen, Hilden, Germany) was used as a positive control for unmethylated reaction, and nuclease-free water was used as a negative control.

Statistical analysis

PTCH1 protein expression across the tumors and normal ovarian and fallopian tube tissue samples was analyzed using the IBM SPSS Statistics, version 21., The distribution of the data was assessed by the Kolmogorov-Smirnov test and Shapiro-Wilk W-test. The difference in total, cytoplasmic and nuclear expression of PTCH1 protein among ovarian tumor samples compared with normal ovarian and fallopian tube tissue and difference in expression of PTCH1 protein among ovarian tumor samples and normal tissue samples were assessed by Mann-Whitney U test. Spearman's correlation was used to test the association between DNA promoter methylation of the *PTCH1* gene and expression of the corresponding protein. $P < 0.05$ was considered statistically significant.

Results

PTCH1 protein expression in HGSCs and LGSCs

Aberrant expression of PTCH1 has been linked to the progression of the ovarian cancer. Therefore, we decided to analyze the expression of this protein in tumor epithelium, connective stroma and whole tissue sections of serous ovarian carcinomas, and in healthy epithelium, stroma and whole tissue sections of ovaries and fallopian tubes (controls). In addition to tumor epithelium, PTCH1 protein expression was also analyzed in the connective stroma of serous ovarian carcinomas to examine its role in the tumor microenvironment. The connective stroma of the tumor is not tumor tissue, but it may interact with tumor cells and promote tumor growth, proliferation, angiogenesis, invasiveness, and metastasis. Analysis of

PTCH1 protein expression indicated increased nuclear expression of this protein in tumor tissues (Fig. 1A-B). In addition to nuclear, cytoplasmic expression of this protein was also observed (Fig. 1A-D). The results of immunohistochemical analysis of nuclear, cytoplasmic, and total PTCH1 protein expression in tumor epithelium and its connective stroma and epithelial and stromal compartments of control samples are shown in Fig. 1E-J. The data suggest that PTCH1 is highly expressed in tumor tissue.

Total expression of PTCH1 protein

PTCH1 protein expression was significantly higher in tumor epithelium of HGSCs and LGSCs compared with healthy OSE ($p = 0.01$ and $p = 0.030$, respectively) and FTE ($p = 0.018$ and $p = 0.018$, respectively) (Fig. 1A-D). There was no statistically significant difference in PTCH1 protein expression in tumor epithelium between HGSCs and LGSCs, nor in healthy epithelium between ovaries and fallopian tubes ($p = 0.574$) (Fig. 1A-D).

In the connective stroma, PTCH1 protein expression was significantly higher in LGSCs than in healthy fallopian tubes ($p = 0.023$). However, there was no statistically significant difference in the expression of this protein in the connective stroma of HGSCs compared with healthy ovarian ($p = 0.993$) and fallopian tube stroma ($p = 0.128$), in the connective stroma of LGSCs compared with the stroma of healthy ovaries ($p = 0.262$), between HGSCs and LGSCs ($p = 0.260$), as well as between ovaries and fallopian tubes ($p = 0.103$) (Fig. 1A-D).

We also wanted to see if we could observe the difference in PTCH1 protein expression between different tissue compartments to examine whether PTCH1 has a greater role in epithelial or stromal tissue. PTCH1 protein expression was significantly higher in tumor epithelium than in connective stroma of HGSCs ($p = 0.047$). At the same time, there was no statistically significant difference in the expression of this protein between tumor epithelial tissue and connective stroma in LGSCs ($p = 0.573$), as well as between epithelial and stromal tissue in healthy ovaries ($p = 0.249$) and fallopian tubes ($p = 0.195$) (Fig. 1A-D).

These data suggest that PTCH1 may play an active role in tumor epithelium of both HGSCs and LGSCs, and in the connective stroma of LGSCs. Since we observed that PTCH1 protein was expressed in both cytoplasm and nucleus of tumor and normal cells, we decided to analyze PTCH1 protein expression in each of these cellular compartments.

Cytoplasmic expression of PTCH1 protein

Cytoplasmic expression of the PTCH1 protein was significantly higher in the tumor epithelium of LGSCs than healthy FTE ($p = 0.023$) (Fig. 1B and D). In tumor epithelium, cytoplasmic expression of PTCH1 protein was higher in LGSCs than in HGSCs ($p = 0.053$) (Fig. 1A-B). There was no statistically significant difference in the cytoplasmic expression of this protein in the tumor epithelium of HGSCs compared with healthy OSE ($p = 0.972$) and FTE ($p = 0.188$) (Fig. 1A, C and D), in the epithelium of LGSCs compared with OSE ($p = 0.149$) (Fig. 1B-C), as well as between OSE and FTE ($p = 0.355$) (Fig. 1C-D).

In the stromal compartment, cytoplasmic expression of PTCH1 protein was significantly higher in healthy ovaries than in healthy fallopian tubes ($p = 0.005$). At the same time, there was no statistically significant difference in cytoplasmic expression of this protein in the connective stroma of HGSCs and LGSCs compared with healthy ovarian ($p = 0.094$ and $p = 0.168$, respectively) and fallopian tube stroma ($p = 0.089$ and $p = 0.165$, respectively), as well as between connective stroma of HGSCs and LGSCs ($p = 0.949$) (Fig. 1A-D).

Cytoplasmic expression of PTCH1 protein was not significantly different between tumor epithelial tissue and connective stroma in HGSCs ($p = 0.918$) and LGSCs ($p = 0.141$), nor between epithelial and stromal tissue in healthy ovaries ($p = 0.182$) and fallopian tubes ($p = 0.702$) (Fig. 1A-D).

Protein expression analysis showed that cytoplasmic expression of PTCH1 was significantly elevated only in tumor epithelium of LGSCs.

Nuclear expression of PTCH1 protein

Nuclear expression of PTCH1 protein was significantly higher in tumor epithelium of HGSCs and LGSCs compared with healthy OSE ($p < 0.0001$ and $p < 0.0001$, respectively) and FTE ($p = 0.010$ and $p = 0.014$, respectively) (Fig. 1A-D). In tumor epithelium, there was no statistically significant difference in nuclear expression of PTCH1 protein between HGSCs and LGSCs ($p = 0.763$). In contrast, in the healthy epithelium, nuclear expression was significantly higher in the fallopian tube than in the ovary ($p < 0.0001$) (Fig. 1C-D).

In the stromal compartment, nuclear expression of PTCH1 protein was significantly higher in healthy ovaries than in healthy fallopian tubes ($p = 0.022$). At the same time, there was no statistically significant difference in nuclear expression of this protein in the connective stroma of HGSCs and LGSCs compared with stroma of healthy ovaries ($p = 0.085$ and $p = 0.627$, respectively) and fallopian tubes ($p = 0.342$ and $p = 0.337$, respectively), as well as between connective stroma of HGSCs and LGSCs ($p = 0.633$) (Fig. 1A-D).

Nuclear expression of PTCH1 protein was significantly higher in tumor epithelium than in connective stroma of HGSCs ($p < 0.0001$) (Fig. 1A), as well as in epithelium of healthy fallopian tubes compared with their stroma ($p = 0.022$) (Fig. 1D). In contrast, in healthy ovaries, nuclear expression was significantly higher in stromal tissue than in epithelium ($p < 0.0001$) (not shown). In LGSCs, there was no statistically significant difference in nuclear expression of the PTCH1 protein between tumor epithelial tissue and tumor connective stroma ($p = 0.166$) (Fig. 1B).

Nuclear localization of PTCH1 protein was confirmed by immunohistochemistry on HGSC and LGSC tissue samples using four different anti-PTCH1 antibodies: anti-PTCH1a, anti-PTCH1b, anti-PTCH1c, and anti-PTCH1d (Table 1; Fig. 2). In addition to nuclear, cytoplasmic expression of this protein was observed in all four cases. In samples treated with anti-PTCH1d antibody, PTCH1 protein was strongly expressed in the cytoplasm, while its nuclear expression was weaker compared with samples treated with anti-PTCH1a, anti-PTCH1b and anti-PTCH1c antibodies (Fig. 2).

Protein expression analysis showed that nuclear expression of PTCH1 was significantly increased in tumor epithelium of both HGSCs and LGSCs, but not in the connective stroma of these carcinomas. These data suggest that PTCH1 may play an active role in the nucleus of serous ovarian carcinoma cells.

PTCH1 protein expression in HGSC cell lines

Since nuclear expression of PTCH1 protein has not been reported in ovarian cancers, we decided to investigate the specificity of PTCH1 nuclear localization by immunofluorescence on HGSC cell lines. In the OVCAR5, OVCAR8, OVSAHO, and FNE1 cell lines, PTCH1 protein expression was detected using two different antibodies, anti-PTCH1a and anti-PTCH1d (Table 1). In both cases, PTCH1 protein expression was higher in cancer than in the control cell line (Fig. 3 and Fig. 4). In addition, a high level of PTCH1 nuclear expression detected by the anti-PTCH1a antibody was observed in all cancer cell lines, whereas in the FNE1 control cell line, the nuclear expression of this protein was low (Fig. 3).

In addition to nuclear, cytoplasmic and membrane expression of the PTCH1 protein was observed in cell lines. In contrast to the anti-PTCH1a antibody, cytoplasmic expression detected by the anti-PTCH1d antibody was highly present in all cancer cell lines with punctate signals observed in the cytoplasm. In contrast, in the control cell line, it was largely absent (Fig. 4). Membranous PTCH1 protein expression detected by the anti-PTCH1d antibody was observed in the FNE1 control cell line (Fig. 4).

PTCH1 protein expression in cell lines was also detected using anti-PTCH1b and anti-PTCH1c antibodies (Table 1). A high level of PTCH1 nuclear expression was observed in all cancer cell lines, while in the FNE1 control cell line, the nuclear expression of this protein was low (Fig. 5 and Fig. 6), as in the case of the use of anti-PTCH1a antibody.

Subcellular localization of PTCH1 protein in HGSC cell lines

To examine the cellular localization of PTCH1 in more detail, subcellular localization of PTCH1 protein in OVCAR5, OVCAR8, OVSAHO, and FNE1 cell lines was analyzed by Western blot. The expression of PTCH1 protein in different cellular compartments (cytoplasmic, membrane, nuclear soluble, chromatin-bound, and cytoskeletal protein fractions) was detected using two different antibodies, anti-PTCH1a and anti-PTCH1d (Table 1). The efficiency of protein isolation from different cellular compartments was analyzed by checking the presence of H3K4me2 protein in the chromatin-bound protein fraction and the presence of GAPDH protein in the cytoplasmic, membrane, and nuclear soluble protein fractions (Fig. 7). Analysis of total proteins confirmed the presence of PTCH1 protein in all cell lines using both antibodies (Fig. 7).

Protein PTCH1 detected by anti-PTCH1a antibody was present in the cytoplasmic, membrane, nuclear soluble, and chromatin-bound protein fractions in all cell lines, with the highest presence in the membrane and nuclear soluble protein fractions. In contrast, in the cytoskeletal fraction, it was present only in the OVSAHO cell line (Fig. 7A).

PTCH1 protein detected by the anti-PTCH1d antibody was present in all cellular fractions in each cell line. PTCH1 was mostly present in the cytoplasmic fraction in OVCAR5 and FNE1 cell lines, in the cytoplasmic

and membrane fractions in the OVCAR8 cell line, and in the nuclear soluble, chromatin-bound, and cytoskeletal fractions in OVSAHO cell line (Fig. 7B).

PTCH1 protein expression in different cellular compartments was also detected using anti-PTCH1b and anti-PTCH1c antibodies (Fig. 8). PTCH1 protein was mostly present in membrane and nuclear soluble fractions when anti-PTCH1a, anti-PTCH1b, and anti-PTCH1c antibodies were used. In contrast, in the case of an anti-PTCH1d antibody, this protein was mostly present in the cytoplasmic fraction. In all four cases, fragments of PTCH1 protein of different molecular weight were detected, which indicates the possibility of proteolytic cleavage of this protein (Fig. 7 and Fig. 8).

DNA promoter methylation status of PTCH1 in serous ovarian carcinomas

DNA promoter methylation of *PTCH1* was exclusively observed in HGSCs (5/37, 13.5%), while no methylation was detected in any of the LGSCs and healthy ovarian and fallopian tube tissues (Fig. 9A). There was no correlation between DNA promoter methylation of the *PTCH1* gene and total expression of PTCH1 protein in HGSCs (analyzed in the whole tissue sections) ($\rho = 0.079$; $p = 0.642$). *PTCH1* gene promoter was unmethylated in OVCAR5, OVCAR8, OVSAHO, and FNE1 cell lines (Fig. 9B).

Taken together, the data from our study suggest that PTCH1 protein may play an active role in the molecular pathogenesis of serous ovarian carcinoma. To our knowledge, this is the first report indicating the importance of nuclear localization of this protein in cancer cells, which can be linked with the development and progression of the serous ovarian carcinomas.

Discussion

The results from our study showed that total expression of PTCH1 protein was significantly higher in the tumor epithelium of HGSCs and LGSCs than in OSE and FTE, as well as in HGSC cell lines compared with the control cell line. Increased expression of this protein in serous ovarian carcinomas suggests its active involvement in the pathogenesis of these cancers, consistent with previous studies where increased expression of the PTCH1 protein is associated with tumor development [28–32]. In this case, PTCH1 protein has a tumor promoter rather than a tumor suppressor role. It should be noted that a significant proportion of serous ovarian carcinomas exhibited nuclear PTCH1 protein expression in the tumor epithelium. This was further confirmed in HGSC cell lines.

Nuclear expression of PTCH1 protein has not been reported in ovarian cancers and is rarely mentioned in the scientific literature. Therefore, we decided to investigate the specificity of PTCH1 nuclear localization by immunohistochemistry on HGSC and LGSC tissue samples and by immunofluorescence on HGSC cell lines, using four different anti-PTCH1 antibodies. Increased nuclear expression of PTCH1 protein (in tumor tissue and cancer cell lines) was observed in the case of three of the four antibodies (anti-PTCH1a, epitope: 1–50 aa; anti-PTCH1b, epitope: 1–50 aa; and anti-PTCH1c, epitope: 1–80 aa), while in the case of the anti-PTCH1d antibody (epitope: 122–436 aa) it was present, but in a much lower amount. In contrast to the other three polyclonal antibodies, anti-PTCH1d is a monoclonal antibody that binds to a

more distant epitope of the PTCH1 antigen. Thus, differences in PTCH1 protein expression may result from differences in the clonality of antibodies and the epitope to which they bind [40]. Polyclonal antibodies recognize the cytoplasmic NTD, whereas a monoclonal antibody recognizes the extracellular ECD1 domain of PTCH1 protein. Since nuclear expression of PTCH1 was observed in HGSCs and LGSCs, and HGSC cell lines, it can be concluded that this protein possibly plays an active tumor promoter role in the nucleus of serous ovarian carcinoma cells.

To examine the subcellular localization of PTCH1 in more detail, we analyzed the presence of PTCH1 protein in different cellular compartments of HGSC cell lines by Western blot using four abovementioned antibodies. PTCH1 protein was mostly present in membrane and nuclear soluble fractions when polyclonal antibodies were used, while in the case of the use of monoclonal antibody, this protein was mostly present in the cytoplasmic fraction, which is in line with immunofluorescence results. In all four cases, fragments of PTCH1 protein of different molecular weights were detected, which indicates the possibility of proteolytic cleavage of this protein, as well as the possibility of posttranslational modifications of the resulting fragments. Although polyclonal antibodies anti-PTCH1a and anti-PTCH1b recognize the same epitope of the PTCH1 antigen, it is not clear why they showed a different pattern of PTCH1 fragments in the same samples.

As already mentioned, increased nuclear expression of PTCH1 protein in HGSC cell lines was detected using polyclonal antibodies, which bind to the cytoplasmic NTD of PTCH1 protein, while increased cytoplasmic expression of this protein was detected using a monoclonal antibody, which binds to the extracellular ECD1 domain of PTCH1 protein. It may be possible that N-terminal cytoplasmic fragments (not recognized by the anti-PTCH1d monoclonal antibody) translocate to the nucleus after proteolytic cleavage, where it performs a hitherto unknown function, while most of the protein remains in the cytoplasm.

Translocation of cytoplasmic domain fragments of transmembrane proteins to the nucleus has already been recognized as an important mechanism of direct signal transfer between these two cellular compartments [41, 42]. Such signal transduction has also been observed in the case of PTCH1 protein [43]. Kagawa et al. showed that PTCH1 protein was subjected to proteolytic cleavage at the C-terminus, resulting in the generation of soluble C-terminal fragment, ICD7. They observed that ICD7 fragments accumulate in the cell nucleus of HeLa cells stably expressing full-length PTCH1, where they modulate the transcriptional activity of the GLI1 protein. At the same time, the N-terminal region remains in the cytoplasmic punctuates, which may correspond to multivesicular bodies and endosomes, and does not travel to the nucleus. In addition, nuclear accumulation of endogenous PTCH1 ICD7 fragments was also observed in mouse embryonic fibroblasts (C3H10T1/2 cells) and mouse embryonic primary cells. Although Kagawa et al. have found that these fragments have certain regulatory roles, their biological importance remains unknown [43]. However, the results of our study indicates the possibility of proteolytic cleavage of the PTCH1 N-terminal cytoplasmic region, resulting in the generation of soluble N-terminal fragments that are translocated to the nucleus. To the best of our knowledge, this is the first study to indicate the presence of N-terminal fragments of the PTCH1 protein in the nucleus. Since nuclear

localization of these fragments was detected in serous ovarian carcinoma cells, it is hypothesized that they could play an active tumor promoter role in the nucleus of these malignant cells. Further studies involving more samples and additional molecular methods are needed to confirm these findings and their clinical relevance.

Since PTCH1 protein expression could be affected by epigenetic changes such as DNA methylation, we have also analyzed the methylation status of the *PTCH1* gene in serous ovarian carcinomas and HGSC cell lines. The results of our study showed that DNA promoter methylation of *PTCH1* was exclusively observed in HGSCs (13.5%). In contrast, no methylation was detected in any LGSC and healthy ovarian and fallopian tube tissues, as well as in control and HGSC cell lines. *PTCH1* gene promoter methylation was found not to affect the PTCH1 protein expression in HGSCs. The relatively small percentage of HGSC samples with the methylated *PTCH1* gene promoter, the absence of methylation of this gene in LGSCs, control tissues, and all cell lines lead to the conclusion that DNA methylation of the *PTCH1* gene promoter is not a prevalent mechanism involved in the pathogenesis of serous ovarian carcinomas. DNA promoter methylation of the *PTCH1* gene has been observed in benign ovarian tumors, such as ovarian dermoids and fibromas [44]. However, this alteration has not been observed as a highly prevalent feature of ovarian cancer [45], which is in line with our results.

Identification of specific molecules and detection of genetic and epigenetic changes associated with the development of ovarian cancer can significantly facilitate the diagnosis and development of new targeted therapies or improve response to currently available therapies.

Conclusions

Our data suggest that PTCH1 protein may play an active role in the molecular pathogenesis of both low- and high-grade serous ovarian carcinomas. For the first time, we indicated the importance of nuclear localization of this protein in cancer cells and possible proteolytic cleavage of this protein, resulting in the generation of soluble N-terminal fragments that are translocated to the nucleus, which can be linked with the development and progression of the serous ovarian carcinomas. Furthermore, we showed that DNA methylation of the *PTCH1* gene promoter is not a potential mechanism involved in the pathogenesis of serous ovarian carcinomas, suggesting that possibly other mechanisms, either epigenetic or posttranslational, regulate *PTCH1* gene expression and protein level in these carcinomas.

Abbreviations

CTD – C-terminal domain

DHH – Desert Hedgehog

DMEM – Dulbecco's modified Eagle's Medium

ECD1 – extracellular domain 1

ECD2 – extracellular domain 2

FFPE – formalin-fixed paraffin-embedded

FTE – fallopian tube epithelium

HGSC – high-grade serous ovarian carcinoma

Hh – Hedgehog

IHH – Indian Hedgehog

LGSC – low-grade serous ovarian carcinoma

LTCC – Live Tumor Culture Core

ML – middle loop

MSP – methylation-specific PCR

NTD – N-terminal domain

OC – ovarian cancer

OSE – ovarian surface epithelium

PTCH1 – Patched 1

PTCH2 – Patched 2

SHH – Sonic Hedgehog

TMD – transmembrane domain

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committees of the School of Medicine, University of Zagreb, and the University Hospital Merkur Zagreb (380-59-10106-20-111/130 and 03/1-11080) and was performed according to ethical standards of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

VKK contributed to conceptualization, interpretation, data acquisition and analysis, performed experimental work, wrote and edited the manuscript, and revised the manuscript for important intellectual content; ACP contributed to conceptualization, interpretation and design of experiments, and revised the manuscript for important intellectual content; AS contributed to data analysis and interpretation, and revised the manuscript for important intellectual content; SV contributed to data analysis and interpretation, and revised the manuscript for important intellectual content; LS conceived the idea, contributed to conceptualization, data collection and analysis and interpretation of the results, and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Figures

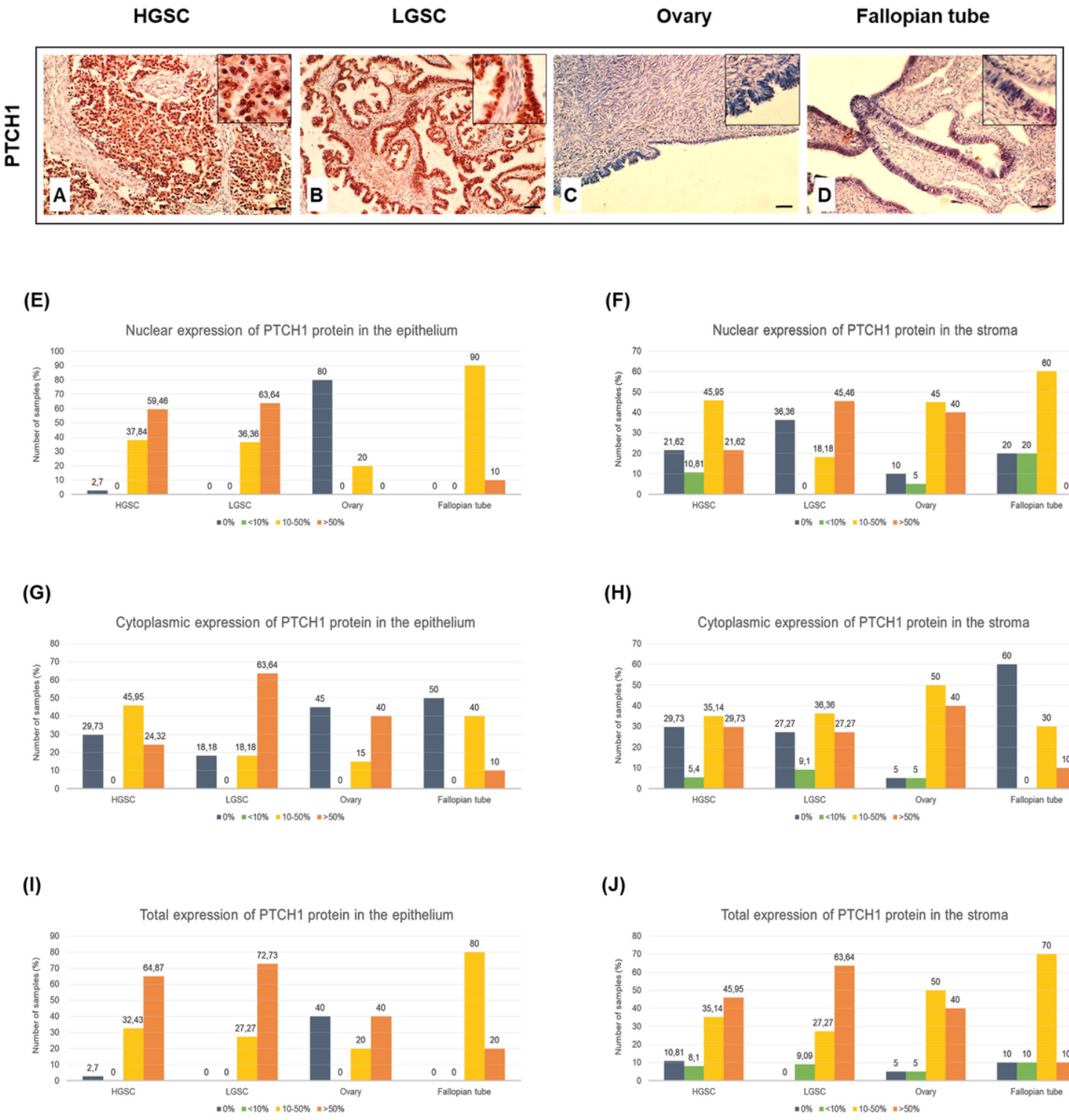

Figure 1

Immunohistochemical staining of PTCH1 protein in high-grade serous ovarian carcinoma (HGSC) (A), low-grade serous ovarian carcinoma (LGSC) (B), healthy ovarian tissue (C), and healthy fallopian tube tissue (D). Scale bar corresponds to 100 µm. Nuclear, cytoplasmic and total expression of PTCH1 protein in tumor epithelium of HGSCs and LGSCs and epithelium of healthy ovaries and fallopian tubes (E, G, I), and in the connective stroma of HGSCs and LGSCs and ovarian and fallopian tube stroma (F, H, J)

determined by immunohistochemical analysis (0%, <10%, 10-50%,> 50% - the percentage of immunopositive cells).

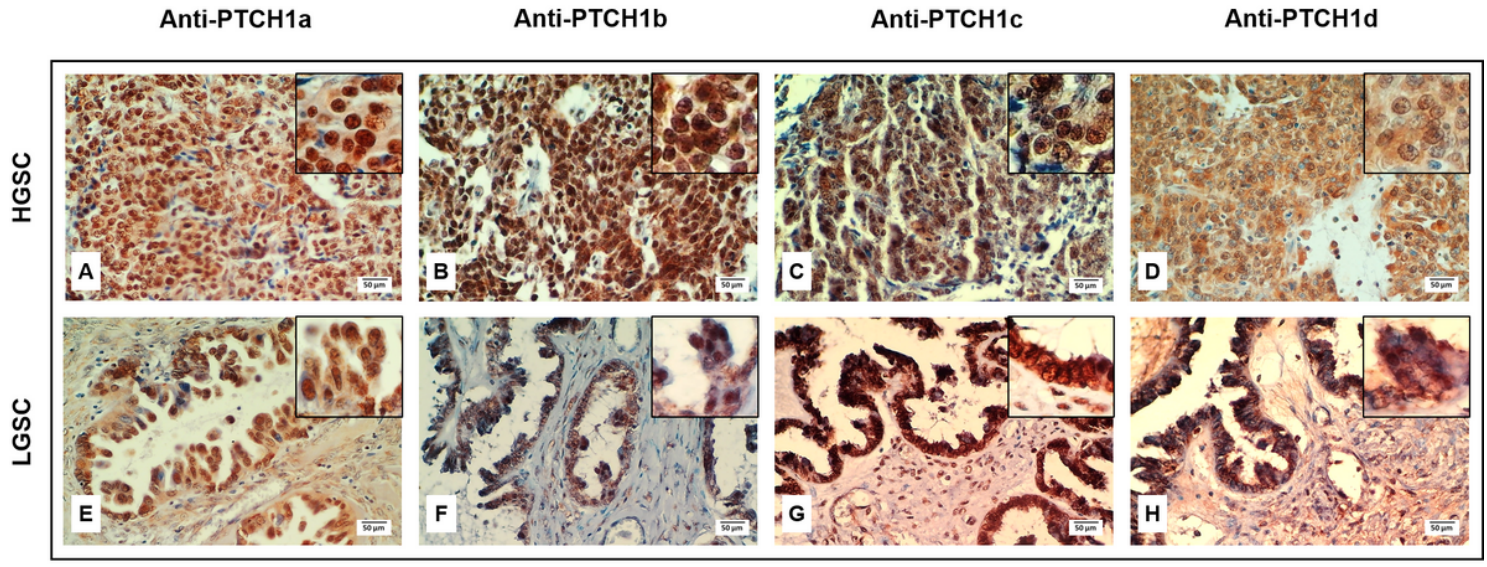

Figure 2

Immunohistochemical staining of PTCH1 protein in high-grade (HGSC; A-D) and low-grade serous ovarian carcinomas (LGSC; E-H) obtained using four different antibodies, anti-PTCH1a (A, E), anti-PTCH1b (B, F), anti-PTCH1c (C, G), and anti-PTCH1d (D, H). Scale bar corresponds to 50 μm.

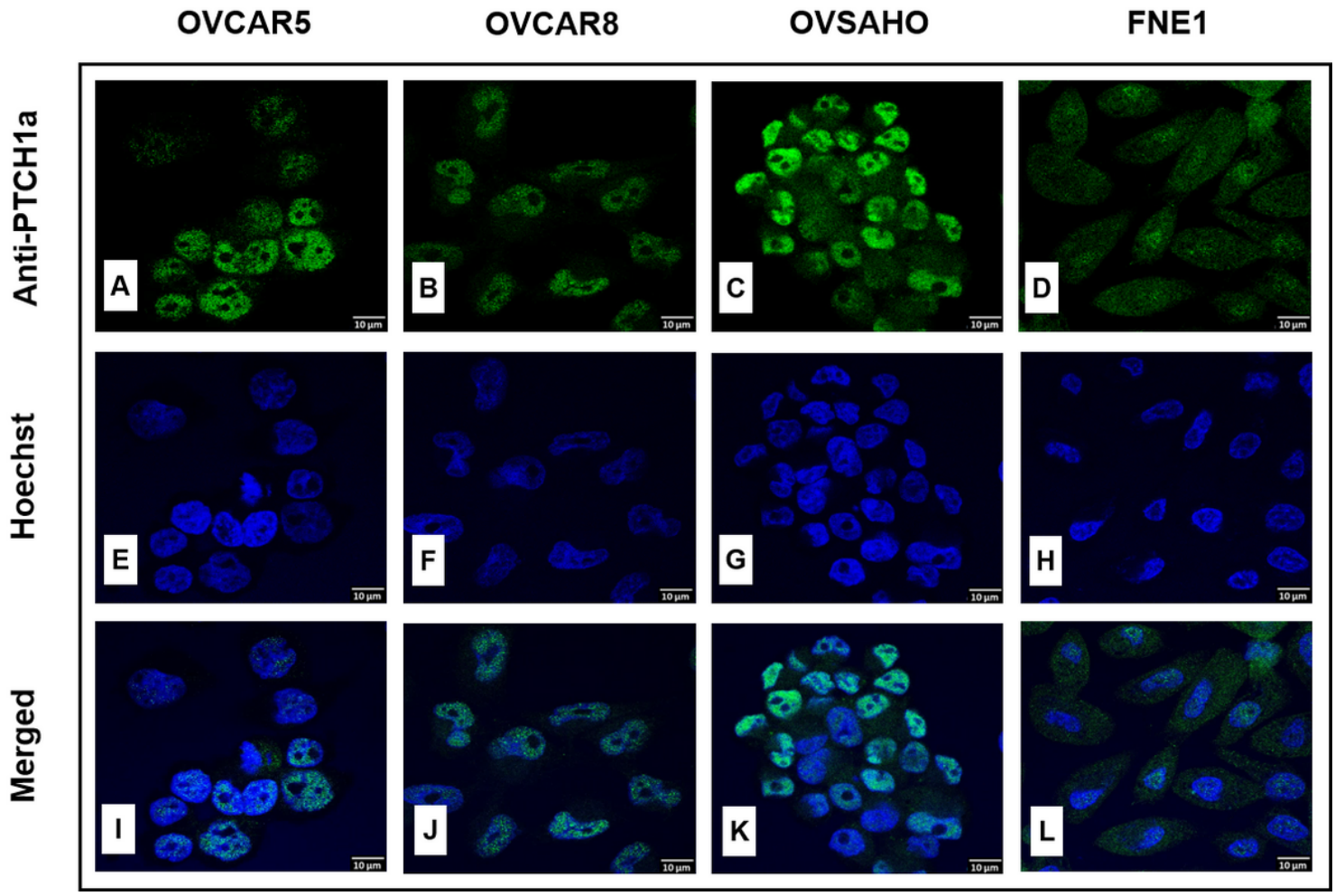

Figure 3

Immunofluorescence staining of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5 (A, E, I), OVCAR8 (B, F, J) and OVSAHO (C, G, K) and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (D, H, L) obtained using an anti-PTCH1a antibody (A-D). Nuclei stained with Hoechst (E-H). Merged images (I-L).

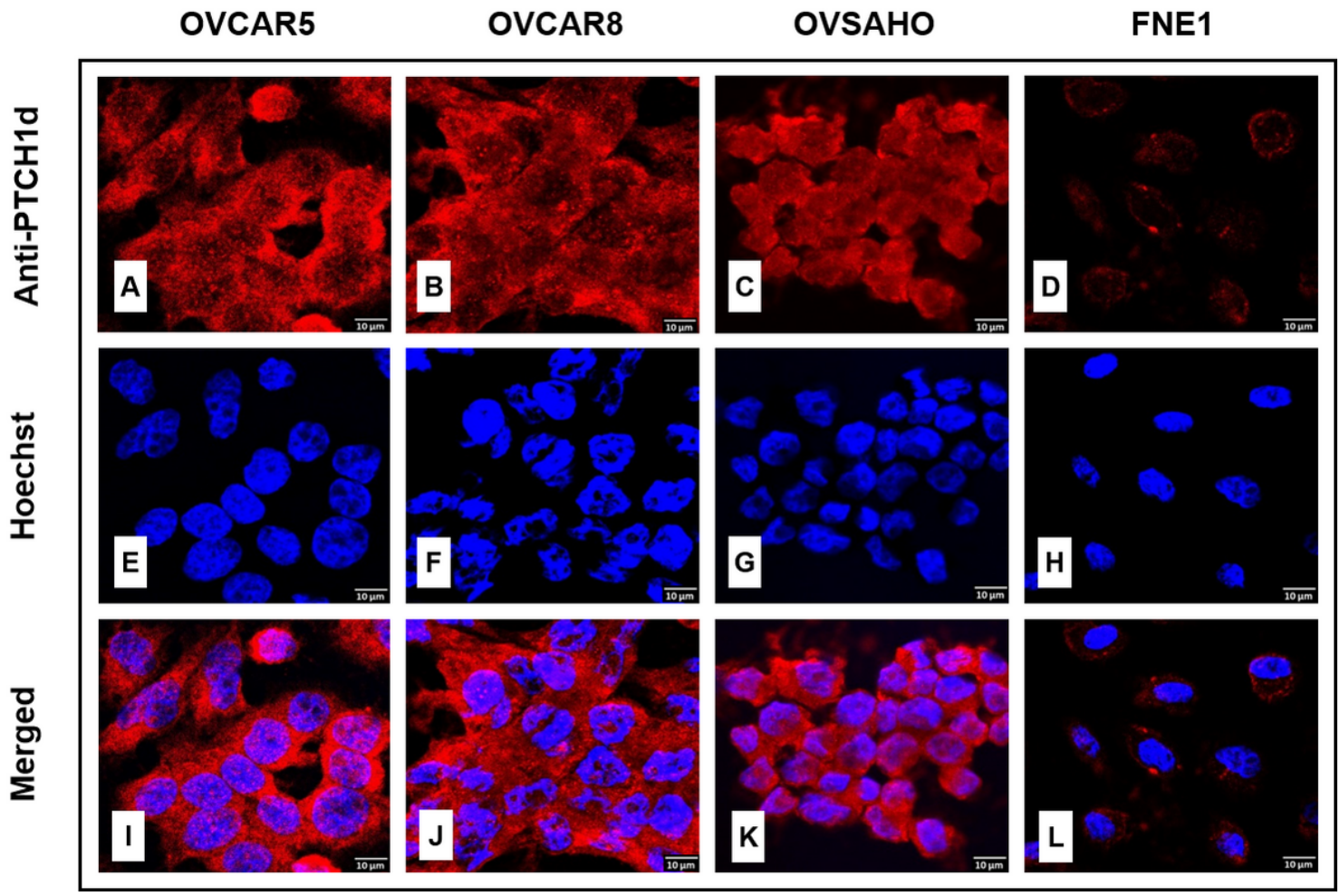

Figure 4

Immunofluorescence staining of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5 (A, E, I), OVCAR8 (B, F, J) and OVSAHO (C, G, K) and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (D, H, L) obtained using an anti-PTCH1d antibody (A-D). Nuclei stained with Hoechst (E-H). Merged images (I-L).

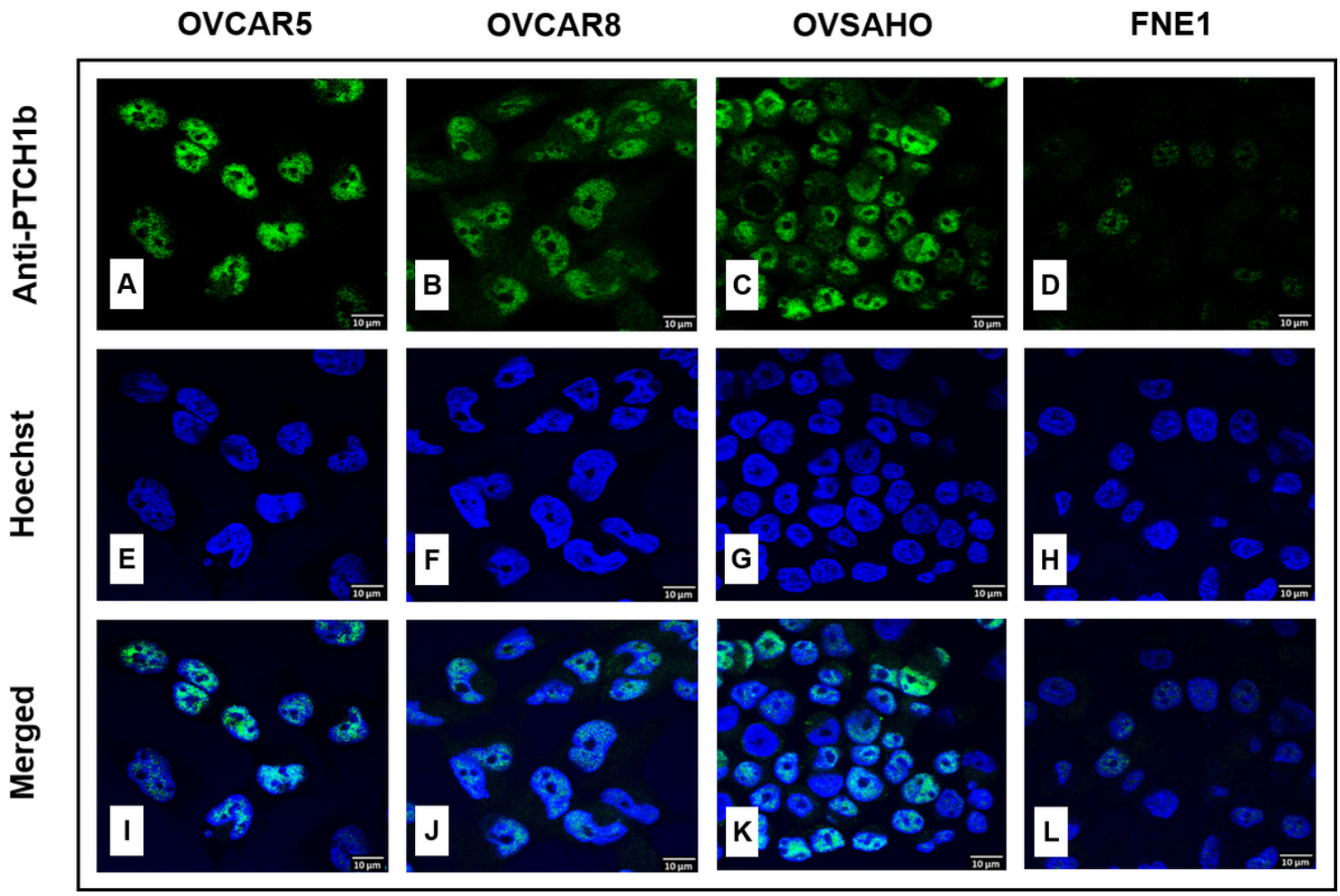

Figure 5

Immunofluorescence staining of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5 (A, E, I), OVCAR8 (B, F, J) and OVSAHO (C, G, K) and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (D, H, L) obtained using anti-PTCH1b antibody (A-D). Nuclei stained with Hoechst (E-H). Merged images (I-L).

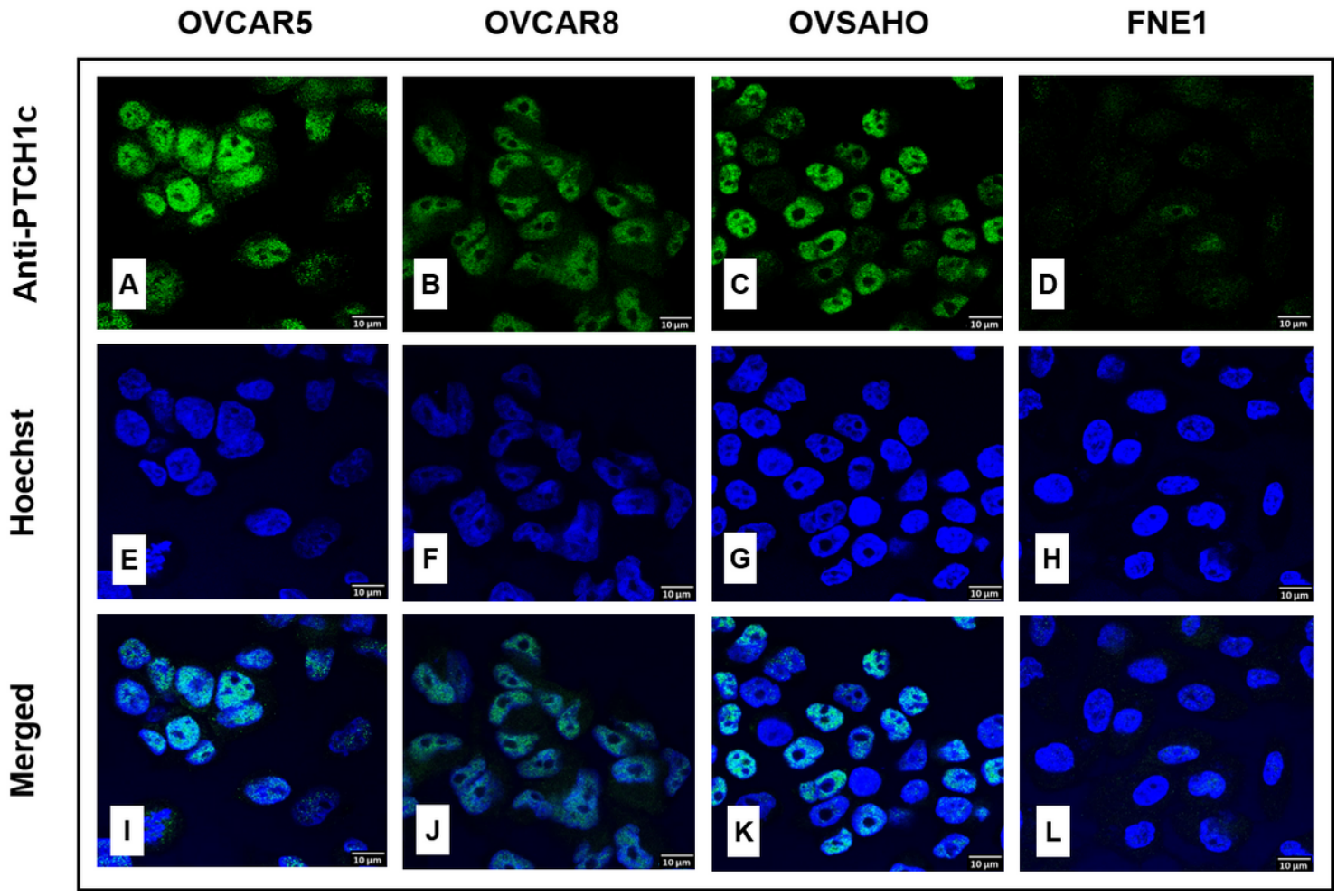

Figure 6

Immunofluorescence staining of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5 (A, E, I), OVCAR8 (B, F, J) and OVSAHO (C, G, K) and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (D, H, L) obtained using an anti-PTCH1c antibody (A-D). Nuclei stained with Hoechst (E-H). Merged images (I-L).

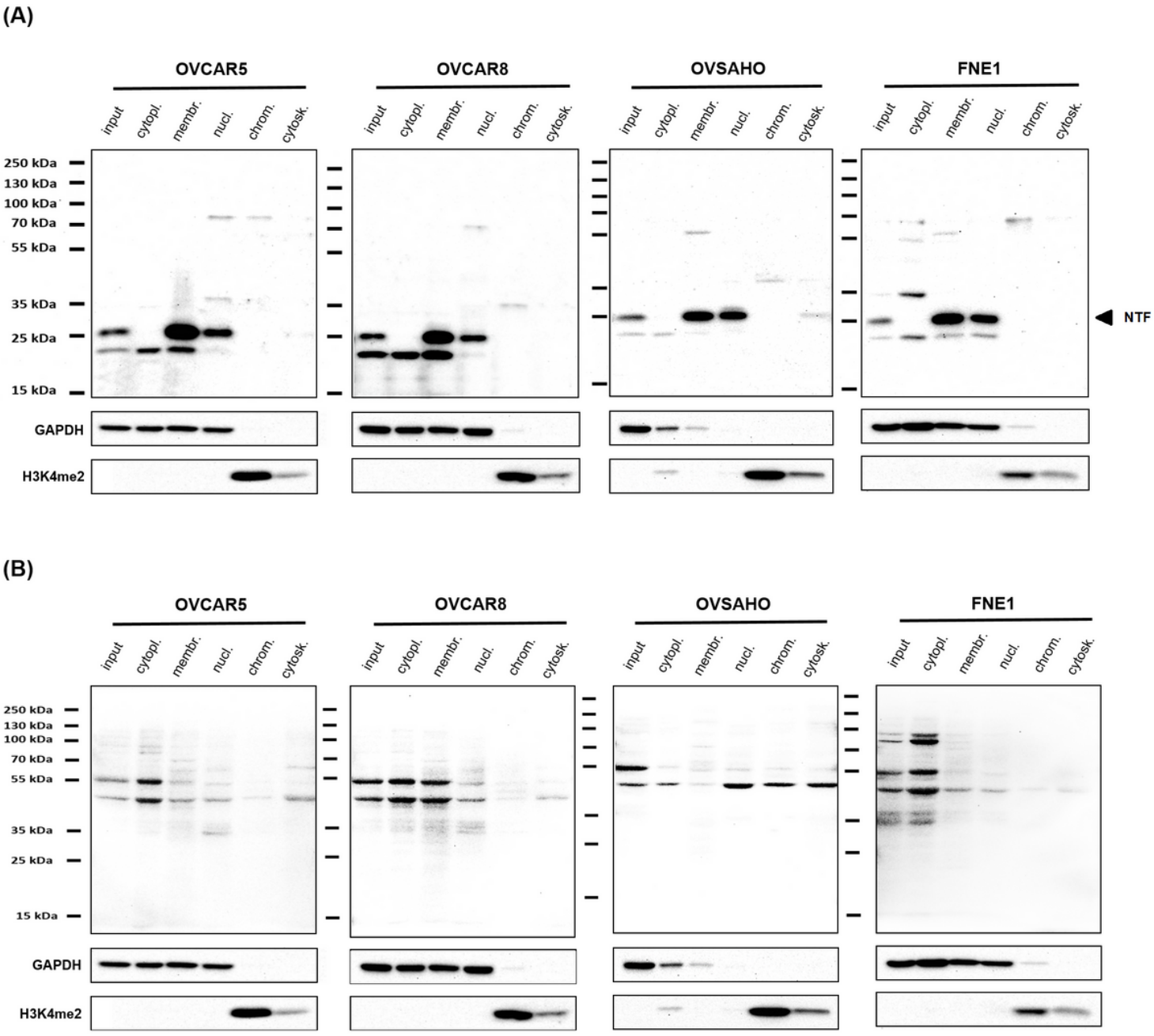

Figure 7

Subcellular localization of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5, OVCAR8, and OVSAHO and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1, detected by Western blot using anti-PTCH1a (A) and anti-PTCH1d (B) antibodies. input - total proteins; cytopl. - cytoplasmic proteins; membr. - membrane proteins; nucl. – nuclear soluble proteins; chrom. - chromatin-bound proteins; cytosk. - cytoskeletal proteins; NTF – N-terminal fragment of PTCH1 protein

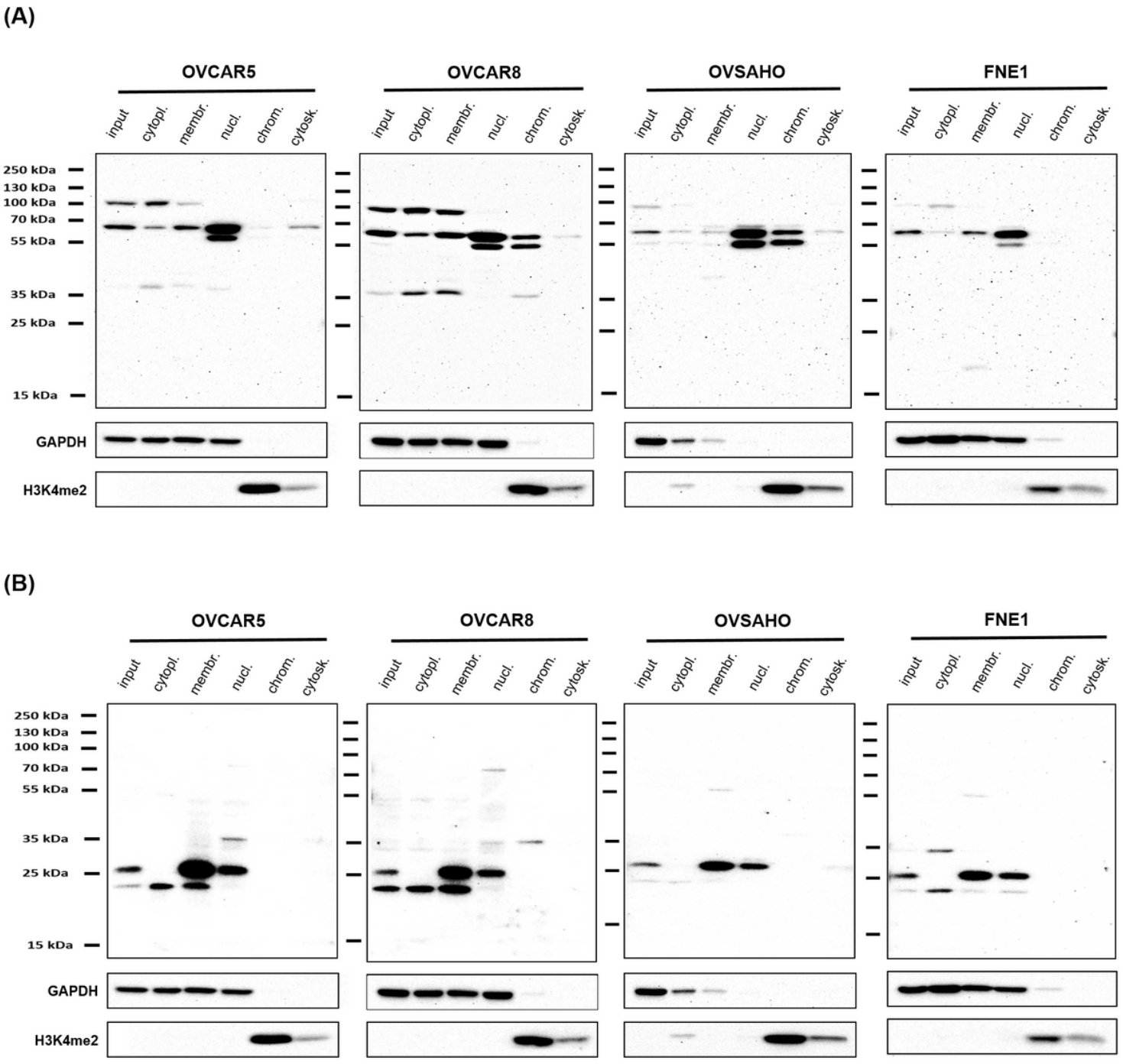

Figure 8

Subcellular localization of PTCH1 protein in high-grade serous ovarian carcinoma cell lines, OVCAR5, OVCAR8, and OVSAHO and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1, detected by Western blot using anti-PTCH1b (A) and anti-PTCH1c (B) antibodies. input - total proteins; cytopl. - cytoplasmic proteins; membr. - membrane proteins; nucl. – nuclear soluble proteins; chrom. - chromatin-bound proteins; cytosk. - cytoskeletal proteins

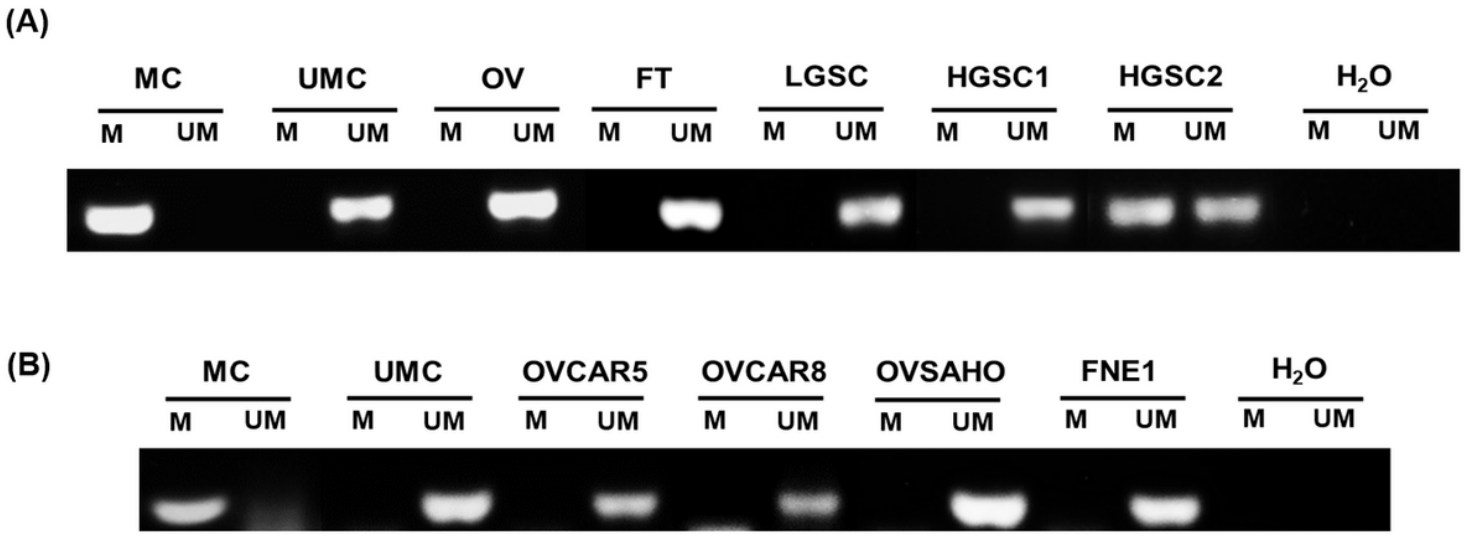

Figure 9

A representative example of methylation-specific PCR analysis for PTCH1 gene promoter in healthy ovarian (OV) and fallopian tube (FT) tissues, low-grade (LGSC) and high-grade serous ovarian carcinomas (HGSC1, HGSC2) (A). DNA promoter methylation status of the PTCH1 gene in high-grade serous ovarian carcinoma cell lines, OVCAR5, OVCAR8, OVSAHO, and normal TERT-immortalized fallopian tube non-ciliated epithelium cell line FNE1 (B). M - methylated reaction; UM - unmethylated reaction; MC - methylated human control, positive control for methylated reaction; UMC - unmethylated human control, positive control for unmethylated reaction; water (H2O) - negative control