Gene Amplification of *ZNF217* Located at chr20q13.2 is Associated with Lymph Node Metastasis in Ovarian Clear Cell Carcinoma

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Abstract. *Background: Recently we reported that amplification of the Zinc Finger Protein 217 (ZNF217) gene adversely affects survival of patients with ovarian clear cell carcinoma. This study sought to determine the mechanism by which ZNF217 amplification affects patient survival. Materials and Methods: Fluorescence in situ hybridization (FISH) was used to detect ZNF217 gene amplification status and ZNF217-specific siRNA was used to inactivate ZNF217 for in vitro biological analyses. Results: We found ZNF217 gene amplification to be significantly correlated with lymph node metastasis ($p<0.05$) in ovarian clear cell carcinoma. Profound inhibition of cell migration and invasion was observed in siRNA-treated cells with ZNF217 amplification, compared to cells without amplification. Conclusion: These findings provide new insight into the biological role of ZNF217 gene amplification in ovarian clear cell carcinoma. Additionally, our observations have an important therapeutic implication for patients with ovarian clear cell carcinomas with ZNF217 amplification, as these patients may potentially benefit from ZNF217 targeted-therapy.*

Ovarian carcinoma is the most lethal gynecological malignancy in the developed countries and the second worldwide (1) and it comprises of several different histological subtypes (2-4). Among the different histological subtypes, ovarian clear cell carcinoma (OCCC) comprises of more than 20% of ovarian carcinoma cases in Japan, although it only represents 8-10% of all ovarian carcinoma cases in the USA (5, 6). Advanced OCCC is associated with poor prognosis, and surgical resection and subsequent platinum-based chemotherapy are the best available treatment at present. However, recurrence and metastasis are still the major causes of mortality in patients treated by surgical resection and chemotherapy. Furthermore, even with the present knowledge of the cellular and molecular mechanisms of OCCC, only a few biological markers can predict the behavior of OCCC (7, 8). We recently reported a numerical chromosome aberration in OCCC (9). In that study, we demonstrated that amplification of the *ZNF217* locus, located in 20q13.2, is the most frequent aberration in OCCC (9). In a subsequent study, we demonstrated that patients with OCCC and *ZNF217* amplification have poor survival (10). However, how *ZNF217* amplification affects the outcome of OCCC is not clear. In the current study, we investigated the mechanism by which *ZNF217* regulates OCCC progression by using *ZNF217* silencing in an OCCC cell line with *ZNF217* amplification.

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Materials and Methods

Tissue samples. Formalin-fixed, paraffin-embedded tissue samples of 60 OCCC cases were used. All OCCC samples used in this study were pathologically pure OCCCs. Samples were obtained from the Department of Obstetrics and Gynecology at the Shimane University Hospital and the Department of Obstetrics and Gynecology at Seirei Hamamatsu General Hospital. Diagnosis was based on conventional morphological examination of sections stained with hematoxylin and eosin (H&E), and tumors were classified according to the WHO classification. Tumor staging was performed according to the International Federation of Gynecology and Obstetrics (FIGO) classification. All patients were first treated with cytoreductive surgery and adjuvant platinum agent- and taxane-based or irinotecan chemotherapy [carboplatin (AUC5) with paclitaxel at 175 mg/m^2, docetaxel at 70 mg/m^2, or irinotecan at 180 mg/m^2 with cisplatin at

60 mg/m^2]. All patients underwent 6-12 courses of chemotherapy. Acquisition of tissue specimens and clinical information was approved by an Institutional Review Board (Shimane University and Seirei Hamamatsu General Hospital). Paraffin tissue blocks were organized into tissue microarrays, each made by removing 3-mm diameter cores of tumor tissue from the block. Selection of the area to core was made by a gynecological oncologist (K.N.) and a pathology technician (K.I.) and was based on review of the H&E slides.

Fluorescence in situ hybridization. BAC clones (RP5-823G15 and RP4-724E16) containing the genomic sequences of the 20q13.2 amplicon were purchased from Bacpac Resources (Children's Hospital, Oakland, CA, USA) and Invitrogen (Carlsbad, CA, USA). BAC clones corresponding to the Ch20P centromere (RP5-1025A1 and RP4-738P15) were used to generate reference probes. The method for fluorescence *in situ* hybridization (FISH) has been previously described (11). The hybridization signals were counted by two individuals. A signal ratio of experimental probe/reference probe greater than three was considered as amplification.

Immunohistochemistry. Expression of ZNF217 was assessed by immunohistochemistry and/or western blot analysis. The antibodies used in this study included a rabbit polyclonal antibody that reacted with ZNF217 (Abcam, Cambridge, MA, USA). The detailed procedures for immunohistochemistry and western blotting were described previously (10).

Cell culture and cell lines. The human ovarian carcinoma cell line MDAH2774 (serous carcinoma) was obtained from the American Tissue Culture Center (Rockville, MD, USA). JHOC9 (clear cell carcinoma) human ovarian cancer cell line was obtained from the Riken Bioresource Center (Ibaragi, Japan). The OVMANA cell line was obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan).

Silencing RNA knockdown of ZNF217 gene expression. Two silencing RNAs (siRNAs) that targeted *ZNF217* were designed with the following sense sequences: GAACAGAACCUCCCAAGGA and GAGGAUGCCUUGUCAAUGA. Control siRNA (luciferase siRNA) (UAAGGCUAUGAAGAGAUAC) was purchased from IDT (Coralville, IA, USA). Detailed procedures for siRNA transfection were described previously (10).

Simulated wound assay to assess cell motility. Cells were seeded in six-well plates and grown to a confluent monolayer. An acellular area was created by scraping the cell surface using a 200-μl pipette tip (time 0). Floating cells were removed by two gentle washes with culture media. The rate at which the defect closed was monitored for 24 h. The numbers of cells invading the acellular area were counted at 4, 8, and 12 h post-scraping. Cells in the monolayer defect were quantified as an average from multiple fields (at least five) at ×200 magnification for each experiment.

Matrigel invasion assay. The invasion study was performed using chemotaxis cell culture chambers, containing a membrane with a 0.8-μm pore size (Kurabo Inc., Osaka, Japan).

Thirty microlitres of serum-free DMEM-diluted Matrigel (1 mg/ml) (BD Bioscience, Bedford, MA, USA) was added to the membrane which was then incubated at room temperature for 4 h to form matrix gels. Chambers without the Matrigel coating were used as control chambers. Control siRNA-transfected JHOC9, OVMANA, and MDAH2774 cells and NAC1 siRNA-transfected cells were seeded individually in wells at a density of 25,000 cells/250 μl in serum-free medium in the upper chamber. In the lower compartment, 750 μl of DMEM containing 5% FBS were added. After 22 h of incubation at 37˚C under 5% CO_2, the Matrigel was carefully removed using a cotton swab. The membranes were fixed with 4% paraformaldehyde and stained with crystal violet. Cells migrating through the membrane and cells invading the Matrigel were counted in five non-overlapping fields at ×200 under a light microscope.

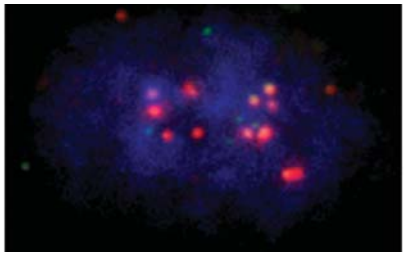

Figure 1. *Dual-color fluorescence in situ hybridization (FISH) demonstrates amplification of the ZNF217 gene in ovarian clear cell carcinoma. FISH analysis reveals a homogeneously stained region in the carcinoma portion with ZNF217 gene amplification.*

Statistical methods for clinical correlation. The Student's t-test (for comparison of two groups) was used to evaluate numeric data. The chi-square test was used for comparisons of categorical data.

Results and Discussion

The *ZNF217* gene amplification was detected in 12 (20.0%) out of 60 OCCC samples, and the *ZNF217* gene amplification significantly correlated with shorter progression-free and overall survival in our previous report (Figure 1) (10). The *ZNF217* amplification is significantly correlated with ZNF217 protein expression (10). In the current study, in order to clarify how ZNF217 gene amplification affects patient survival in OCCC cases, we searched for clinicopathological parameters correlated with *ZNF217* gene amplification. Interestingly, the *ZNF217* gene amplification significantly correlated with lymph node metastasis in OCCC (Table I) ($p<0.05$). Therefore, we focused our analysis on the relationship between *ZNF217* gene amplification and cell migration and invasion. In our previous reports, we identified two *ZNF217* gene-amplified cell lines, OVMANA and JHOC9. Therefore, we used these two cell lines as models of *ZNF217*-gene-amplified OCCC. *ZNF217* siRNA treatment significantly reduced ZNF217 protein expression compared with control siRNA treatment (10). Cell motility was then investigated with a wound-healing assay. *ZNF217*-knockdown in OVMANA and JHOC9 cells resulted in an 83% and an 87% decrease, respectively, in cell motility in comparison with cells transfected with the control siRNA ($p<0.01$) (Figure 2A-C). In the Matrigel invasion assay, a 48% and a

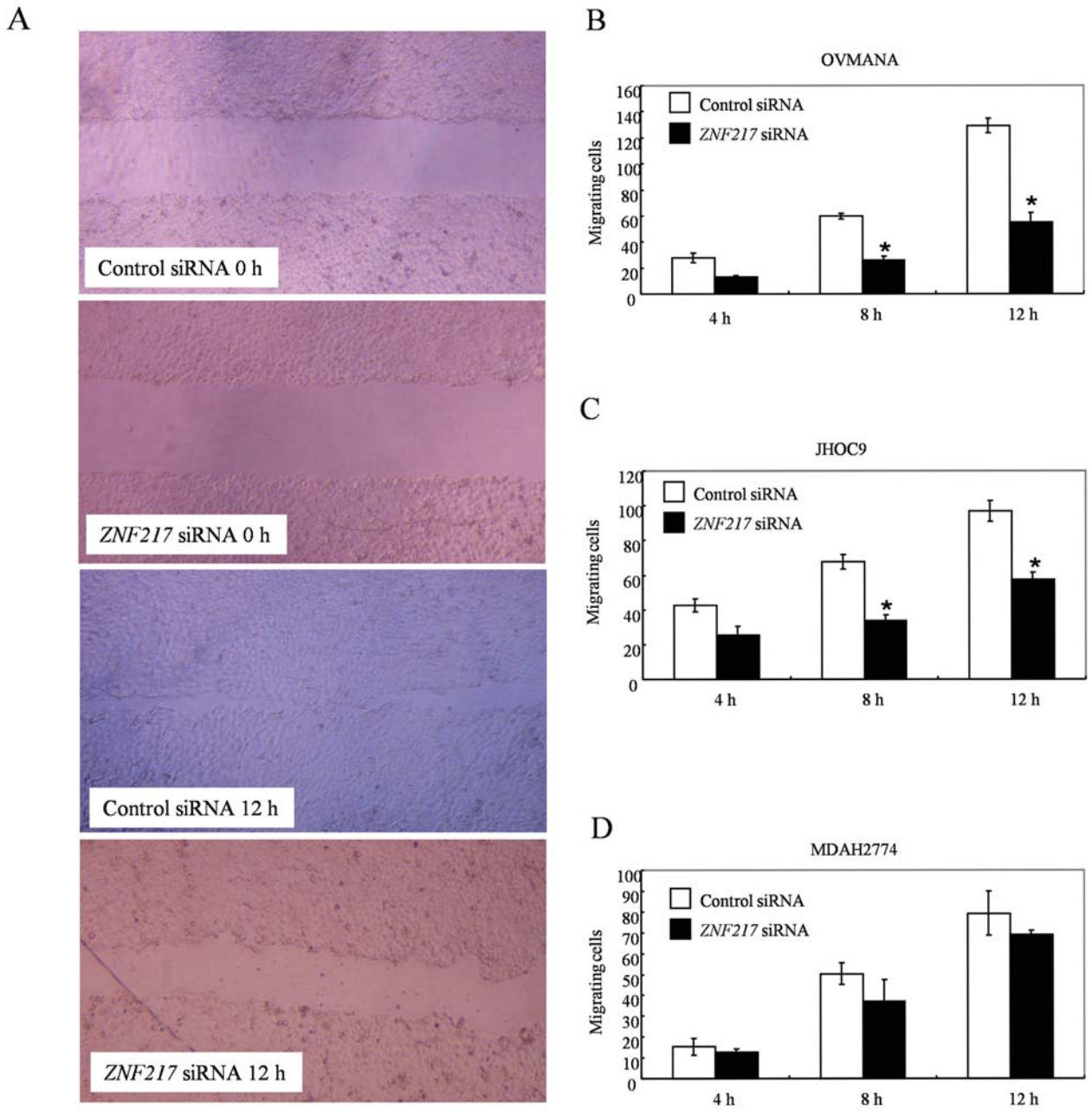

Figure 2. *A: Effect of ZNF217 knockdown on cell migration in a simulated wound-healing assay. A simulated wound was created by scraping a confluent monolayer of JHOC9 cells. Compared to the JHOC9 cells treated with control siRNA, there was a significant reduction in the number of ZNF217 siRNA-treated JHOC9 cells migrating into the wound area. The number of migrated cells was significantly lower in ZNF217 siRNA-transfected JHOC9 (B) and OVMANA (C) cells than in control siRNA-transfected cells in a time course assay. *$p<0.05$. The number of migrated cells was not significantly lower in ZNF217 siRNA-transfected MDAH2774 cells (D) than in control siRNA-transfected cells in a time course assay.*

31% decrease in cell invasion was observed in *ZNF217*-knockdown OVMANA and JHOC9 cells, respectively, in comparison with control siRNA-transfected cells ($p<0.01$) (Figure 3A and B). In contrast, the same treatment did not affect cell growth of MDAH2774 cells, which have no *ZNF217* gene amplification (Figure 2D and Figure 3B), suggesting that OCCCs with the *ZNF217* gene amplification are more dependent up-on activation of the ZNF217-related pathway for cell migration and invasion than those without the *ZNF217* gene amplification. These results are consistent with a previous report that *ZNF217* silencing inhibits cell migration and invasion in the ovarian carcinoma cell line HO-

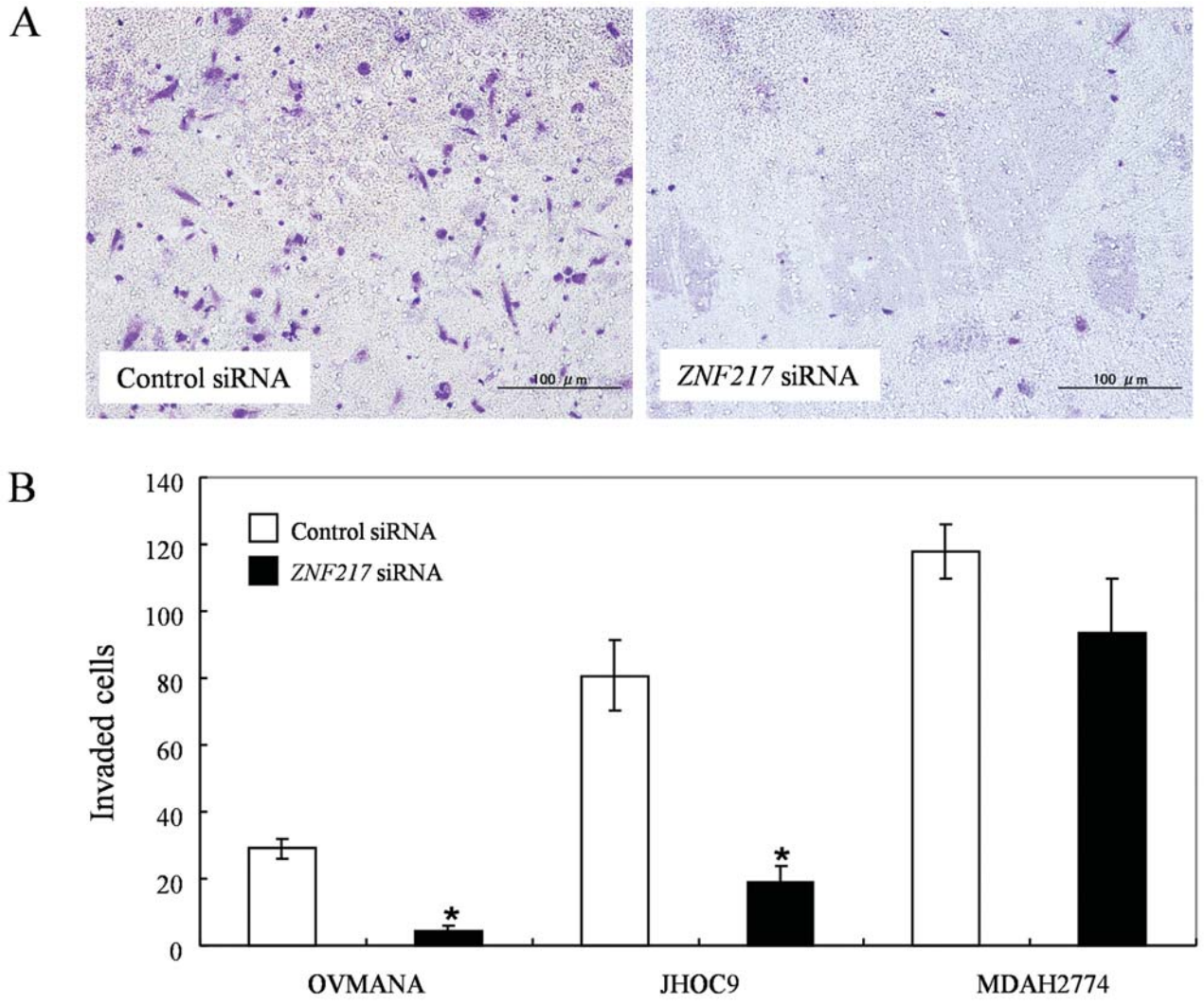

Figure 3. *Matrigel invasion chamber assay. The invasion abilitiy of JHOC9, OVMANA, and MDAH2774 cells were evaluated by their ability to invade and penetrate through the Matrigel-coated pores on membrane inserts in transwells. A: Control siRNA-treated JHOC9 cells had a significantly higher invasion capacity than the ZNF217 siRNA-treated JHOC9 cells. B: JHOC9 and OVMANA ZNF217 siRNA-transfected cells exibited significantly lower invasion capacity than control siRNA-transfected JHOC9 and OVMANA cells. *p<0.05. The error bars represent the standard deviation. MDAH2774 cell were not affected by ZNF217 siRNA.*

8910 (12). Taken together, current and previous findings suggest that the *ZNF217* amplification may affect lymph node metastasis of OCCC, by increasing cell invasion ability.

The key question is how *ZNF217* amplification regulates cell invasion and migration in OCCC. ZNF217 contains eight Cys_2-His_2 zinc-fingers and belongs to the large family of Kruppel-like transcription factors (13). Although the exact function of ZNF217 has not been established, biochemical purification studies have found ZNF217 to be a constituent of several related transcriptional co-repressor complexes that contain the histone deacetylases HDAC1 and HDAC2 (14-16). ZNF217 has also been identified as a component of the *C*-terminal binding protein (CtBP1) complex (17). CtBP1 can serve as a co-repressor for several classes of transcription factors in both an HDAC-dependent and -independent fashion (18). Chromatin immunoprecipitation (ChIP) assays have shown that CtBP1 and several of its associated proteins target the *E-cadherin* promoter in various breast cancer cell lines (17). Recently, Cowger *et al*. reported that ZNF217 negatively regulates the expression of E-cadherin, which might result in the activation of cell invasion and migration in cancer cells (19).

Genetic events that arise during tumor progression may become essential for tumor survival, a phenomenon generally

Table I. *The relationship between ZNF217 gene amplification and lymph node (LN) metastasis.*

	LN		*p*-Value
	Negative	Positive	
Normal	43 (90%)	5 (10%)	<0.05
Amplification	8 (67%)	4 (33%)	

described as oncogene addiction (20). This addiction may be targeted in the treatment of human cancer. The best illustration is that of breast cancer with (*HER2*; *ERBB2*) gene amplification on chromosome arm 17q (21), which is sensitive to Herceptin, an antibody targeting the receptor (22). Our recent study (10) and current findings suggest that *ZNF217* gene amplification may be an effective target for the development of novel therapeutics for blocking transformed growth and metastasis of OCCC.

Conflict of Interest Statement

The Authors declare that there are no conflicts of interest.

Acknowledgements

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References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin *61*: 69-90, 2011.
2 Gilks CB: Molecular abnormalities in ovarian cancer subtypes other than high-grade serous carcinoma. J Oncol *2010*: 740968, 2010.
3 Cho KR and Shih Ie M: Ovarian cancer. Annu Rev Pathol *4*: 287-313, 2009.
4 Kobel M, Kalloger SE, Boyd N, McKinney S, Mehl E, Palmer C, Leung S, Bowen NJ, Ionescu DN, Rajput A, Prentice LM, Miller D, Santos J, Swenerton K, Gilks CB and Huntsman D: Ovarian carcinoma subtypes are different diseases: implications for biomarker studies. PLoS Med *5*: e232, 2008.
5 Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I and Taguchi K: Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer *88*: 2584-2589, 2000.
6 Kennedy AW, Biscotti CV, Hart WR and Webster KD: Ovarian clear cell adenocarcinoma. Gynecol Oncol *32*: 342-349, 1989.
7 Kobel M, Xu H, Bourne PA, Spaulding BO, Shih Ie M, Mao TL, Soslow RA, Ewanowich CA, Kalloger SE, Mehl E, Lee CH, Huntsman D and Gilks CB: IGF2BP3 (IMP3) expression is a marker of unfavorable prognosis in ovarian carcinoma of clear cell subtype. Mod Pathol *22*: 469-475, 2009.
8 Katagiri A, Nakayama K, Rahman MT, Rahman M, Katagiri H, Nakayama N, Ishikawa M, Ishibashi T, Iida K, Kobayashi H, Otsuki Y, Nakayama S and Miyazaki K: Loss of ARID1A expression is related to shorter progression-free survival and chemoresistance in ovarian clear cell carcinoma. Mod Pathol *25*: 282-288, 2012.
9 Kuo KT, Mao TL, Jones S, Veras E, Ayhan A, Wang TL, Glas R, Slamon D, Velculescu VE, Kuman RJ and Shih Ie M: Frequent activating mutations of *PIK3CA* in ovarian clear cell carcinoma. Am J Pathol *174*: 1597-1601, 2009.
10 Rahman MT, Nakayama K, Rahman M, Nakayama N, Ishikawa M, Katagiri A, Iida K, Nakayama S, Otsuki Y, Shih IM and Miyazaki K: Prognostic and therapeutic impact of the chromosome 20q13.2 ZNF217 locus amplification in ovarian clear cell carcinoma. Cancer doi: 10.1002/cncr.26598, 2011.
11 Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman RJ, Shih Ie M and Wang TL: Amplicon profiles in ovarian serous carcinomas. Int J Cancer *120*: 2613-2617, 2007.
12 Sun G, Zhou J, Yin A, Ding Y and Zhong M: Silencing of *ZNF217* gene influences the biological behavior of a human ovarian cancer cell line. Int J Oncol *32*: 1065-1071, 2008.
13 Iuchi S: Three classes of C2H2 zinc finger proteins. Cell Mol Life Sci *58*: 625-635, 2001.
14 You A, Tong JK, Grozinger CM and Schreiber SL: CoREST is an integral component of the CoREST- human histone deacetylase complex. Proc Natl Acad Sci USA *98*: 1454-1458, 2001.
15 Hakimi MA, Dong Y, Lane WS, Speicher DW and Shiekhattar R: A candidate X-linked mental retardation gene is a component of a new family of histone deacetylase-containing complexes. J Biol Chem *278*: 7234-7239, 2003.
16 Lee MG, Wynder C, Cooch N and Shiekhattar R: An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature *437*: 432-435, 2005.
17 Shi Y, Sawada J, Sui G, Affar el B, Whetstine JR, Lan F, Ogawa H, Luke MP, Nakatani Y and Shi Y: Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature *422*: 735-738, 2003.
18 Chinnadurai G: CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol Cell *9*: 213-224, 2002.
19 Cowger JJ, Zhao Q, Isovic M and Torchia J: Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: identification of a ZNF217 consensus recognition sequence. Oncogene *26*: 3378-3386, 2007.
20 Weinstein IB and Joe A: Oncogene addiction. Cancer Res *68*: 3077-3080, 2008.
21 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL: Human breast cancer: correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science *235*: 177-182, 1987.
22 Pauletti G, Dandekar S, Rong H, Ramos L, Peng H, Seshadri R and Slamon DJ: Assessment of methods for tissue-based detection of the *HER-2/neu* alteration in human breast cancer: a direct comparison of fluorescence *in situ* hybridization and immunohistochemistry. J Clin Oncol *18*: 3651-3664, 2000.

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