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# Overexpression of CHP2 Enhances Tumor Cell Growth, Invasion and Metastasis in Ovarian Cancer

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**Abstract.** *Background: Calcineurin B homologous protein isoform 2 (CHP2) was identified to be expressed in various malignant cell lines including ovarian cancer, but not in the normal tissue counterpart. The biological function of CHP2 related to cancer progression is still unknown. Materials and Methods: A CHP2-negative human epithelial ovarian cancer cell line OVCAR3 was used for this study. CHP2 was analyzed before and after gene transfection. Cell proliferation, adhesion, motility, and invasion capacities were assessed in parental and transfected OVCAR3/CHP2 cell lines to explore the possible functions of CHP2 in ovarian cancer progression. Results: With RT-PCR analysis, CHP2-transfected OVCAR3/CHP2 cancer cells showed high CHP2 gene expression, whereas non-transfected clones did not produce detectable CHP2 mRNA. CHP2-transfected OVCAR3/CHP2 cells showed increased proliferation rates and exhibited increased activities of cell adhesion, migration and invasion. The current study provides the first evidence that overexpression of the CHP2 gene affects the biological behavior of ovarian cancer cell line OVCAR3 and is one of key mechanisms for ovarian carcinoma progression, suggesting that CHP2 may be an attractive target for biological anticancer therapy.*

Ovarian carcinomas is the leading cause of death by gynecological cancer in women (1). Although 5-year survival rates have increased over the past several decades to ~40%, overall survival rates remain relatively constant (2, 3). Metastatic spread of self-regulated growth ovarian cancer cells *via* invasion to distant tissues is the primary cause of treatment failure and subsequent death in cancer patients. It is critical to characterize the genetic defects that underlie key steps in the development and spread of ovarian cancer if advances are to be made in diagnosis, prevention, and treatment.

Recently, Wang *et al.* (4) identified 55 independent cDNA sequences potentially encoding hepatocellular carcinoma (HCC) tumor antigens using serological analysis of recombinant cDNA expression libraries (SEREX) from four HCC patients. Of these genes, HCA520 (NCBI nucleotide accession number NM_022097 with designation of hepatocellular carcinoma antigen gene 520; GenBank number AF_146019) was identified as being expressed in an ovarian cancer cell line, but not in the normal tissue counterpart (4). Pang *et al.* (5) indicated that HCA520 is an isoform of calcineurin B homologous protein (CHP) and nominated it as CHP2. CHP2 is located on chromosome 16p12.2. The full length of the CHP2 is 2396 bp, which is composed of seven exons and has an open reading frame of 591 bp, encoding 196 AA with N-terminal myristoylation site and four EF-hand $Ca^{2+}$ binding motifs. The expected molecular weight of CHP2 is 23 kDa. CHP2 is an essential cofactor for Na+/H+ exchanger isoform 1 (NHE1) (6, 7), and protects cells from serum deprivation-induced death (8). CHP2 distinctive ovarian cancer cell line expression and its role as cofactor for Na+/H+ exchanger prompted us to study its biological function in ovarian cancer.

In this study, in order to determine the biological functions of CHP2, a CHP2-negative ovarian carcinoma cell line was used to investigate if CHP2 affects cell proliferation, adhesion, motility, or invasion by stable gene transfection.

## Materials and Methods

*Cell culture.* Human epithelial ovarian cancer cell line OVCAR3 was obtained from Basic Medicine Research Institute, Qilu Hospital, Shandong University, PR China. OVCAR3 cells were grown as a

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monolayer in RPMI-1640 culture medium (Gibco Life Technologies, USA) with 10% heat-inactivated fetal calf serum (FCS; Gibco, USA) at 37˚C in a 5% $CO_2$, humidified atmosphere.

*Plasmid constructs.* A 601 bp fragment of the CHP2 cDNA, which contained the entire open reading frame without termination codon, was obtained by RT-PCR from total RNA of hepatocellular carcinoma tissues. The primers were determined from the published sequence (GenBank number AF_146019): the forward primer was 5'-GAGCTCATGGGGTCGCGCAGCTCCCAC GCCGCGGTCATT-3' (including Sac I site at 5'), and the reverse primer 5'-GAATTCGCTTCAGGATCCGGATGCTCAT-3' (including EcoR I site at 5'). The amplified fragment was run out on a 1% agarose gel. The 601 bp fragment was then excised from the gel and purified using the Agarose Gel DNA Purification Kit Ver. 2.0 (TaKaRa, Japan) and cloned into the pMD18-T Simple vector (TaKaRa, Japan). After DNA sequencing, the pMD18-T-CHP2 was treated with restriction endonucleases EcoRI and Sac I (TaKaRa, Japan).

The green fluorescent protein eukaryotic expression vector pEGFP-N1 (Becton–Dickinison, USA) was prepared to create pEGFP-N1-CHP2 with insertion of the CHP2 gene by digestion with EcoRI and Sac I for 4 h. The resultant digest was purified by Agarose Gel DNA Purification Kit Ver.2.0. The vector and CHP2 gene insert were combined in a 1:3 ratio (vector:insert) in Ligation Mix (TaKaRa, Japan). The resultant mixture was then used to transform *E. coli* Competent Cell DH5 α (TaKaRa, Japan). DNA was harvested from the broth and prepared using an Endo-free Plasmid Mini Kit I (OMEGA, USA). The digest was run on 1% agarose gel to confirm the presence of the 601 bp CHP2 fragments. Once successful CHP2 gene insertion was confirmed, the colony was cloned.

*Transfection and production of stable clones.* Cells were trypsinized and plated at a density of $3x10^5$ cells/ well in 6-well tissue culture plates (Corning, USA) the day before tansfection. When the density of cells was greater than 90% confluent, cells were rinsed twice with serum-free RPMI-1640, pEGFP-N1-CHP2 or empty control vector and lipofectamine 2000 (Invitrogen, USA) mixtures prepared in Opti-MEM (Invitrogen, USA) added. After the cells were incubated for 5 h, the plasmid-lipofectamine 2000 mixture was then removed and RPMI-1640 plus 10% FCS and 800 μg/ml G418 (Invitrogen, USA) were added. After incubation for 48 h, fluorescent images were acquired with an OLYMPUS IX81 inverted fluorescence biomicroscope (Olympus Corp, Tokyo, Japan) equipped with a DP30BW intensified charge-coupled device (ICCD) for examination of the cellular localization of the encoded protein. Images were captured using a UPLSAPO objective and ICCD camera, and subsequently processed using Image-Pro software (MediaCybernetics, USA). Approximately 2 weeks later, independent clones were selected for G418 resistance and detected with fluorescence biomicroscopy.

*RT-PCR analysis of CHP2 and NHE1 expression.* Total RNA from CHP2 transfected and untransfected cells were extracted using RNAiso Reagent (TaKaRa, Japan), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed by using RNA LA PCRTM Kit (AMV) Ver. 1.1 (TaKaRa, Japan). One μl aliquots from the reverse transcription were used as templates for CHP2 and NHE1 amplification, respectively. To analyze the expression of CHP2, the following primer pairs were used: sense primer 5'-GAGCTCATGGGGTCGCGCAGCTCCCACGCC GCG GTCATT-3', and the antisense primer 5'-GAATTCGCT TCAGGATCCG GATGCTCAT-3' (GenBank number AF_146019). PCR was performed under the following reaction conditions: 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 90 sec with a thermal cycler. NHE1 gene amplification was performed with sense primer 5'-CCAGC TCATT GCCTT CTACC-3' and antisense primer 5'-TGTGT CTGTT GTAGG ACCGC-3' (GenBank M81768), as described previously (9). β-actin amplification was used as internal control; the β-actin primer pairs were as follows: sense primer 5'-ATCATGTTTGAGACCTTCAACA-3', and the antisense primer 5'-CATCTCTTGCTCGAAGTCCA-3'.

*Cell proliferation assay.* Cells were resuspended in complete medium, plated at $1x10^4$ cells/well in 6-well plates and incubated at 37˚C and 5% $CO_2$. At 12, 24, 36, 48, 60, and 72 h, cells were carefully trypsinized and subsequently counted using a Vi-cell XR automated cell viability analyzer (Beckman Coulter, USA) according to the trypan blue dye exclusion method. Three independent experiments were performed.

*Adhesion assay.* Six-well plates were coated overnight with fibronectin at a concentration of 10 μg/ml at room temperature under sterile conditions. Wells were washed with phosphate buffered saline (PBS) and nonspecific binding sites were blocked with 2 ml 0.1% bovine serum albumin (BSA) in RPMI-1640 for 1 h at 37˚C. Cells were trypsinized and washed twice with RPMI-1640 and plated onto the fibronectin-coated 6-well plates at a density of $4x10^5$ cells/well, then incubated for 15 min, 30 min, 60 min, or 90 min. At the end of incubation, unbound cells were carefully removed by aspiration, and wells were washed twice with PBS. The number of adherent cells was determined using a Vi-cell XR automated cell viability analyzer. Three independent experiments were performed.

*Wound healing assay.* To study directional cell migration, the wound healing experiment was performed (10). Briefly, cells were plated on fibronectin (10 μg/ml) pre-coated 6-well plates at the same density of $2x10^5$ cells/well, respectively. Confluent monolayers were washed twice with PBS and artificial wounds were created by scraping with a pipette tip. After cell debris was removed, the cultures were incubated in RPMI-1640 containing 0.5% FCS. Wound closure was monitored at 0 h and 36 h after wounding. Images of the wounds were taken digitally with an inverted microscope (Olympus, Japan) from exactly the same position at various time points (x10 field). The dimensions of the wound areas were measured by Image-Pro software, set at 100% for 0 h, and the mean percentage of the total dimensions of the wound areas were calculated. Data were taken from six independent experiments.

*Invasion assay.* Membranes with 8 μm pores of Transwell chambers (Corning, USA) were coated with 100 μl of two-fold-diluted Matrigel (Sigma, USA) in cold RPMI-1640, and then incubated for 2 h at 37˚C. After prehydration with serum-free media according to the manufacturer's instructions, matrigel membranes were set on 24-well cluster plates (Corning, USA) and 600 μl conditioned medium from NIH3T3 cells was used as chemoattractant in the lower chamber. Cells were washed twice and resuspended in serum free RPMI-1640 and inoculated in the upper chambers at doses of $2x10^5$ cells/chamber. After 12 h incubation at 37˚C in a humidified 5% $CO_2$ atmosphere, the chambers were extracted, remaining noninvasive cells were removed with a cotton-tipped applicator, and the cells that had penetrated through to the bottom of the chamber were fixed with methanol and stained with haematoxylin

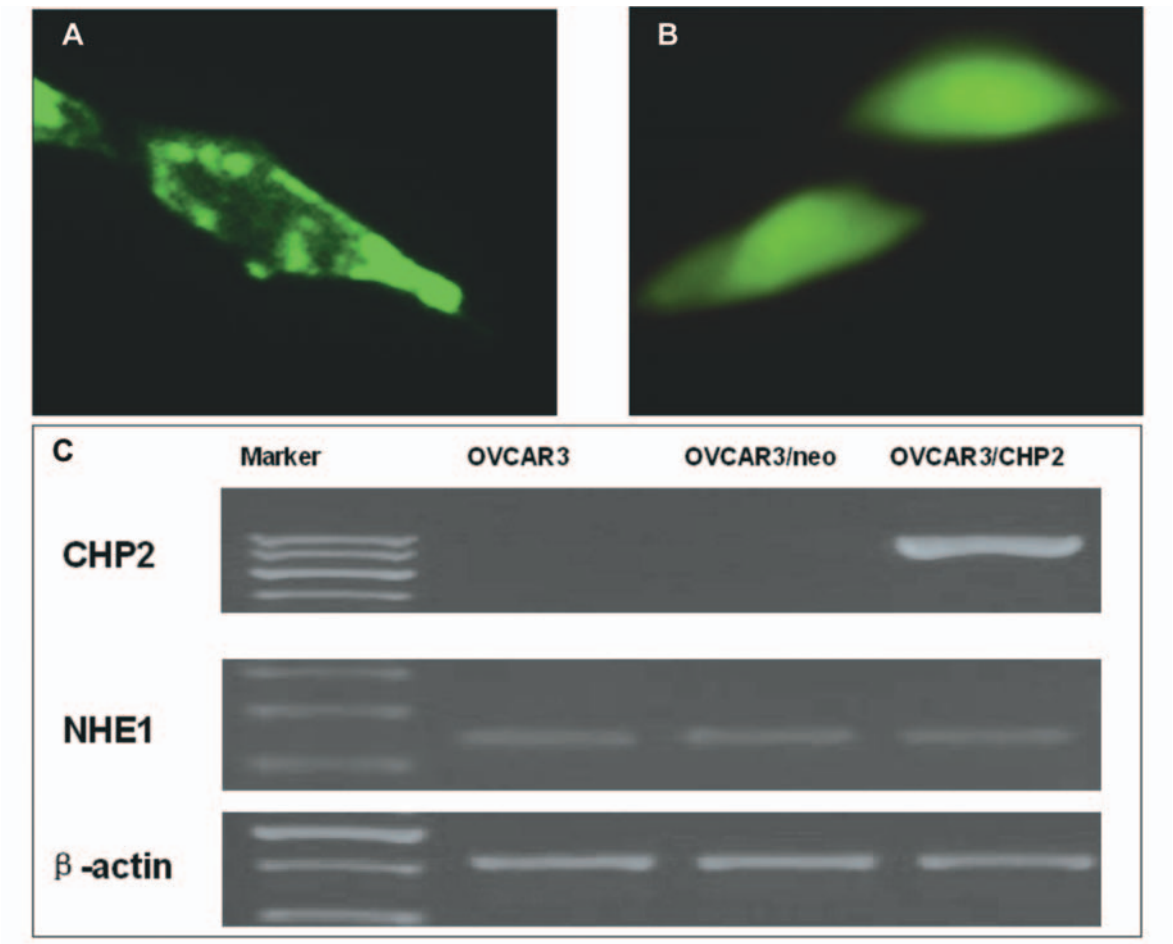


Figure 1. *Localization of CHP2 in transfected OVCAR3 cells (x 40). (A) With the Green Fluorescent Protein reporter gene, fluorescence microscopy confirmed that most of the EGFP/CHP2 fusion protein was localized in the plasma membrane of OVCAR3 cells. (B) GFP was uniformly distributed in the whole cells of empty vector transfected OVCAR3 cell. (C) CHP2 and NHE1 expression in parental and transfected OVCAR3 cells. CHP2 was only positive in OVCAR3/CHP2 cells, no change was observed in NHE1 expression before or after CHP2 transfection.*

and eosin (HE) solutions. Invasion was quantified by blind counting cells in 10 microscope fields (x40 field). Each of two replicate membrane filters was counted and data are presented as the number of cells penetrating through the membrane per field (x40 field); all experiments were repeated twice.

*Statistical analysis*. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Neuman-Keuls analysis as a *post hoc* test, using SPSS 11.5 for Windows (SPSS, USA). In all statistical comparisons, a $p$-value of $<0.05$ was considered to be statistically significant.

## Results

*CHP2 and NHE1 expression.* The human ovarian carcinoma OVCAR3 cells were transfected with plasmid pEGFP-N1-CHP2 or with the empty control vector; stable transfectants were selected for G418 resistance. With the green fluorescent protein (GFP) reporter gene, fluorescence microscopy confirmed that most of the EGFP/CHP2 fusion protein was localized in the plasma membrane of OVCAR3 cell (Figure 1A) while GFP was uniformly distributed over whole cells of empty vector transfected OVCAR3 cells (Figure 1B). With RT-PCR analysis, we further demonstrated that CHP2 gene expression was negative in parental OVCAR3 and control OVCAR3/neo cells. In contrast, high CHP2 gene expression was detected in the transfected OVCAR3/CHP2 cells. Strong NHE1 expression was seen in the parental OVCAR3 cell line; no obvious change was observed after CHP2 transfection (Figure 1C).

*Effects of CHP2 on cell proliferation.* In order to explore the biological function of CHP2, the effects of overexpression of CHP2 on cell proliferation was examined. The cell proliferation rates were not different between OVCAR3/neo cells and the parental cells. Compared with OVCAR3/neo and

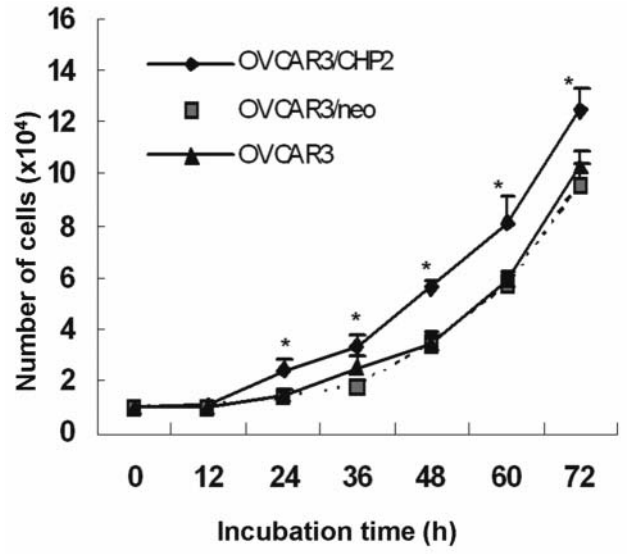


Figure 2. *Cell growth of parental and transfected OVCAR3 cells. Compared with OVCAR3/neo and parental OVCAR3 cell groups, OVCAR3/CHP2 cells showed increased cell proliferative activity. Asterisks denote data is significantly different ($p<0.05$; ANOVA with Neuman-Keuls post hoc test).*

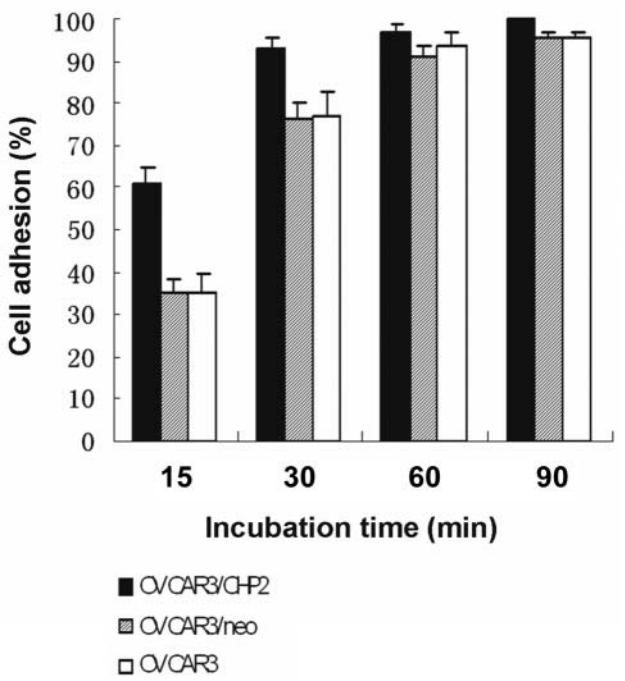


Figure 3. *Adhesion of parental and transfected OVCAR3 cells to fibronectin. Approximately $4x10^5$ cells were seeded onto the 6-well plates coated with fibronectin. After 15 min, 30 min, 60 min, or 90 min of incubation the number of adherent cells was counted. Increased adhesive activity was seen in OVCAR3/CHP2 cells compared with the other two groups ($p<0.05$; ANOVA with Neuman-Keuls post hoc test).*

the parental OVCAR3 cell groups, OVCAR3/CHP2 cells showed significantly higher cell proliferative activity (Figure 2).

*Effects of CHP2 on cell adhesion.* When the ability to adhere to tissue culture plates coated with fibronection was examined, the OVCAR3/CHP2 cells showed significantly more adhesion to fibronectin at all times after incubation compared with the empty vector transfectants ($p<0.05$) and control cells ($p<0.05$) (Figure 3).

*Effects of CHP2 on cell migration.* The effect of CHP2 on cell migration was investigated using the wound-healing assay (Figure 4). The CHP2-overexpressing OVCAR3 cells had a significantly lower percentage than the other cell lines ($p<0.05$, Figure 4A, B). These results suggested that the transfected OVCAR3/CHP2 cells resulted in enhanced migration of OVCAR3 cells to the wound area. CHP2-overexpressing OVCAR3/CHP2 cells exhibited marked increased invasive potential compared with OVCAR3/neo cells ($p<0.05$) and parental OVCAR3 cells ($p<0.05$) (Figure 4C).

## Discussion

Three CHP isoforms have been identified so far. CHP1 is expressed ubiquitously and is an essential cofactor for NHE1 to NHE3 (5, 11), which attenuates the stimulation of NHE1 by serum and a mutationally activated GTPase (12). CHP3 is restricted to the heart, brain, stomach and testes (13-15). CHP2 has 61% nucleotide level homology with CHP1 and interacts with the CHP1-binding site in NHE1-3, enhancing their exchange activities (8). Recently, Pang *et al.* have demonstrated that the interaction of NHE1 with CHP2 but not with CHP1 leads to serum-independent permanent activation of NHE1, which is a well documented property found in malignantly transformed cells (8). CHP2 is restricted to cancer tissues or cells, such as hepatic carcinoma, colon adenocarcinoma, cervical carcinoma, leukemia, ovarian cancer cell line G1-102 but not their normal tissue counterpart or other normal tissues. These results implied that CHP2 expression may represent a useful biomarker for the detection and diagnosis of certain types of cancer (4, 8). In this study, we successfully transfected the full open reading frame of CHP2 gene into OVCAR3 cells and examined the biological functions of CHP2 in human epithelial ovarian cancer cell line OVCAR3.

Our observation of the increased proliferative capacity in transfected ovarian cancer OVCAR3/CHP2 cells suggests that CHP2 may have unexpected functions in ovarian cancer. Since CHP2 was detected in colon tumor metastatic cells (GenBank™ nucleotide accession number EST370271), we investigated whether CHP2 was related with the invasive and metastatic capacity of ovarian cancer in the current study. Tumor metastasis is comprised of multiple steps and tumor

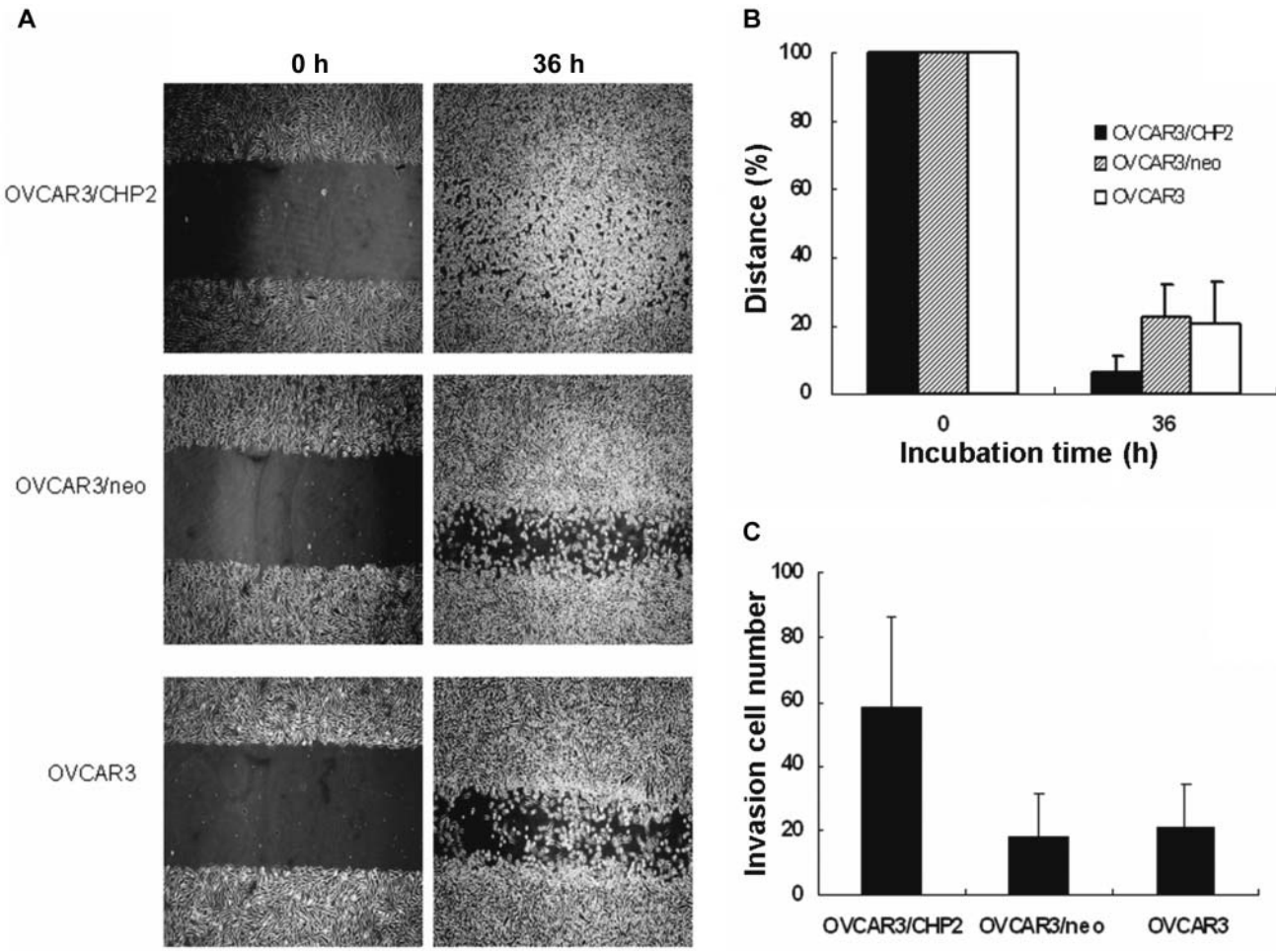


Figure 4. *Migration and invasion assays of parental and transfected OVCAR3 cells. (A) Confluent OVCAR3/CHP2, OVCAR3/neo, and control cells were wounded by scratching with a pipette tip and the cells were incubated with RPMI-1640 containing 0.5% FBS. Digital photographs were taken immediately and 36 hours later by a UPLSAPO objective and ICCD camera with an inverted microscope. Images are from one representative experiment. (B) Quantification of the wound-healing assay. The distances between the edges of the wound were measured and normalized to 100% at 0 h. The figure is plotted using the mean percentage ±SD of 6 independent experiments. (C) Matrigel invasion assay. OVCAR3/CHP2 cell exhibited significantly increased invasive capacity relative to OVCAR3/neo cells ($p<0.05$) and control cells ($p<0.05$).*

cells complete the metastatic process through altered adhesion, increased motility and invasive capacity (16). In order to explore the possible biological functions of CHP2 in OVCAR3 cells, we analyzed each one of these steps in parental and transfected cell lines.

The effect of CHP2 expression on cell adhesion to fibronectin was examined, CHP2 led to a moderate, but significant increase of adhesion of OVCAR3 cells to a fibronectin-coated surface after incubation. This result indicated that the overexpression of CHP2 in human OVCAR3 ovarian carcinoma cells contributed to an increased binding ability of the tumor cells to fibronectin. Migration away from the primary tumor is a major step in metastasis; a wound-healing assay was used for migration assessment in our study. With this assay, CHP2 transfection led to a significant increase in migration of OVCAR3/CHP2 cells to the wound area. Adhesion of the metastatic cells to the microvascular wall and migration into the host organ are essential in the multistep metastatic process. A Matrigel invasion assay was further applied to assess cancer cell adhesion and motility, two processes of tumor invasion. We demonstrated that CHP2 significantly increased invasion ability of OVCAR3 cells. Since CHP does not appear to be a $Ca^{2+}$-sensor but rather acts as a critical regulator of pH-sensing activity in the exchangers (6), increased ovarian cancer cell invasion and metastasis capacities after transfection may be resulted from increased pHi by CHP2.

It is well documented that malignantly transformed cells maintain abnormally high pHi, because activation of NHE1 has been observed in various malignantly-transformed cells, such as human leukemic (17), human malignant glioma (18),

and human breast cancer cells (19). Notably, CHP2 appears to be almost exclusively expressed in these transformed cells. The activation of NHE1 by bound CHP2 may be a key mechanism for the maintenance of serum-independent high pHi in these abnormal cells. A recent study has reported that NHE1 inhibitor markedly retarded the development of tumors in nude mice (20). In our current study, NHE1 was detected in the CHP2-transfected OVCAR3 cell line; increased proliferative, invasive and metastatic capacities were observed after CHP2 transfection. Our results suggest that an intact CHP2-NHE1 pathway is essential for cancer progression, which is consistent with a recent study that CHP2 serves as an obligatory subunit required both for supporting the basic activity and regulating the pH-sensing of NHE1 *via* interactions between distinct parts of these proteins (6).

## Conclusion

We have demonstrated that CHP2 overexpression *in vitro* increased the proliferative, invasive and metastatic activities of OVCAR3 ovarian cancer cells. This is the first evidence that overexpression of the CHP2 gene affects the biological behavior of ovarian cancer. Our findings suggest that CHP2 may be a new biological anticancer therapy target for ovarian cancer.

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