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Original Article

ZNF217 is associated with poor prognosis and enhances proliferation and metastasis in ovarian cancer

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Abstract: ZNF217 is an alternatively spliced Kruppel-like transcription factor that has recently been implicated to play a role in human carcinogenesis. Here, we used immunohistochemistry (IHC) to show that ZNF217 protein is overexpressed in nearly 60% of ovarian tumor samples. The disease-free survival time was shorter in patients with positive ZNF217 expression than in ZNF217-negative patients (P=0.042). Fluorescence in situ hybridization (FISH) analysis showed ZNF217 genomic amplification in the poorly differentiated tumors, suggesting that ZNF217 is associated with the progression of ovarian cancer. Invasion was enhanced in HO-8910 cells stably transfected with constructs carrying full-length ZNF217 relative to cells transfected with the empty vector. To confirm our findings *in vivo*, we performed a tumorigenicity assay in nude mice inoculated with the HO-8910 overexpressing ZNF217 cells. As expected, tumors grown in the ZNF217 group were more invasive and prone to metastasis than those formed control groups. Based on these clinical and laboratory observations, we conclude that ZNF217 may contribute to ovarian cancer invasion and metastasis, and associated with worse clinical outcomes.

Keywords: Ovarian cancer, ZNF217 gene, gene expression, proliferation and invasion, tumor metastasis

Introduction

Ovarian epithelial carcinoma is one of the most lethal gynecological malignancies found in humans. Due to a lack of good biomarkers, early diagnosis is difficult, and the disease often reaches an advanced stage before diagnosis. The mortality rate is among the highest of the gynecological tumors, with a 5-year survival rate of only about 30% [1].

ZNF217 is a putative oncogene believed to encode an alternatively spliced Krüppel-like transcription factor. It was originally identified in breast cancer and was thought to be directly involved in human carcinogenesis. Expression of ZNF217 appears to be an early event, and is correlated with poor prognosis [2, 3]. Overexpression of *ZNF217* leads to increased phosphorylation of Akt, and inhibition of the phosphatidylinositol 3 kinase pathway and Akt phosphorylation decreases *ZNF217* protein levels and increases sensitivity to doxorubicin [4]. *ZNF217* may promote survival of preneoplastic cells with dysfunctional telomeres early on and confer resistance to chemotherapy later [4, 5]. Recently, it has been reported that ZNF217 amplification occurs in a variety of tumor types, such as breast, gastric, ovarian, lung, prostate, and colon cancer, and is associated with aggressive tumor behavior [2, 6-9].

We evaluated ZNF217's role as a biomarker of ovarian carcinogenesis and tumor progression in patient samples and explored possible molecular mechanisms in promoting tumor growth and invasion.

Methods

Patient characteristics

A total of 44 patients with a median age of 43 years (range: 16 to 61 years) were enrolled in the study. All were treated at the Nanfang Hospital, Southern Medical University. Informed consent was obtained from all patients in advance.

Cell culture

Ovarian serious cystadenocarcinoma OVCaR3 and SKOV3 cell lines were obtained from the

American Type Culture Collection (ATCC). The HO-8910 cell line was obtained from KEY GEN Biotech Corporation (China). ALL cell lines were cultured in in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% FBS. Cells were incubated at 37°C in a humidified 5% CO_2 incubator.

Immunohistochemistry and scoring

Tissue array sections (4-μm thick) were deparaffinized in xylene, rehydrated in graded alcohols, and treated with 3% hydrogen peroxide in methanol at room temperature to block endogenous peroxidase activity. ZNF217 antibody (1:400 dilutions) staining was visualized using the avidin-biotin-peroxidase technique followed by chromogen detection with diaminobenzidine (DAB). Negative controls were created by omitting the primary antibody.

The immunohistochemically stained tissue sections were scored separately by two pathologists [10, 11]. Whole tissue sections were scanned to assign the scores. Staining intensity was scored as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%), according to the relative sizes of the positive staining areas in relation to the entire tumor section. The sums of the intensity and extent scores were used as final staining scores (0-7). This scoring method gives highly consistent results across independent evaluators and has been used in previous studies. For the purpose of statistical evaluation, tumors with a final staining score ≥3 were considered positive.

Fluorescence in situ hybridization analysis

Forty-four ovarian cancer samples (paraffin-embedded and fresh tissue) that expressed ZNF217 were used for FISH analysis. Paraffin sections (6-μm thick) were deparaffinized and cell suspensions were made from fresh tissues by enzymatic digestion. A dual-color, locus-specific probe set containing spectrum-orange-labeled ZNF217 (map to 20q13.2) and a spectrum-green-labeled 18 centromere probe was obtained from Vysis (Downers Grove, IL, U.S.). Hybridization signals on 100 interphase cells on DAPI-counterstained slides were scored using a Nikon fluorescence microscope (Nikon Co, Tokyo, Japan). The signal was measured under ×1000 magnification.

Plasmid constructs and production of stable cell lines

Full-length human *ZNF217* cDNA from SKOV3 cells was amplified by polymerase chain reaction (PCR) using primers for ZNF217. It was then inserted into pEGFP-N1 at the EcoR I and Bgl II sites. Clones were confirmed by restriction analysis and by DNA sequencing of both strands. All transfectants were selected with 1000 μg/mL G418 for 3 weeks. Selected clones were screened with Western blot using ZNF217 antibody (Abcam, UN) and/or RT-PCR. When confluent, cells were subcultured by trypsinization (0.05% trypsin, 0.53 mM EDTA in Hanks' balanced salt solution). The pictures showing the morphologies of various stable cells expressing different constructs were taken through phase contrast microscopy (ECLIPSE TE300, Nikon Co., Tokyo, Japan).

Western blotting

Cells were lysed in buffer (1% Triton X-100, 50 mM Hepes pH 7.4, 150 mM NaCl, 1.5 $MgCl_2$, 1 mM EGTA, 10 mM NaF, 100 mM NaPPi, 10% glycerol, 1 mM PMSF, 1 mM Na_3VO_4, and 10 lg/ml aprotinin). Protein concentration was measured using a Bio-Rad protein assay kit. To analyze HO-8910, PEGFP-N1/HO-8910, and PEGFP-N1-ZNF217/HO-8910 cells, protein (60 ug) was separated on 10% SDS-PAGE gels followed by transfer to nitrocellulose membranes. After blocking with 5% nonfat milk for 90 minutes, blots were probed overnight at room temperature with goat polyclonal antibody against ZNF217 at a 1:200 dilution or with mouse β-actin monoclonal antibody at a 1:1000 dilution. After extensive washing with TBST, the immune complexes were detected with horseradish peroxidase conjugated to rabbit anti-goat or goat anti-mouse secondary antibodies (1:5000). An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL, U.S.) was used for detection.

MTT assay

The number of attached cells in each well was examined by MTT assay and quantified in a 96 wells plate. One hundred milliliters of 1640 medium lacking fetal bovine serum and 20 ml MTT (5 mg/mL) (Sigma, U.S.) were added to each well. After incubation at 37°C in 5% CO_2 for 24, 48, or 72 hours, the medium was discarded. Two hundred milliliters of 0.04 mol/L

hydrochloric acid in isopropanol was added to each well. The amount of MTT formazan product, which reflects the number of cells adhering to FN, was determined by measuring absorbance with a microplate reader at a test wavelength of 570 nm.

Cell cycle assay

HO-8910 cells, PEGFP-N1/HO-8910 cells, and PEGFP-N1-ZNF217/HO-8910 cells were seeded into 6-well plates. After being allowed to culture for 24, 48, or 72 hours, all cells were digested with 0.25% trypsin and 0.02% EDTA, washed twice with PBS, and fixed with 1000 uL/L ice-cold alcohol. Thirty minutes later, the cells were centrifuged and rinsed three times with PBS. The precipitation was suspended in PBS with 10 mg/L PI and 1.0 g/L RNase. After 20 minutes, the cell cycle was evaluated by flow cytometry.

In vitro invasion assay

Cell migration was assayed using a Transwell chemotaxis chamber (8-μm pore filters). The HO-8910 cells, PEGFP-N1/HO-8910 cells, and PEGFP-N1-ZNF217/HO-8910 cells (10^5/well) were starved for 24 hours. Then these cells were trypsinized and loaded into the top chamber. Ten percent fetal bovine serum was then placed into the bottom chamber as a chemoattractant. Cells were incubated at 37°C in 5% CO_2 for 12 or 24 hours and allowed to migrate through the chemotaxis chamber. After incubation, the cells remaining at the upper surface were completely removed. The cells that had migrated to the bottom of the chemotaxis chamber were stained with hematoxylin and eosin. The experiments were repeated in triplicate wells and the migrated cells were counted microscopically (200×) in five different fields per filter. Cell invasion capabilities were assessed using a Matrigel invasion chamber (Becton-Dickenson, Bedford, MA, U.S.) according to the manufacturer's instructions. The procedures were essentially the same as those for the migration assay using Transwell chemotaxis chambers.

Tumorigenicity assays

Three- to four-week-old female nude mice were purchased from Southern Medical University. (Guangzhou, China). Animal studies were conducted in strict accordance with the principles and procedures approved by the Committee on the Ethics of Animal Experiments of Southern Medical University. There were four animals in each group. These were hypodermically injected with 3.5×10^6 cells in 100 μl medium. After 30 days, the animals were killed and examined for tumor formation. 1 mm^3 tumor tissues were harvested and transplanted into the ovaries of the next passage of female nude mice. After 30 days, these animals were sacrificed and examined for tumor formation and metastasis. Tumor volume was calculated using V=1/2 ($width^2\times length$). The expression of ZNF217 was detected by immunohistochemistry.

Statistical analysis

Statistical analyses were performed using the chi-square test with SPSS software 13.0. The patients' mean ages and tumor sizes were evaluated by t-test. Survival curves were produced using the Kaplan-Meier test for the risk of cancer death. Prognostic factors were examined by both univariate and multivariate analyses. In multivariate analysis, age, histological grade, and tumor stage were included as variables. All *p*-values were two-sided. $P<0.05$ was considered statistically significant.

Results

Relationship between clinicopathologic characteristics and ZNF217 expression in ovarian cancer patients

In ovarian cancer, ZNF217 overexpression is associated with poor prognosis. Significant expression of ZNF217 protein was observed in 59.09% (26/44) of our ovarian cancer samples. (Immunostaining scores ≥3 were defined as positive). As many as 35.3% (14 samples) had immunostaining scores over 5. ZNF217 overexpression was observed predominantly in ovarian cancer (**Figure 1A-C**). Another statistically significant correlation was found between ZNF217 expression and demographic and pathologic factors, such as age, histological differentiation, and tumor stage (**Table 1**). Due to the small sample size of metastatic cases (n=3), no further statistical correlation study on metastasis could be performed.

ZNF217 overexpression was significantly negatively correlated with overall survival. Patients

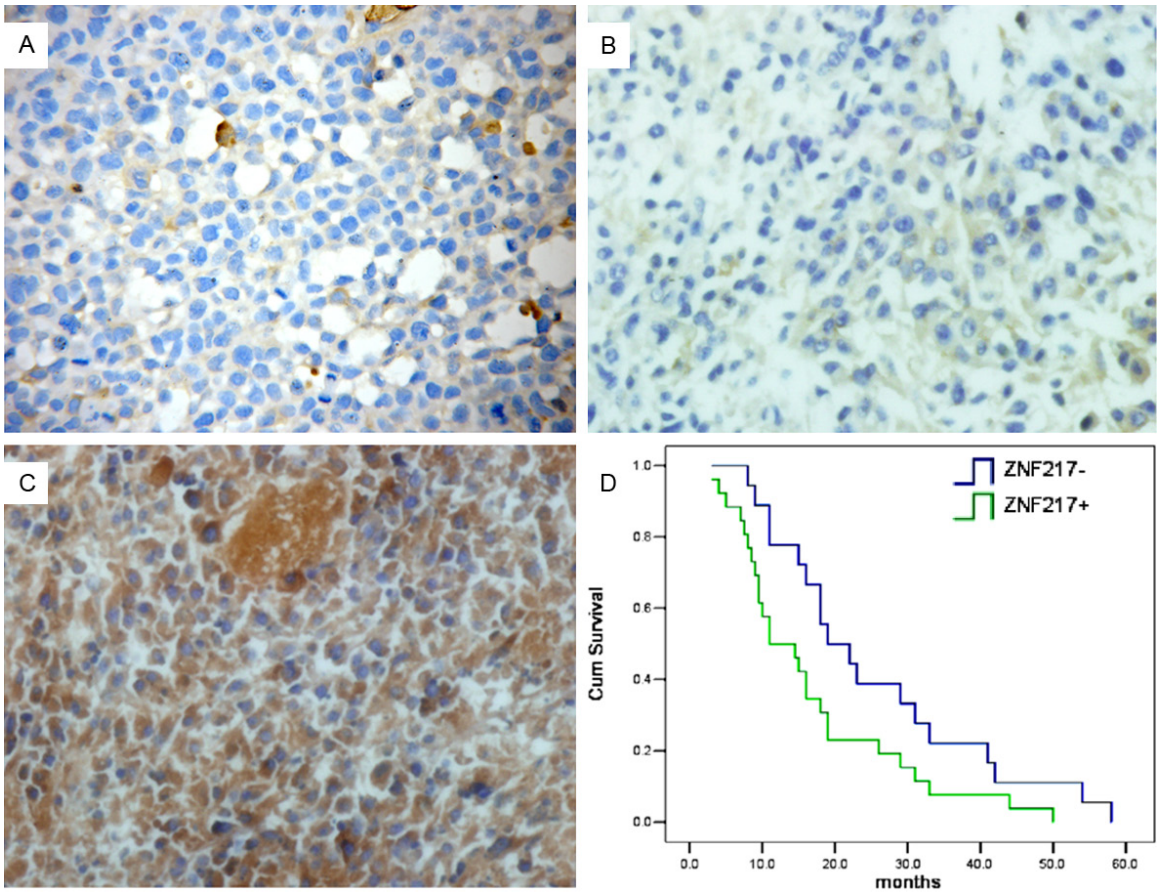

Figure 1. ZNF217 is overexpressed in ovarian cancer. A-C. ZNF217 expression in tumor specimen from ovarian cancer patients was assessed by immunohistochemical staining. Representative images were shown. Examples of weak (≤3), moderate (+4), and strong (+5) immunoreactivity in ovarian cancer (A through C, respectively). Original magnification ×400. D. Kaplan Meier Curve in ovarian cancer patients. The median ZNF217+ and ZNF217- for all patients were 18 months (range, 3-56 months) and 27 months (range, 8-56 months), respectively. The medians for groups 1 and 2 were 12 and 29 months, respectively (P<0.05). Kaplan-Meier analysis with the log-rank test of disease-free survival (DFS) between groups 1 (26 cases) and 2 (18 cases) (P=0.042). Multivariate Cox's proportional hazard analysis for clinical prognostic factors affecting disease-free survival (DFS) of patients with ZNF217 overexpression in advanced-stage epithelial ovarian cancer.

with ovarian cancer had worse disease-free survival rates if their ZNF217 levels were high (log rank test, p=0.042) (**Figure 1D**). ZNF217 overexpression was significantly associated with advanced disease progression (p=0.039). ZNF217 expression appears to be an independent molecular marker associated with poor clinical outcomes.

ZNF217 overexpression is associated with genomic amplification

To elucidate the molecular mechanisms underlying ZNF217 overexpression, we investigated the presence of genomic amplification through FISH analysis. A clear pattern of genomic amplification was detected across 44 interpretable cases of ovarian cancer. This suggests that overexpression of ZNF217 is significantly associated with disease progression events. When immunostaining scores exceeded 5, FISH showed positive results. FISH was not more sensitive than immunohistochemistry. Rather, the results show that the tissue was more malignant and associated with worse clinical outcomes.

Over-expressed ZNF217 enhanced the proliferation and invasion of ovarian cancer cells in vitro

To study the biological function of ZNF217, an ovarian cancer cell line with ZNF217 overexpression was constructed. Firstly, we detected

Table 1. Clinicopathologic characteristics of groups 1 (ZNF217+) and 2 (ZNF217-)

Characteristics	Group 1 (*n*=26)	Group 2 (*n*=18)	*P* value
Age (mean±SD, year)	47.26±3.04	50.55±1.63	0.366
FIGO stage (n, %)			0.039
I	0 (0)	5 (27.78)	
II	8 (30.76)	5 (27.78)	
III	16 (61.54)	7 (38.89)	
IV	2 (7.69)	1 (5.56)	
Pathologic tissue differentiation (n, %)			0.034
High	0 (0)	4 (22.22)	
Moderate	5 (19.23)	4 (22.22)	
Low	21 (80.77)	10 (55.56)	

the expression of ZNF217 in three ovarian cancer cell lines, HO-8910, OVCaR3 and SKOV3 cells by western blot. Significantly less protein expression of ZNF217 was found in HO-8910 cell (**Figure 2A**). So a stable HO-8910 cell line with ZNF217 overexpression was constructed as described in the Methods. The cell was named as HO-8910/ZNF217-PEGFP-N1. Further, increased expression of ZNF217 protein was confirmed by western blotting in HO-8910/ZNF217-PEGFP-N1 cells compared to PEGFP-N1/HO-8910 and HO-8910 cells (**Figure 2D**). Using an MTT assay, we found that the parental ovarian HO-8910 cells had a similar growth rate as PEGFP-N1/HO-8910 cells over a seven-day period, the growth of HO-8910/ZNF217-PEGFP-N1 cells was significantly faster than the former two lines from day 3 (P<0.05) (**Figure 3A**).

Moreover, we performed matrigel invasion assay of HO-8910/ZNF217- PEGFP-N1 and control cell lines. Cells with overexpressing ZNF217 showed the greatest degree of cell invasion among all three groups (**Figure 3B**). To exclude the possibility that this is a unique phenomenon in HO-8910 cells, we conducted the same experiment using the OVCaR3 cell line. We observed similar results (data not shown). These findings indicate that ZNF217 is an important factor in the induction of cell invasion.

Over-expression of ZNF217 increased cell cycle progression

We measured the alteration of cell cycle progression after ZNF217 over-expression. Using flow cytometry analysis, HO-8910/ZNF217-PEGFP-N1 showed that 72.4, 5.2, 22.4, and 1.9% of cycling cells were in G1, G2, S and G2/G1, respectively. PEGFP-N1/HO-8910 showed 78.0, 6.3, 15.7, 1.9%, the parental HO-8910 showed 88.8, 6.2, 13.0 and 1.9% respectively (**Figure 3C**). HO-8910/ZNF217-PEGFP-N1 cells showed a significant we found that HO-8910/ZNF217-PEGFP-N1 cells showed a significant increase in S phase population cells compared to the PEGFP-N1/HO-8910 and the parental HO-8910 cells (P<0.05).

ZNF217 mediates ovarian cancer metastasis

To investigate whether ZNF217 can mediate ovarian cancer metastasis, we transplanted tumor tissues which come from subcutaneous tumor of nude mice into the ovaries of female nude mice. After 30 days, these animals were sacrificed and examined for tumor metastasis. The tumor form PEGFP-N1-ZNF217/HO-8910 cells showed a significant increase in peripheral tissue metastatic activity. No metastases were found in the other two groups (**Figure 4**). The result suggested that ZNF217 may be important in promoting tumor metastasis in ovarian cancer.

Discussion

In view of the similarities among ZNF217-induced genomic aberrations in ovarian carcinomas, these cell lines can be said to represent a unique model for investigating relationships between the genomic and phenotypic aspects of ovarian epithelial neoplastic progression.

In contrast to previous reports, we showed that ZNF217 may promote neoplastic progression of ovarian epithelial carcinomas. This is based in part on our previous study, which showed not only the oncogenic properties of ZNF217 in HO-8910 cells but also described the expression in ovarian cancer. Recently, it was demonstrated that ZNF217 overexpression induces activation of the p53-p21-pRB pathway leading

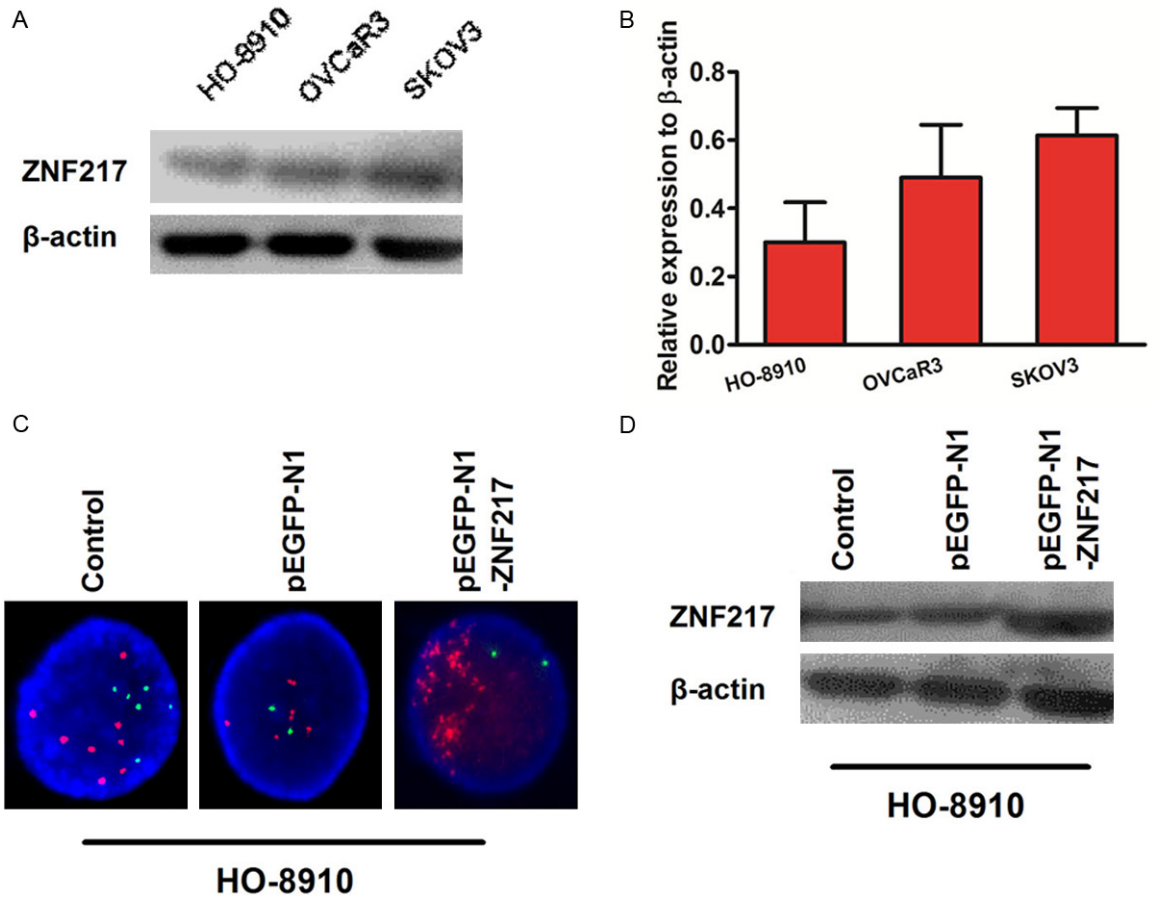

Figure 2. A stable overexpressed cell line was established. A. ZNF217 expression were detected in three ovarian cancer cell lines using Western blot. B. Immunosignals were quantified by densitometric scanning. ZNF217 expression in the individual cell samples was calculated as ZNF217 expression relative to β-actin expression. Data are mean±SD from three independent experiments. C. Compared to HO-8910 and PEGFP-N1/HO-8910 cells, ZNF217/HO-8910 showed obvious genomic amplification using FISH assay. D. Compared to HO-8910 and PEGFP-N1/HO-8910 cells, ZNF217/HO-8910 showed obvious ZNF217 protein expression using Western blot.

to cellular proliferation, ZNF217 may be a contributing factor to the immortalization and neoplastic progression of ovarian cancer [12].

In MTT and flow cytometry assay, human ovarian epithelial cells stably transfected with ZNF217 demonstrated significantly higher proliferation rates than control groups. In both cases, constitutively active ZNF217 was introduced into cell lines that later experienced extended effective proliferative traits and lifespans relative to cells without ZNF217 gene overexpression.

Based on our initial observations of ovarian cancer samples in which ZNF217 expression was detected in patients with colon metastasis, we hypothesized that ZNF217 is associated with cell invasion. We used HO-8910 cell lines stably transfected with a ZNF217 overexpression construct. The HO-8910 cell line is a transformed immortalized cell lines originating from ovarian cancer. It has seen extensive use as a model cell line in the examination of the molecular events leading to ovarian cancer. One purpose of this study was to examine the effects of ZNF217 on the cell invasion activity of HO-8910. Both controls failed to induce any significant cell invasion in either HO-8910 cells or PEGFP-N1/HO-8910 cells. Our results suggest that this is likely to be critical to the enhanced cell invasion induced by ZNF217.

To confirm our findings *in vivo*, we performed a nude mouse tumorigenicity assay in mouse ovarian cancer tissue. As expected, ZNF217+ promoted tumor invasion and transfer over control groups. In contrast, ZNF217 overex-

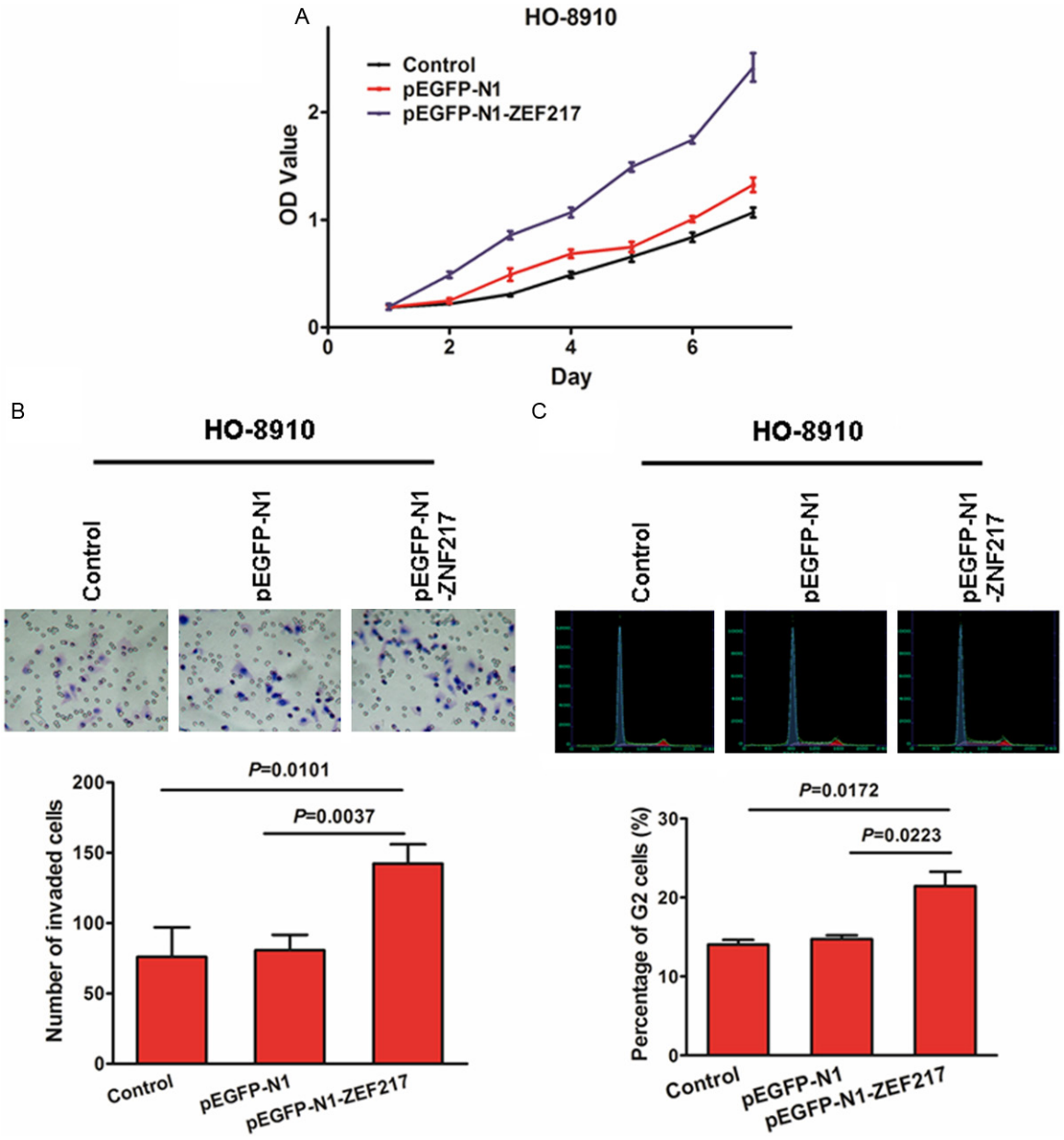

Figure 3. ZNF217 overexpression has a significant effect on the biological behaviors in ovarian cancer cells. A. MTT assay were used to detect the potential of cell proliferation *in vitro*. B. Matrigel invasion assay was performed with HO-8910 cells, EGFP/HO-8910 cells, and ZNF217+/HO-8910 cells. Both of the control groups induced cell migration and invasion less than the ZNF217+/HO-8910 cell groups did. C. Cell cycles were detected with flow cytometry analysis. Each bar represents the mean±SD. Results were representatives of three independent experiments.

pression induced earlier tumor formation and enhanced transfer ability, which suggests that ZNF217 may important to activating or promoting tumor invasion. An expression assay of epithelial proteins in cells transfected with ZNF217 showed both morphological modifications and molecular profile changes, suggesting that ZNF217 overexpression promotes cell invasion and metastasis.

In order to determine the clinical implications of ZNF217 overexpression in ovarian epithelial cancer, we gathered resected human ovarian epithelial cancer tissue samples with a five-year-average follow-up. We performed immunochemistry to examine the expression level of ZNF217. We identified three expression profiles of ZNF217 protein in ovarian cancer, all of which had correlations with clinical outcomes.

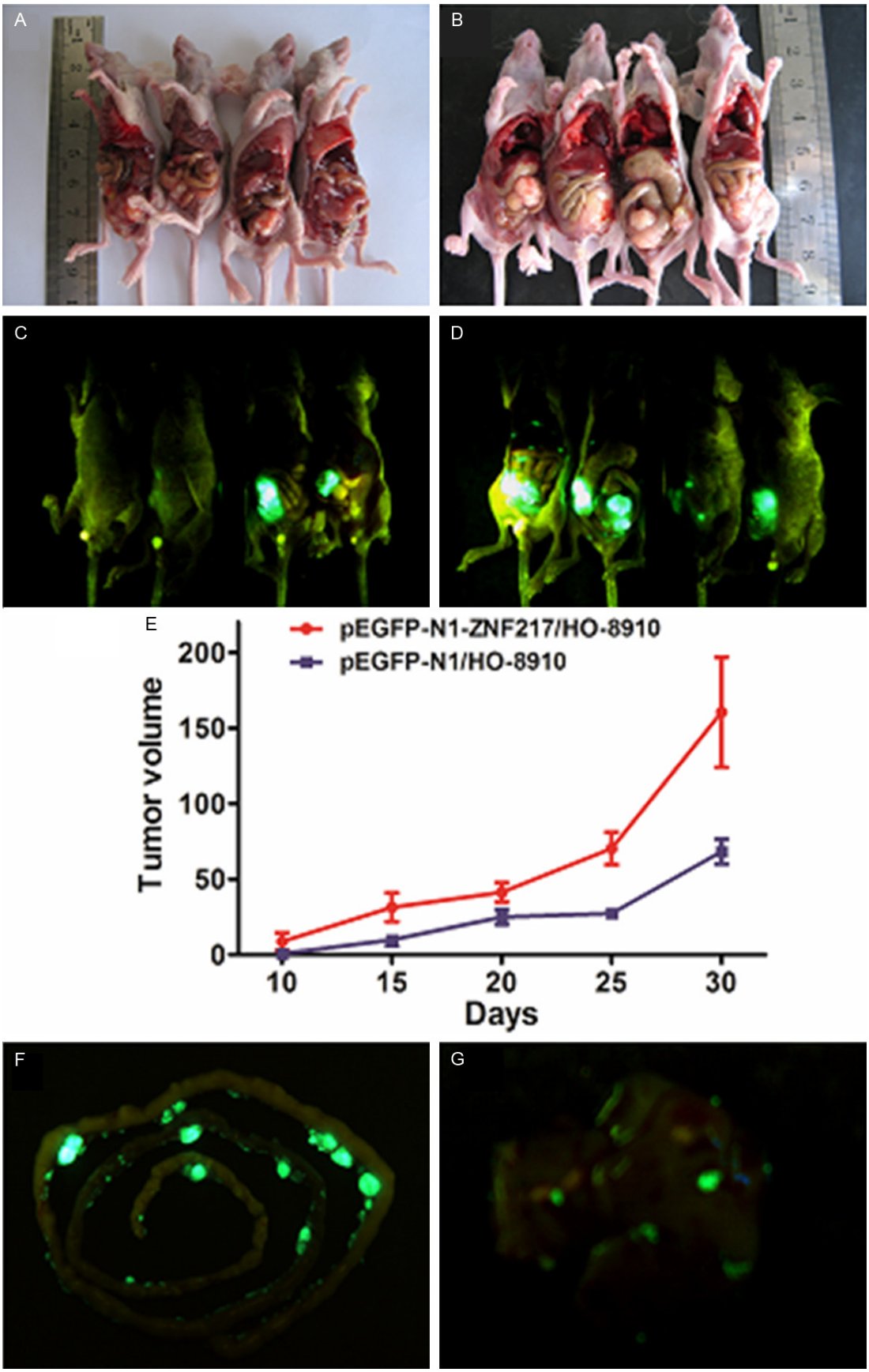
A
B
C
D
E
pEGFP-N1-ZNF217/HO-8910
pEGFP-N1/HO-8910
Tumor volume
200
150
100
50
0
10
15
20
25
30
Days
F
G

Figure 4. ZNF217 promoted tumour growth and metastasis *in vivo*. A&C. pEGFP-N1/HO-8910 cells; B&D. pEGFP-N1-ZNF217/HO-8910 cells. E. Nude mice tumorigenicity assay. ZNF217+ promoted greater tumor formation ability in the experimental group than in the control group. F&G. Lymph node and liver metastasis were obtained in the tumor of pEGFP-N1-ZNF217/HO-8910 cells.

First, expression of ZNF217 protein was observed in 59.09% (26/44) of the resected ovarian epithelial cancer tissue samples (**Table 1**). Second, ZNF217-positive ovarian epithelial cancer showed little pathologic tissue differentiation relative to negative patients (80.77% vs. 55.56%, p=0.034). Third, ZNF217 overexpression in ovarian epithelial cancer was significantly associated with advanced disease progression (stage III: 61.54% vs. 38.89%, stage IV: 7.69% vs. 5.56%, p=0.039) and worse disease-free survival rates (log rank test, p=0.042). We believe that this is the first clinical evidence to indicate a strong correlation between ZNF217 expression and prognosis of ovarian cancer patients. Moreover, the fact that this study is based on a substantial number of patient samples with follow-ups covering an average of 5 years suggests that our findings are accurate.

A prognostic marker for ovarian cancer is crucial because there are on-going controversies regarding the best post-operative management and follow-up strategies for ovarian cancer patients. Although the role of post-operative chemotherapy is relatively well established, being able to identify the highest risk patients will further improve the therapeutic ratio of chemotherapy. Biomarkers will help personalize the use of chemotherapy for ovarian cancer patients. However, there are only a few candidate prognostic biomarkers upon which decisions for chemotherapy can be based. Part of this problem stems from the fact that most studies of ovarian cancer have been based on far fewer than 44 samples apiece with an average duration of follow-up well under 5 years. The 50% increase in risk of recurrence risk that we observed in our ZNF217-positive group over our negative group suggests that ZNF217 status alongside other prognostic markers, may provide guidance for better-informed decisions regarding postoperative management in ovarian cancer patients.

In summary, our clinical and molecular findings support the hypothesis that ZNF217 may play an important role in the progression of ovarian cancer and that it does so through enhanced cell invasion. This is in contrast to other reports, which have only shown the role of ZNF217 on cell proliferation. It also presents ZNF217 as an attractive molecular marker for predicting cancer outcomes. The presence of significant ZNF217 expression in ovarian cancer samples and its association with poor clinical outcomes may warrant further study into ZNF217 as a therapeutic target for the management of ovarian cancer. However, these results are preliminary and would require further validation in future studies.

Disclosure of conflict of interest

None.

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