Tumor-enhancing activity of Basigin expression in gallbladder carcinoma and its prognostic role

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Abstract. Basigin (EMMPRIN/CD147) is a multifunctional membrane glycoprotein that is overexpressed in many solid tumors and is involved in tumor invasion and angiogenesis. The main purpose of this study was to investigate the tumor-enhancing activity of Basigin in a gallbladder carcinoma (GC) cell line and in primary GC tissues. A system that blocks Basigin in the human primary GC cell line GBC-SD was developed using RNA interference. GBC-SD cells were transfected with the small interfering RNA that target Basigin, then the proliferative, invasive and migration activities of the cells were assayed *in vitro*. Additionally, tissue samples from 98 patients with GC and 26 patients with chronic cholecystitis were stained with anti-Basigin antibody for immunohistochemical analysis. Furthermore, the association of Basigin expression with the clinicopathological characteristics and prognosis of the patients was analyzed. siRNA-treated GBC-SD cells exhibited significantly decreased growth ability, invasion and migration capacities compared to control cells *in vitro*. Moreover, clinicopathological analysis demonstrated that the intensity of Basigin staining in cancerous tissue was significantly associated with the histological type (p=0.02), distant metastasis (p<0.01) and Nevin stage (p<0.01) of GC. A proportional hazards model revealed the survival rate of patients with stronger Basigin expression to be the lowest (p<0.01). These results suggest that Basigin is a prognostic marker and potential therapeutic target for patients with GC.

Introduction

Gallbladder carcinoma (GC) is the most common malignancy of the biliary tract. Metaplastic changes have recently attracted great attention as potential key factors underlying the mechanism of GC carcinogenesis. Although radiological and surgical techniques have been rapidly developed, the long-term survival of GC patients is poor. Compared to other common types of cancer, the identification of GC prognostic markers has not been extensively pursued, as this disease has a relatively low incidence and is usually diagnosed during its later stages (1,2). Molecular markers, such as p53, MUC1 and MGMT, have been demonstrated to be related to GC progression (3-5). However, most have only been identified in single studies comprising a small number GC cases, and thus have limited value in GC diagnosis. Consequently, the identification of reliable molecular markers may provide important prognostic information and facilitate the identification of adequate therapeutic targets.

Basigin, also known as extracelluar matrix metalloproteinase inducer (EMMPRIN/CD147), is a highly glycosylated member of the immunoglobulin superfamily that is expressed on the surface of many malignant tumor cells and functions as an upstream modulator of matrix metalloproteinase (MMP) production in the local tumor environment (6-8). Recently, it was demonstrated that Basigin may bind and regulate several tumor-related signalling proteins and stimulate cell proliferation and survival (9). Here, we show that the overexpression of Basigin in a GC cell line enhanced cell growth, invasion and migration capacities. We also demonstrate that Basigin is expressed in a heterogeneous pattern in GC tissues compared with chronic cholecystitis tissues. Furthermore, we show that the analysis of Basigin expression can be utilized as a prognostic indicator for GC, independent of several commonly used markers.

Materials and methods

Cell culture. The human GC cell line GBC-SD and the normal human epithelial cell line HGBEC were obtained from the National Cell Bank of the Cell Institute of Shanghai, China, and were cultured in 90% DMEM (Gibco) supplemented with antibiotics (1x penicillin/streptomycin 100 U/ml, Gibco) and 10% FBS (Gibco). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Patient and tissue samples. A total of 98 fresh GC tissues were obtained from 58 male and 40 female patients (aged 36-78 years, mean ± SD 52.66±11.06 years; Nevin stages I-V) immediately after cholecystectomy and were paired with 26 chronic cholecystitis tissues obtained from Xiangya Hospital of Central South University of Changsha, China. None of the patients recruited had undergone chemotherapy or radiotherapy prior to the surgery. Samples obtained from carcinoma tissue

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were fixed in 10% formalin and routinely processed for paraffin embedding. Histological sections (4-μm thick) were stained with hematoxylin and eosin, as well as immuno-peroxidase procedures. The histological sections were independently reclassified by two experienced pathologists according to histological type and Nevin staging. The 98 patients were subject to a 5-year follow-up. Patients that succumbed to diseases other than GC or to unexpected events were excluded from the sample.

The study was approved by the Research Ethics Committee of Xiangya Hospital of Central South University of Changsha, China. Informed consent was obtained from the patients. The specimens were handled in a blinded manner according to ethical and legal standards.

Construction of small interfering RNA targeting Basigin. Based on the Basigin cDNA sequence in GenBank, we used BLAST to design a pair of nucleotide oligos containing endonuclease restriction sites at both ends. Synthesis was performed by Shanghai Pharmnet Biotechnologies (Shanghai, China). Oligos were annealed and ligated with linearized pSUPER using T4DNA ligase. The recombinant plasmid was named pSUPER/Basigin small interfering RNA (siRNA) and was sequenced and identified by PCR and restriction endo-nuclease digestion.

Transfection of siRNA. The transfection of siRNA was performed to silence Basigin expression. GBC-SD cells were incubated in appropriate antibiotic-free medium with 10% fetal bovine serum (Gibco), then transferred to a 6-well tissue culture and incubated at 37°C in a CO_2 incubator to obtain 60-80% confluence. The cell cultures were transfected with recombinant plasmid pSUPER/Basigin siRNA or empty vector in serum-free medium using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), prepared according to the manufacturer's protocol. Cells were collected 72 h after transfection. Specific silencing of the targeted gene was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Semiquantitative RT-PCR analysis. For the semiquantitative RT-PCR analysis of Basigin mRNA levels, total RNA was isolated from the GBC-SD and HGBEC cells using TRIzol (Invitrogen), and cDNA was synthesized using an RT-PCR kit (Takara, Japan) according to the manufacturer's instructions. The primers sequences were as follows: Basigin, F: 5'-GAG AGCTTGCGAAACTGGTC-3', R: 5'-AACCAACACAGG ACCCAG-3' (492 bp); ß-actin, F: 5'-ACTGGAACGGTG AAGGTGACAG-3', R: 5'-GGTGGCTTTTAGGATGGC AAG-3' (161 bp). PCR analysis was performed under the following conditions: denaturation at 94°C for 1 min, then 30 cycles of denaturation for 20 sec at 97°C, annealing for 20 sec at 64°C, and extension for 20 sec at 72°C. The amplified products were analyzed by agarose gel electrophoresis on a 1.0% gel, followed by ethidium bromide staining. PCR products (1 μl) were electrophoresed on 1.5% agarose gels, and the gray scale ratio of Basigin/ß-actin was calculated.

MTT assay. GBC-SD, control RNAi and Basigin/RNAi cells in 200 μl DMEM were seeded in duplicate into each well of 96-well culture plates. After 24, 48, 72 and 120 h of culture,

100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT, 5 mg/ml, Sigma) solution (5 mg/ml in PBS) was added. After a 4-h incubation at 37°C in 5% CO_2, 100 μl DMSO (Gibco) was added to each well. The dark blue crystals of MTT-formazan were dissolved by shaking the plates at room temperature for 10 min, then absorbance was measured on a Bio-Rad Microplate Reader (Bio-Rad, Hercules, CA) using a test wavelength of 490 nm and a reference wavelength of 630 nm. Each experiment was performed in triplicate. Growth rate (%) = A490 (transfectants)/A490 (RNAi control) x100%.

Invasion and migration assays. GBC-SD, control RNAi and Basigin/RNAi cells were labeled with the fluorescent lipophilic tracer DiI (Molecular Probes, Eugene, OR) and seeded at a density of 50,000 per 6.4-mm diameter well in BD Falcon Individual HTS Fluoroblok Cell Culture inserts in 24-well companion plates (BD Biosciences, San Jose, CA). The percentage of cells moving through the 8 μmol/l pore Fluoroblok membrane was estimated by measuring the fluorescence generated from below at various time points after seeding. For invasion measurements, the membrane was coated with 100 μg Matrigel in a 30-μl volume per well. Experiments were carried out in triplicate on three separate occasions.

Immunohistochemical staining and assessment. Immuno-histochemical staining was carried out using the avidin-biotin method and a commercially available kit (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). One paraffin-embedded block of tissues was selected from each patient and cut into 4-μm sections. The sections were deparaffinized and treated with methanol containing 3% hydrogen peroxide for 10 min, then antigen retrieval was conducted in a microwave oven at 95°C for 5 min with cooling at 25°C for 2 h. After washing in PBS, blocking serum was applied for 10 min. The sections were incubated with anti-Basigin monoclonal anti-body (Santa Cruz Biotechnology, CA) overnight at 4°C. After washing in PBS, a biotin-marked secondary antibody was applied for 10 min followed by a peroxidase-marked streptavidin for an additional 10 min. The reaction was visu-alized using 3,3'-diaminobenzidine tetrahydrochloride. The nuclei were counterstained with hematoxylin. Positive and negative immunohistochemistry controls were routinely used. Reproducibility of staining was confirmed by reimmuno-staining via the same method in multiple randomly selected specimens.

To evaluate the expression of Basigin, three independent observers without knowledge of the clinicopathologic data examined the immunostained slides. The number of Basigin-positive cells that showed immunoreactivity on the cell membranes and cytoplasm in 10 representative microscopic fields were counted, and the percentage of positive cells was calculated. The criteria used for assessment were as pre-viously reported (10). In brief, expression was classified as 0 (negative), <5% of tumor cells stained; 1 (low), 5-75% of tumor cells stained; and 2 (high), >75% of tumor cells stained.

Statistical analysis. SPSS13.0 software for Windows (SPSS Inc., USA) and SAS 9.1 (SAS Institute, NC) were used for
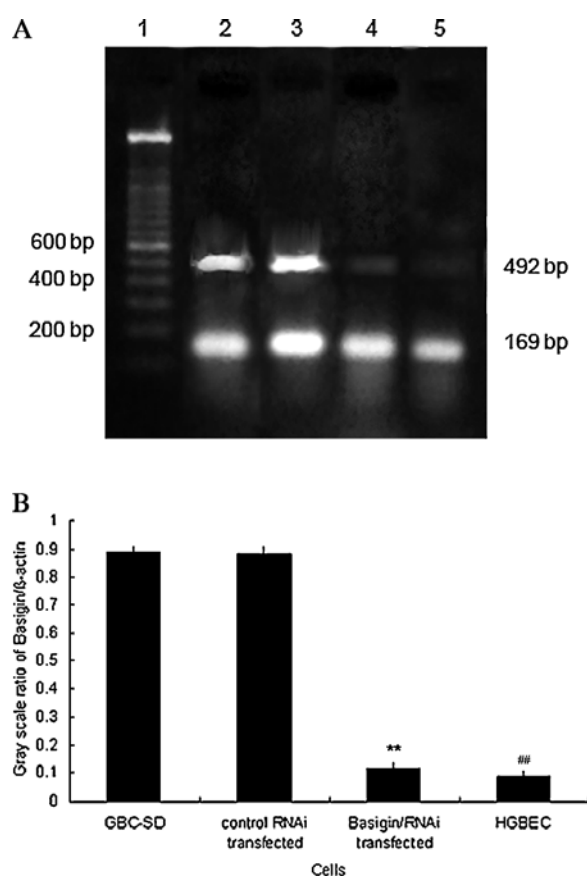
Figure 1. Semiquantitative RT-PCR of Basigin. (A) RT-PCR products of total RNA in GBC-SD, control RNAi, Basigin/RNAi and HGBEC cells were electrophoresed on 1.5% agarose gels as described in Materials and methods (Basigin, 462 bp; ß-actin, 169 bp). Lane 1, marker; lane 2, GBC-SD cells; lane 3, control RNAi transfected GBC-SD cells; lane 4, Basigin/RNAi transfected GBC-SD cells; lane 5, HGBEC cells. (B) Comparison of gray scale ratio of Basigin/ß-actin in GBC-SD, control RNAi, Basigin/RNAi and HGBEC cells. **p<0.001 compared to untransfected GBC-SD cells and control RNAi transfected GBC-SD cells; ##p<0.001 compared to GBC-SD cells.

statistical analysis. Continuous variables were expressed as $\overline{X}\pm s$. Statistical analysis was performed with Fisher's exact test for 2x2 tables, and with Pearson's χ^2 test for non-2x2 tables. Time-to-event curves were estimated by the Kaplan-Meier method and compared using the log-rank test. Cox regression analysis was used to assess the prognostic value of Basigin. p<0.05 was considered to be statistically significant.

Results

Semiquantitative RT-PCR analysis of Basigin. Total RNA was extracted from the GBC-SD, control RNAi and Basigin/RNAi cells, and the RT-PCR products were electrophoresed on 1.5% agarose gels. As shown in Fig. 1A, the PCR product of Basigin was 492 bp. ß-actin was used as the internal control and is the signal seen at 169 bp. The gray scale ratios of Basigin/ß-actin are shown in Fig. 1B. These indicate that the Basigin mRNA level was significantly higher in GBC-SD cells than in normal HGBEC cells (p<0.001). Moreover, pSUPER/Basigin siRNA transfected into GBC-SD cells markedly reduced Basigin gene expression levels compared with the untransfected or control RNAi transfected cells (both p<0.001).
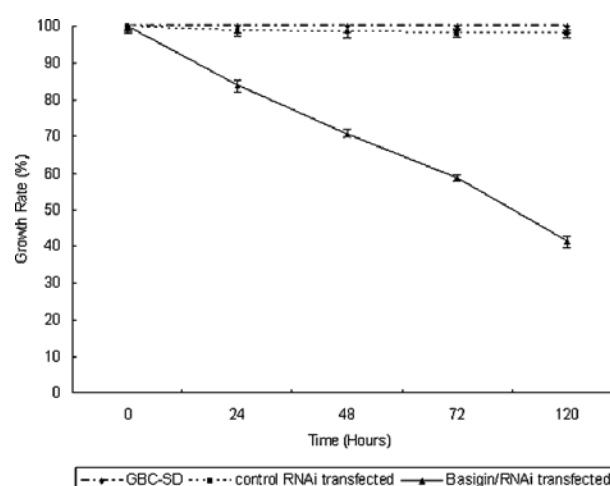

Figure 2. Silencing of Basigin impedes GBC-SD cell proliferation *in vitro*. Cells were harvested at 24, 48, 72 and 120 h post-transfection, and cell proliferation was measured by MTT assay. Decreased growth ability was detected in Basigin/RNAi transfected cells compared with untransfected GBC-SD and RNAi control transcfected cells.

Silencing of Basigin inhibits GBC-SD cell proliferation. Because of the importance of the proliferative ability of tumor cells in tumor progression, the *in vitro* proliferative ability of Basigin-deficient cells was determined by MTT assay. The results indicate that the silencing of Basigin suppressed tumor cell growth. As shown in Fig. 2, Basigin/RNAi cells experienced an ~50% inhibition rate at the 72 h time point. Meanwhile, the growth of GBC-SD cells transfected with RNAi control was not affected. Thus, silencing Basigin could inhibit the proliferative ability of GBC-SD *in vitro*.

Silencing of Basigin inhibits GBC-SD cell invasion and migration. To determine the possible role of Basigin in tumor progression, the capacity of GBC-SD cells for invasion or migration after RNAi treatment was assessed using a modified Bowden chamber assay with a Fluoroblok membrane. Only cells that migrated or invaded across the membrane were fully recorded, in order to quantify the invasive and metastatic capacity of the cells in real time. As shown in Fig. 3, there was a sharp increase in fluorescence during the first 12 h for the GBC-SD, control RNAi transfected and Basigin/RNAi transfected cells. From 12 to 48 h, the fluorescence generated by the Basigin/RNAi transfected cells decreased significantly, whereas that of the GBC-SD and control RNAi transfected cells increased (p<0.01). For the invasion assay, the fluoroblok membrane was coated with Matrigel. As with the migration assay, the fluorescence increased sharply during the first 12 h, then leveled off in the case of the Basigin/RNAi transfected cells, the invasion capacity of which appeared to be dramatically reduced compared to the controls (p<0.01).

Association between Basigin expression and the clinico-pathological characteristics of GC. Basigin was mainly detected in cancerous tissues, but was also expressed in some chronic cholecystitis patients. Of the 98 patients, 80 (81.63%) with GC exhibited Basigin-positive expression, whereas only 2 of 26 patients (7.69%) with chronic cholecystitis did.
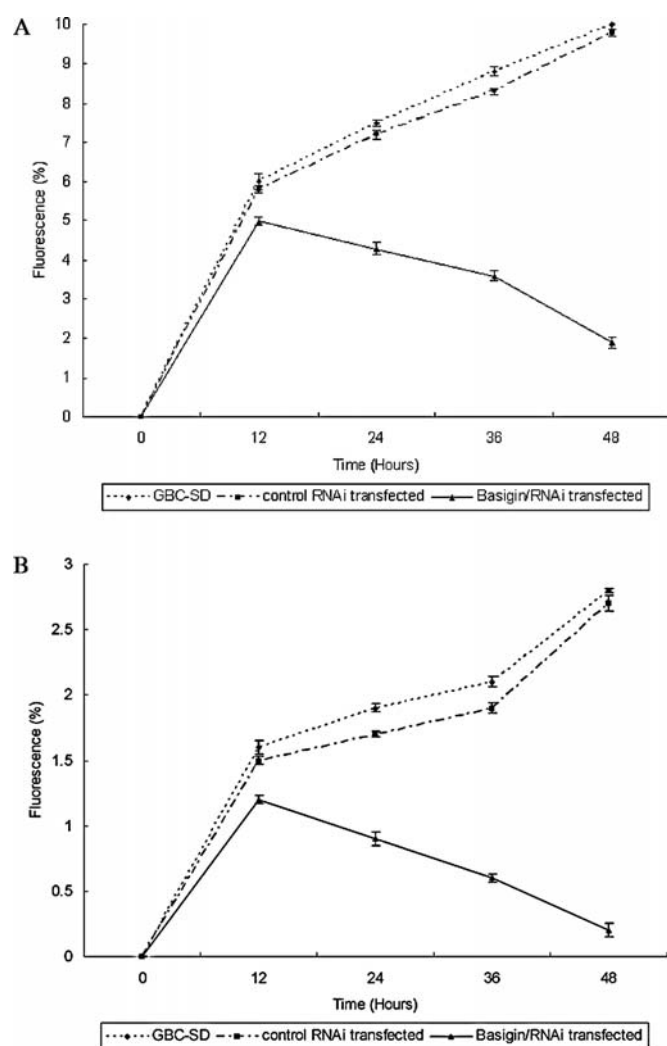
Figure 3. Silencing of Basigin inhibits GBC-SD cell (A) migration or (B) invasion. Each experiment was repeated three times, and each point represents the mean ± SD of three separate determinations.

The association between Basigin expression and the clinicopathological features of the GC patients is shown in Table I. The positive expression of Basigin was not related to the gender or tumor size of the GC patients. Positive Basigin expression tended to be positively associated with the Nevin stage of the tumor tissue ($p < 0.01$), as well as with the degree of histological differentiation ($p = 0.02$). In particular, the incidence of Basigin-positive expression was significantly higher in tumor tissues with distant metastasis than in those without it ($p < 0.01$).

The prognostic role of Basigin expression patterns in patients with gallbladder carcinoma. The 98 patients were subject to a 5-year follow-up. In terms of the Nevin stages of GC, the 5-year survival rate of patients with Basigin expression was lower than that of those without its expression ($p < 0.01$, Table II). Moreover, in the carcinomas with distant metastasis, patients with positive Basigin expression had a significantly lower 5-year survival rate than those with negative expression ($p < 0.01$, Table II). However, there was no significant association between Basigin expression and the 5-year survival rate of patients with all three degrees of histological differentiation ($p > 0.05$).

The association between the 5-year survival rates and the expression levels of Basigin was analyzed using the Kaplan-Meier method. According to their Basigin expression levels, the patients were classified into three groups: 0, 1 and 2. The χ^2 value by the log-rank test (Mantel-Cox) indicated a significant difference among the groups with regard to the expression status of Basigin ($p < 0.01$, Table III). In all three groups, patients with stronger Basigin-positive expression had the poorest prognosis. Cox regression analysis revealed the expression of Basigin to be an apparent independent prognostic indicator (Fig. 4).

Table I. Association between Basigin expression and the clinicopathological features of the gallbladder carcinoma patients.

Factor	No.	Basigin expression (%)			p-value
		0	1	2	
Gender					
Male	58	17.24	44.83	37.93	0.16
Female	40	20.00	50.00	30.00	
Tumor size (cm)					
≥5.0	55	18.18	51.86	30.96	0.38
<5.0	43	18.60	50.91	30.91	
Nevin stages					
I-III	57	21.05	60.88	18.07	<0.01
IV-V	41	14.63	41.46	43.90	
Distant metastasis					
M0	56	21.43	60.21	20.14	<0.01
M1	42	14.29	45.24	38.10	
Histological type					
Well	39	23.08	51.28	25.64	0.02
Moderate	31	22.58	54.84	22.58	
Poor	28	7.14	32.14	60.71	

Table II. Kaplan-Meier analysis of the postoperative survival rate according to Basigin expression in the cancer tissues.

Clinicopatho-logical features	No.	5-year survival rate (%)		p-value
		Basigin⁻	Basigin⁺	
Nevin stage				
I-II	57	81.26	42.18	0.02
IV-V	41	50.23	10.86	<0.01
Distant metastasis				
M0	56	82.08	41.02	0.03
M1	42	36.11	9.26	<0.01

Table III. Kaplan-Meier analysis of the prognostic value of Basigin expression.

Group	Total no.	5-year survival rate	
		No.	Percentage
0	18	15	83.33
1	46	25	54.35
2	34	10	29.41
Overall	98	50	51.02

Discussion

Basigin has tumor-enhancing potential in gallbladder carcinomas. The specific knock-down of target genes without affecting other genes is critically important in research on gene function. RNAi is a specific gene-silencing technology (11). In the present study, we constructed the Basigin siRNA expression vector pSUPER/Basigin siRNA for transfection into the malignant gallbladder carcinoma (GC) cell line GBC-SD, which can be effectively and stably inhibited to express the Basigin gene.

The presence of malignant invasion and metastasis, either at diagnosis or later in the clinical course, is one of the most frequent clinical findings in GC. The growth and invasion of GC cells involves the breakdown of the extracellular matrix by proteinases including matrix metalloproteinases (MMPs), which are involved in tumor progression, growth, invasion, angiogenesis and metastasis (12,13). Previous studies have demonstrated that Basigin promotes tumor cell invasion by stimulating stromal cells to produce elevated levels of MMPs (14-16). As shown in the present study, interference with Basigin expression suppressed the proliferative potential of GC cells *in vitro*. In agreement with a recently published study (17,18), we found up-regulated expression of Basigin mRNA in GC cells, suggesting its participation in the process of prolif-eration as an essential component of cell surface signaling transduction. Li *et al* (19) and Xu and Hemler (20) showed a strong association between cell proliferation and the Basigin-CD98 cell surface super complex, which plays a critical role in energy metabolism. Furthermore, we demonstrated that
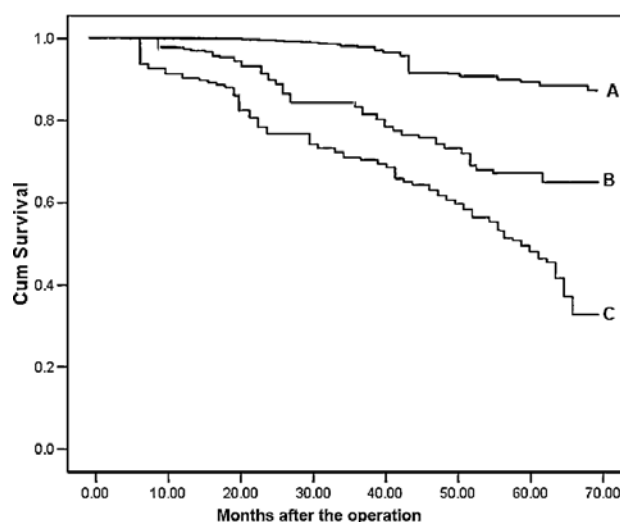

Figure 4. Kaplan-Meier survival curves for Basigin expression in GC. (A) Categorized by negative Basigin expression (18 cases); (B) categorized by weak-moderate positive Basigin expression (46 cases); (C) categorized by stronger positive Basigin expression (34 cases). Survival was significantly poorer for patients with stronger positive Basigin expression than for the other two groups (p< 0.01).

Basigin siRNA inhibits the invasiveness as well as the meta-stasic capacity of GC cells *in vitro*. Our findings indicate that Basigin is involved in the invasion and metastasis of GC cells.

Basigin has a poor prognostic role in gallbladder carcinomas. In the present study, we determined the expression of Basigin in cancerous tissue by immunohistochemical staining, and explored the association between this expression, clinicopatho-logical findings and the 5-year survival rate in 98 patients with GC. GC tissues had a heterogeneous staining pattern of weakly-, moderately- and strongly-expressed Basigin, while chronic cholecystitis tissues did not. These abnormal categories did not show a statistically significant association with several commonly assayed factors, including tumor size, age and patient gender, but the association with histological type, Native stage and lymphatic metastasis status was significant.

In this study, the prognosis for Basigin-positive patients with GC was found to be significantly worse than the prog-nosis for Basigin-negative ones. The results are consistent with a previous study on other types of carcinoma. The positive association of Basigin expression with Nevin stage, distant metastasis and the degree of histological differentiation found in this study may explain the poor prognostic outcome conferred by Basigin expression.

Multivariate analysis of Basigin revealed that the classi-fication of Basigin expression had independent prognostic value when the other factors considered, including Nevin stage, distant metastasis and the degree of histological differentiation, were taken into account. In the present study, we analyzed the association of Basigin expression with the prognosis of GC. Significant differences in prognosis were found among the three groups with different Basigin expres-sion. Patients without Basigin expression had the best outcome, while those with strong Basigin expression had the worst.

In conclusion, our findings indicate that Basigin-targeting siRNA could significantly downregulate the Basigin mRNA

level in GC cells, and that it inhibits their proliferation, invasiveness and metastatic activity *in vitro*. The suitability of Basigin as a prognostic marker and therapeutic target for early-stage GC was also evaluated.

Acknowledgements

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