

***Hepsin* shows inhibitory effects through apoptotic pathway on ovarian cancer cell lines**

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Abstract. In light of the poor prognosis for ovarian cancer patients, the research continues for innovative and efficacious treatment modalities. Along with surgical and chemotherapeutic regimens, gene therapy has emerged as one of the leading contenders in the treatment armamentarium. *Hepsin* is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone. However, the biological function of *Hepsin* expression remains unclear. Apoptotic cell was increasingly common among higher proportions of *Hepsin* transfectants. We discovered that first-time stable transfectants of *Hepsin* significantly inhibited cell growth in the monolayer, anchorage-independent cell growth in the soft agar *in vitro*, and tumorigenicity *in vivo* in ovarian cancer cell lines. These results demonstrated that *Hepsin* has a potential therapeutic effect that inhibits through up-regulation of p53-dependent apoptosis and caspase-3, -6, and -7 activations. Our findings in this study identify *Hepsin* as a target for new approaches in ovarian cancer treatment.

Introduction

Despite its pervasive impact, the etiology of ovarian cancer and the factors that promote its progression are not yet well understood. Although chemotherapy remains a major treatment modality for human ovarian cancer, chemoresistance is a clinical problem that severely limits the success of the

treatment. It is now widely accepted that the apoptotic capacity of ovarian cancer cells is pivotal in determining their responses to chemotherapeutic agents.

Hepsin is a type II transmembrane serine protease originally identified in the human liver as a cDNA clone (1,2). A 1.85-kb *Hepsin* mRNA is highly expressed in normal liver and kidney tissues and is poorly expressed in other tissues, including the normal prostate and lung. In normal ovarian tissue, *Hepsin* mRNA is not expressed, and *Hepsin* overexpression has been reported in ovarian, renal, and prostate carcinomas (3-5). However, the biological functions of *Hepsin* remain unknown.

The targets of p53-regulated genes or p53-interacting proteins have provided critical information to the understanding of the biochemical and biological functions of the p53 tumor suppressor gene. The purpose of this study is to examine the role of *Hepsin* in the wild-type (wt) p53-dependent apoptosis pathway and caspase family. We studied the p53 apoptotic-mediated factors and performed cell growth in the monolayer, anchorage-independent cell growth in the soft agar *in vitro*, and tumorigenicity *in vivo*. We provided new insights into the biological functions and gene therapeutic target in ovarian cancer.

Materials and methods

Cell culture and media. BG-1, OVCAR-3, CaOV-3 cell lines were derived from human epithelial ovarian carcinoma. These ovarian cancer cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The LNCaP cell line was derived from human prostate carcinoma. The prostate cancer cell lines were maintained in RPMI-1640 (Life Technologies, Inc., Rockville, MD) with 10% FBS. Cells were maintained at 37°C in an atmosphere with 5% CO₂ in the air.

Reverse transcription-PCR analyses. In reverse transcription-PCR (RT-PCR), we used primers 5'-TGTCCTGGATGGCGA GTGTTT-3' (*Hepsin*-F) and 5'-CCTGTTGGCCATAGTACT GC-3' (*Hepsin*-R), as described (3). The number of PCR cycles resulting in PCR products in the linear logarithmic phase of amplification curve was determined. PCR samples were electrophoresed on 2% agar gel and visualized by ethidium bromide.

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Abbreviations: p53AIP, p53 apoptosis-inducing protein 1; wt, wild-type

Key words: *Hepsin*, ovarian cancer, p53-dependent apoptotic pathway, caspase

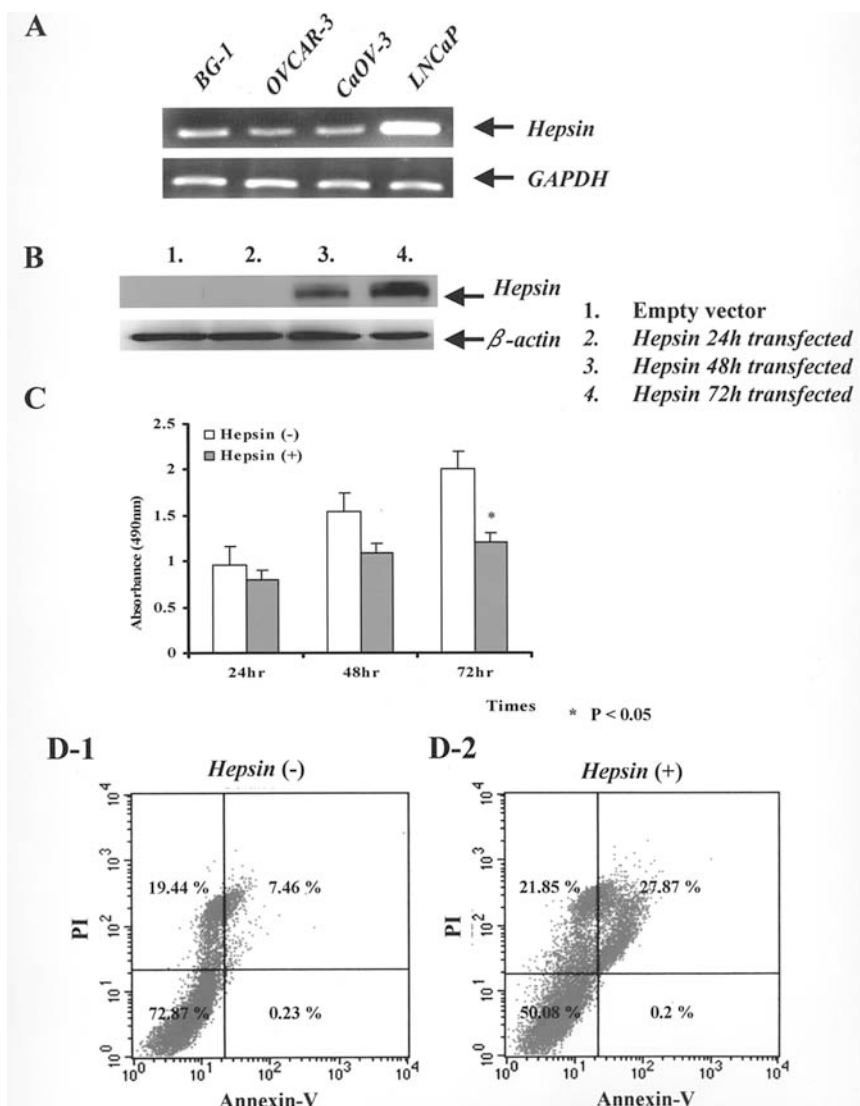


Figure 1. (A) BG-1, OVCAR-3 and CaOV-3 ovarian cancer cells and LNCaP prostate cancer cells were analyzed for *Hepsin* by RT-PCR. (B) Total cell lysates of *Hepsin* transiently transfected were treated for 0, 24, 48 and 72 h. BG-1 cells were analyzed for *Hepsin* protein using Western blotting. As a loading control, a β -actin antibody was used in the same blot. (C) Cell proliferation of MTS assay was analyzed either with or without *Hepsin* transient transfection for 24, 48 and 72 h using BG-1 cell lines. The absorbance was read at a wavelength of 490 nm using an ELISA plate-reader. The assays were carried out through quadruplicate transfection experiments. (D) Representative flow cytometric data for apoptosis in the BG-1 cell line either with or without *Hepsin* transient transfection after 72 h of treatment [D-1, *Hepsin*(-); D-2, *Hepsin*(+)].

Western blot analysis. Cell lysates were collected and estimated using a Protein Assay system (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The protein from each cell line was subjected to SDS-PAGE and was transferred onto the nitrocellulose membrane. The polyclonal and monoclonal antibodies and working dilution ratios used for immunoblotting were as follows: *Hepsin* (Cayman Chemical, Ann Arbor, MI), p53, Puma- α , Puma- β/δ , Noxa, Bcl-2, Bax, Bak, p53AIP1, p53R2, caspase-3, -6, and -7 (Santa Cruz Biotechnology, Santa Cruz, CA), and β -actin (Sigma Chemical Co., St. Louis, MO). The membranes were then incubated with each second antibody. The expressions of antigen-antibody complexes were detected using an enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Generation of transient transfectant. A full-length *Hepsin* cDNA construct encoding the *Hepsin* protein was transiently transfected in the BG-1 cell lines using a TransFast transfection

reagent (Promega, Madison, WI). BG-1 cell lines were treated either with or without *Hepsin* for 24, 48 or 72 h.

Cell proliferation assay and FITC-conjugated Annexin V. In order to evaluate the growth effects of *Hepsin*, MTS assay (Promega) was performed. Cells were seeded into 96-well plates to obtain a density of 5×10^4 cells/well and transfected for 24, 48 or 72 h. The absorbance was read at a wavelength of 490 nm using an ELISA plate-reader (Bio-Rad Systems). Moreover, apoptosis was measured by staining with FITC-conjugated Annexin V using a Mebcyto apoptosis kit (MBL, Nagoya, Japan) according to the manufacturer's recommendations with flow cytometric analysis.

Generation of stable transfectant. *Hepsin* stable transfectant in the BG-1 cell lines was generated using a TransFast transfection reagent. Zeocin (0.8 mg/ml) (Invitrogen, Carlsbad, CA)-resistant colonies were selected after 4 weeks. Drug

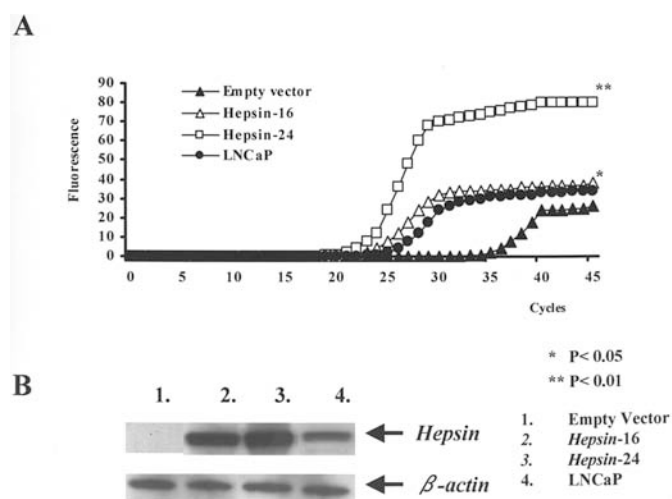


Figure 2. *Hepsin* was stably transfected in the BG-1 cell line using a TransFast transfection reagent. Zeocin (0.8 mg/ml)-resistant colonies were selected after 4 weeks. Cells derived from the individual colonies after transfection and drug selections were obtained by cloning cylinders and were then further expanded. (A) Stable BG-1 cell transfectants of *Hepsin* cells (BG-1/*Hepsin*-16, 24), an empty vector type of BG-1 ovarian cancer cells, and LNCaP were analyzed for *Hepsin* by real-time quantitative PCR. As a loading control, a GAPDH was used. (B) Total cell lysates were prepared from stable transfectants of *Hepsin* cells, an empty vector type of BG-1, and LNCaP were analyzed. As a loading control, a β -actin antibody was used in the same blot.

selections of individual colonies were performed and then further expanded.

Real-time PCR. Real-time quantitative RT-PCR was performed using a LightCycler rapid thermal cycler instrument (Roche Diagnostics, LightCycler, Mannheim, Germany) under the conditions recommended by the manufacturer. The real-time PCR used the primers: 5'-CCAAGGACACCCTCC CTC-3' (*Hepsin*-F), 5'-AAGAGCATCCCATCATCAGG-3' (*Hepsin*-R); 5'-TTCCTAGCACTGCCCAACA-3' (p53-F), 5'-GACTGGAAACTTTCCACTTG-3' (p53-R); 5'-CAACTAC ATGTTTACATGTTC-3' (GAPDH-F), and 5'-GCCAGTG GACTCCACGAC-3' (GAPDH-R), as described (6,7). The products were checked by melting point analysis and electrophoretic mobility. Standard curves for the calculation of the number of transcripts were elaborated using plasmids containing the respective amplified fragment as an insert, and were adjusted to use GAPDH as the reference gene.

Cell growth in monolayer. Cells were plated at a density of 2.5×10^4 cells/well in 6-well plates in DMEM with 10% FBS. Cell proliferations were followed by counting the cell numbers after 1, 3, 5, and 7 days. All points were results of triplicate experiments by a hemocytometer, and the mean values were analyzed.

Cell growth in soft agar. A cell suspension (1×10^4 cells/well) in 1 ml of 0.2% Noble agar with DMEM with 10% FBS was overlaid into a 35-mm dish containing a 0.5% agar base. Colonies >0.2 mm in diameter were counted on day 21.

Tumorigenesis in nude mice. Cells (2.5×10^7) suspended in 200 μ l of sterile PBS were injected subcutaneously above the

hind leg of 5-week-old male BALB/c nude mice (Charles River Japan, Yokohama, Japan). The established tumor volume was measured weekly as a spheroid from three-dimensional diameters.

Immunohistochemistry. Formalin-fixed paraffin-embedded sections, 4- μ m thick, were deparaffinized with xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked by methanol containing 0.3% hydrogen peroxidase for 15 min. The sections were then treated at room temperature with a primary antibody for p53, Puma- α , Puma- β/δ , Noxa, Bax, Bak, p53AIP1, and p53R2, followed by staining using a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were counterstained with hematoxylin.

Results

The transient transfection of *Hepsin* increased the progress of apoptosis. The mRNA of *Hepsin* was determined using quantitative RT-PCR (3). Among the established ovarian cancer cell lines, *Hepsin* expression was detected in the CaOV-3, OVCAR-3, and BG-1 cell lines. As a positive control, we used the LNCaP prostate cancer cell line (Fig. 1A). Among these three, the BG-1 cell line exhibited the strongest transformation on anchorage-independent cell growth. For this reason, the BG-1 transfectant was chosen for further experiments. Several previous reports also showed that *Hepsin* acts during apoptosis and in the G₂ phase of the cell cycle in prostate cancer (8). We used both MTS and FITC-conjugated Annexin V assays that examined apoptosis expression transient transfection of *Hepsin* treatment in the BG-1 ovarian cancer cell line. Protein expression of *Hepsin* transient transfectant was detected using Western blotting. *Hepsin* expression was significantly detected only after 72-h treatment (Fig. 1B). Proliferation was significantly suppressed in 72-h transfectant in accordance with the pattern of protein expression ($p < 0.05$) (Fig. 1C). Representative flow cytometric data revealed that transient transfection of *Hepsin* for 72 h showed increased FITC-positive and propidium iodide (PI)-positive signals (Fig. 1D). Therefore, *Hepsin* was likely to regulate the apoptosis induced by transient transfection of *Hepsin*.

Evaluation of *Hepsin* expression stably transfected BG-1/*Hepsin* cells. mRNA from stably transfected cells was measured by real-time quantitative RT-PCR and was adjusted to the GAPDH levels. As a positive control, we used the LNCaP prostate cancer cell line. Both the mRNA and protein levels of *Hepsin* were similarly and highly expressed by BG-1/*Hepsin*-24 and BG-1/*Hepsin*-16 cells. However, the empty vector type weakly expressed the mRNA levels of *Hepsin*. Furthermore, BG-1/*Hepsin*-24 cells were significantly over-expressed compared to BG-1/*Hepsin*-16 cells (Fig. 2).

Inhibition of cell growth in monolayer culture. The effect of *Hepsin* expression on cell proliferation was analyzed in BG-1/*Hepsin*-16 and BG-1/*Hepsin*-24. We found a significant inhibitory effect of *Hepsin* expression on cell growth in the monolayer cultures of BG-1/*Hepsin*-16 and BG-1/*Hepsin*-24 ($\sim 70\%$ inhibition) ($p < 0.05$) compared to the empty vector type of BG-1 (Fig. 3).

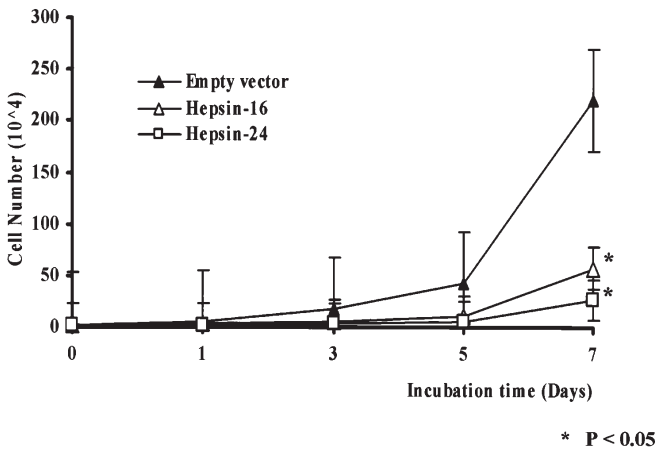


Figure 3. Monolayer growth of *Hepsin* stable transfectant cells (BG-1/*Hepsin*-16, 24) and an empty vector type in the BG-1 ovarian cancer cell line. Cells were plated at a density of 2.5×10^4 cells/well in 6-well plates and were incubated for 7 days in DMEM supplemented with 10% FBS. The number of cells was determined after an additional 1, 3, 5 and 7 days.

Table I. Colony formation on soft agar of *Hepsin* stable transfectant cells and the empty vector type on BG-1 ovarian cancer cell lines.

Cell line	Average no. of colonies/dish ^a	Inhibitory effect (%)
Empty vector	125.3	0
<i>Hepsin</i> -16	25.6	79.6 ^b
<i>Hepsin</i> -24	8.3	93.4 ^c

^aThe number represents through triplicate experiments. ^b $p < 0.05$, ^c $p < 0.01$.

Increased accumulation of p53-dependent apoptosis and caspases in stably transfected BG-1/Hepsin cells. To determine whether the p53 tumor suppressor gene regulates *Hepsin* expression in ovarian cancer cells, p53 mRNA and protein expression were analyzed in BG-1/*Hepsin*-16 and BG-1/*Hepsin*-24 cells. The mRNA and protein levels of BG-1/*Hepsin*-24 and BG-1/*Hepsin*-16 cell showed overexpression of p53 compared with the BG-1 empty vector. BG-1/*Hepsin*-24 cells showed the highest overexpression levels compared with BG-1/*Hepsin*-16 cells (Fig. 4A and B).

Accordingly, we experimented on the promoters of apoptotic cell death, which are pro-apoptotic Bcl-2 protein (Bax-, Bak- and BH-3-only proteins such as Noxa, Puma- α , Puma- β/δ), and p53-regulated apoptosis-inducing proteins (p53AIP1), the repair of damaged DNA proteins (p53R2), and anti-apoptotic Bcl-2 protein (Bcl-2). In BG-1/*Hepsin*-24, Noxa, Puma- α , Puma- β/δ , Bax, Bak, p53AIP1, and p53R2 proteins were up-regulated. Subsequently, down-regulation of Bcl-2 resulted in p53-dependent apoptosis. The p53-related apoptosis factors were highly up-regulated in BG-1/*Hepsin*-24, unlike BG-1/*Hepsin*-16 and the empty vector type (Fig. 4B).

To investigate the specific apoptotic regulated by *Hepsin*, we examined the effect of *Hepsin* on the caspase family by Western blotting. The protein levels of BG-1/*Hepsin*-24 and

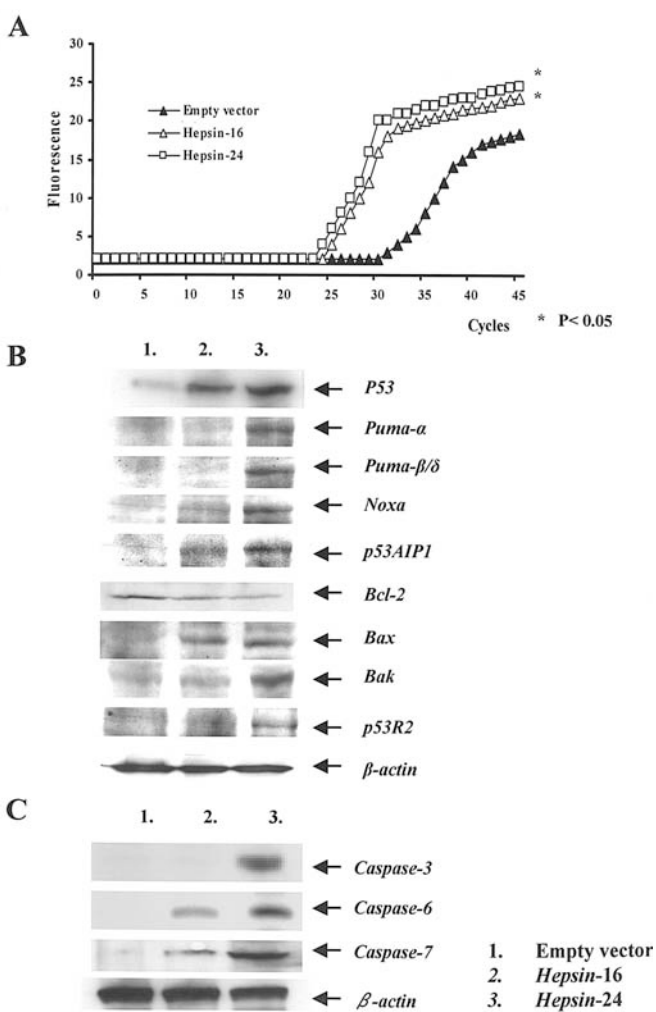


Figure 4. Effect of stable transfection of *Hepsin* on the expression of the p53-dependent apoptosis protein. (A) Stable BG-1 cell transfectants of *Hepsin* cells (BG-1/*Hepsin*-16, 24) and an empty vector type of BG-1 ovarian cancer cells were analyzed for p53 by real-time quantitative PCR, as described. As a loading control, a GAPDH was used. (B) Total cell lysates were prepared from BG-1 *Hepsin* stable transfectants (BG-1/*Hepsin*-16, 24) and the empty vector type was analyzed for p53, Puma- α , Puma- β/δ , Noxa, p53AIP1, Bcl-2, Bax, Bak and p53R2 proteins using Western blotting. As a loading control, β -actin antibody was used in the same blot. (C) BG-1/*Hepsin*-16, -24 and the empty vector type were analyzed for caspase-3, -6, and -7 proteins using Western blotting. As a loading control, β -actin antibody was used in the same blot.

BG-1/*Hepsin*-16 showed significant overexpression of caspase-3, -6, and -7 (Fig. 4C), but not caspase-1, -2, -8 and -9 (data not shown), compared with the BG-1 empty vector. Moreover, caspase-3, -6, and -7 of BG-1/*Hepsin*-24 cells showed the highest overexpression levels compared with BG-1/*Hepsin*-16 cells.

Inhibition of cell growth in soft agar. The inhibitory effect in colony formation was approximately 80% higher than the empty vector type. Furthermore, BG-1/*Hepsin*-24 cells produced a smaller number of colonies compared to BG-1/*Hepsin*-16 cells and the empty vector type ($p < 0.05$ or $p < 0.01$) (Table I).

Inhibition of tumor growth in vivo. We examined the tumorigenicity of BG-1/*Hepsin*-24 or the empty vector type in nude

mice. Characteristically, there was a noticeable suppression of tumor growth in a statistically significant manner by BG-1/*Hepsin*-24. The extent of reduction in the tumor volume

compared to the empty vector type was more than 95% ($p < 0.01$) (Fig. 5).

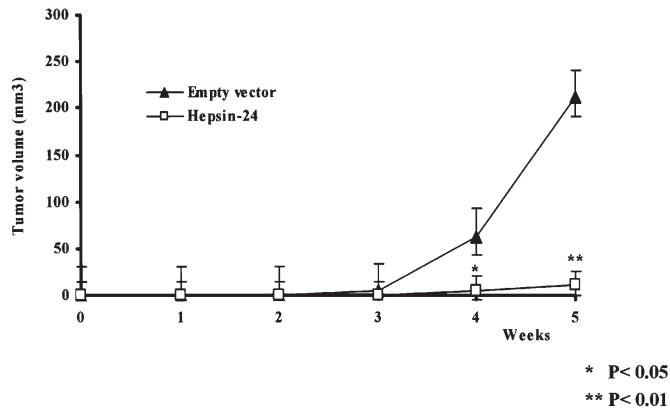


Figure 5. Tumorigenesis of *Hepsin* stable transfectant cells (BG-1/*Hepsin*-24) and the empty vector type of the BG-1 ovarian cancer cell line. After 24 h of serum starvation, each clone (2.5×10^7 cells) was resuspended in 200 μ l of sterile PBS and injected s.c. above the hind leg of 5-week-old male BALB/c nude mice. The established tumor volume was measured weekly as a spheroid from three-dimensional diameters. Each point represents the mean and SD from 5 mice. The mean tumor $\pm$ SD are shown at the times that tumor measurements were made ($n=5$).

In vivo expression of p53-dependent apoptosis protein. To confirm the p53-dependent apoptosis protein overexpression by immunohistochemistry staining, we used an empty vector and *Hepsin* transfectants (BG-1/*Hepsin*-24). The representative staining of the p53-dependent apoptosis factors (Bax, Bak, Puma- α , Puma- β/δ , Noxa, p53AIP1, and p53R2) were also densely stained in BG-1/*Hepsin*-24 tissue. Furthermore, p53AIP1 was more densely stained than the other p53-dependent apoptosis factors (Fig. 6).

Discussion

In light of the poor prognosis for ovarian cancer, the research continues for innovative and efficacious treatment modalities. Along with surgical and chemotherapeutic regimens, gene therapy has emerged as one of the leading contenders in the treatment armamentarium.

Data from the present study show that *Hepsin* is essential for the functional activity of ovarian cancer. Previous reports have documented overexpression of *Hepsin* in ovarian cancer; however, its biological functions remain to be understood. In the current study, we demonstrated that the overexpression of *Hepsin* inhibited cell proliferation, colony formation *in vitro*,

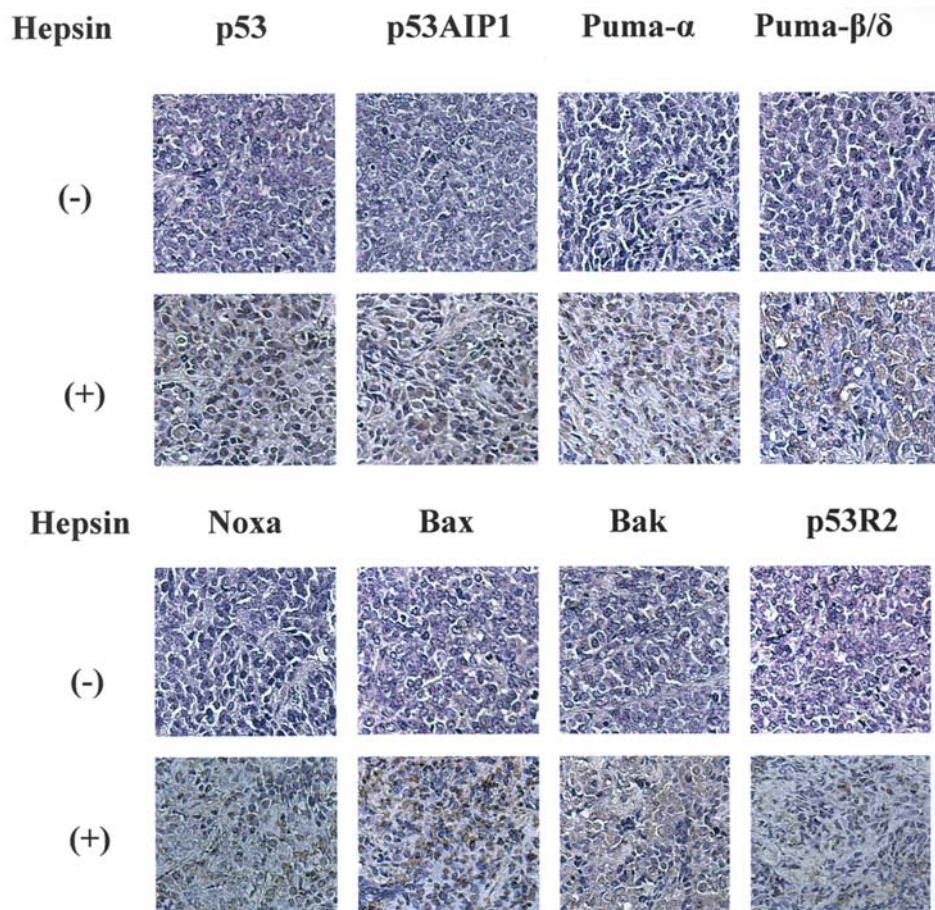


Figure 6. Immunohistochemical analysis of the p53-dependent apoptosis protein transfectants of *Hepsin* cells (BG-1/*Hepsin*-24) and the empty vector type in the BG-1 ovarian cancer cell line. After 5 weeks, each tumor was removed, fixed or embedded in paraffin, and 4- μ m sections were performed using the antibody for p53, p53AIP1, Puma- α , Puma- β/δ , Noxa, Bax, Bak, and p53R2 (original magnification, $\times 200$).

and tumorigenicity *in vivo*. Prostate cancer studies showed that *Hepsin* acts during apoptosis and in the G₂ phase of the cell cycle (8).

DNA damage and oncogene expression lead to the stabilization and activation of the p53 tumor suppressor protein. The wild-type p53 (wt p53) protein exerts inhibitory effects toward the growth of abnormal cells, and has been considered as a guardian of the genome by preventing cancer development (9). To our knowledge, the present study is the first report of *Hepsin*-induced activation of the p53-dependent pathway. Further research is needed to define the mechanism involved in the upstream activation of p53 by *Hepsin*.

Depending on the cellular context, p53 activation can cause cell cycle arrest or apoptosis, and both responses contribute to tumor suppression (10). In this study, we discovered that *Hepsin* induced activation of p53 leading to a p53-dependent apoptosis.

Several promoters of apoptotic cell death, such as promoters of pro-apoptotic Bcl-2 protein: Puma (11,12), Noxa (13,14), Bax (15), Bak (16), and other promoters such as p53AIP1 (17), and p53R2 (18), were considered to regulate p53-dependent apoptosis. In this study, we used protein analysis to study the p53-dependent apoptosis pathway. Our results showed that *Hepsin*-induced expression up-regulated the Puma, Noxa, Bax, Bak, p53AIP1, and p53R2 proteins. This confirms the fact that Bcl-2, which is a death antagonist and has the capacity to suppress caspase-dependent pathways of apoptosis, plays a potentially important role in p53-dependent apoptosis (13,19-22).

To date, there is no evidence for a regulatory role for *Hepsin* in apoptotic-mediated caspase signaling complex activity. Our data indicates that p53-dependent apoptosis, which mediated the activation of caspase-3, -6, and -7, was enhanced by *Hepsin* expression.

In the present study, we discovered that the inhibitory effect of *Hepsin* overexpression isn't only present in experimental cell growth in the monolayer but also in anchorage-independent cell growth *in vitro*. In the present study, we first reported the vigorous inhibitory effect of tumorigenesis expression *in vivo*. *In vivo* studies showed that the extent of reduction in the tumor volume compared to the empty vector type was more than 95% (Fig. 5). Combining these results, it is suggested that *Hepsin* has a potentially therapeutic effect through growth inhibition of the ovarian cancer cell line.

In conclusion, we propose that *Hepsin* induces activation of apoptosis and inhibits cell growth *in vitro* as well as tumorigenicity *in vivo*. Our findings identify *Hepsin* as a potential therapeutic target gene for new approaches to treating ovarian cancer.

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