Involvement of cancer-derived IgG in the proliferation, migration and invasion of bladder cancer cells

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Abstract. It is widely accepted that immunoglobulin (Ig), the classical immune molecule, is extensively expressed in many cell types other than B-cells (non-B-IgG), including some malignant cells. The expression of Ig in malignant cells has been associated with a poor prognosis. In the present study, immunohistochemical analysis detected strong positive staining of IgG in three bladder cancer cell lines, the cancer cells in 77 bladder cancer patient samples and the cells in 3 cystitis glandularis tissue samples, while negative staining was observed in 4 specimens of normal transitional epithelial tissues. Importantly, functional transcripts of IgG with unique V<sub>H</sub>D<sub>J</sub> rearrangement patterns were also found in bladder cancer cells. The knockdown of IgG in bladder cancer cell lines using small interfering RNA significantly inhibited the proliferation, migration and invasion of the cells. Notably, high IgG expression, as determined by immunostaining, was significantly correlated with a high histological grade and recurrence. The results of the present study suggested that IgG expression is involved in the malignant biological behavior and poor prognosis of bladder cancer. Therefore, IgG may serve as a novel target for bladder cancer therapy.

Keywords: bladder cancer, immunoglobulin G, cancer progression, prognosis

Introduction

Bladder cancer is a major public health concern, accounting for 90% of all primary bladder tumors, and is the fourth most common cancer in men (1). The estimated number of new cases of bladder cancer and associated mortalities for 2016 were 76,960 and 16,390, respectively, in the USA (2). In 2004, the World Health Organization recommended a new histological grading system based on malignancy (3), as follows: Papillary urothelial neoplasms of low malignant potential (PUNLMP), low-grade and high-grade urothelial carcinomas. The independent prognostic factors for survival and local tumor control have been shown to involve the entire urethra, tumor size, histological grade, stage, nodal status and disease site (3). Therefore, a high histological grade is associated with a worse prognosis.

Immunoglobulin (Ig) is the most established family of immune molecules. Over the past 500 million years, Ig has become increasingly complex, resulting in the formation of complicated and diverse structures (4,5). Furthermore, infinite variations are found within the variable region (V) of Ig. It was originally thought that Ig acts strictly as an antibody and is only secreted by B lineage cells. However, increasingly, evidence has suggested that Igs, including IgM, IgG and IgA, are expressed in many cell types other than those of the B-cell lineage, including epithelial cells, germ cells and neurons (6-10). Importantly, the non-B-cell-derived Igs, particularly IgG, have recurrently been shown to be overexpressed in the cells of many cancers, including breast, colon, lung, liver and stomach cancers (6,11-14). Furthermore, unlike classical IgG, which has an antibody function, cancer cell-derived IgG is predominantly involved in the survival and progression of cancer cells (6,15).

In order to address the multitude of distinct antigens in the environment, the mechanism of Ig gene rearrangement yields a vast repertoire of antigen receptor-binding specificities. This process involves two stages of rearrangement characterized by the assembly of the V, diversity (D) and joining (J) gene segments of the Ig heavy chain (H), and the V and J gene segments of the light chain (γ or κ), in the developing
B-cell (16). Therefore, B-cell-Igs typically display a huge diversity within a given individual. In our previous studies, V\textsubscript{\textkappa}D\textkappa\textsubscript{\textkappa} or V\textsubscript{\textlambda}J\textlambda\textsubscript{\textlambda} rearrangements in non-B-cell-Igs isolated from neurons, epithelial cells from the breast and colon, and spermatogenic cells from humans or mice were analyzed, and were compared with those in B-cell-Igs (17-19). The results suggested that, unlike B-cell-Igs, non-B-cell-Igs display a restricted and unique V\textsubscript{\textkappa}D\textkappa\textsubscript{\textkappa} or V\textsubscript{\textlambda}J\textlambda\textsubscript{\textlambda} recombination pattern, and that the antigen epitopes of non-B-cell-derived IgG may be different from those of classical IgG.

In our previous study, commercial antibodies against IgG were able to recognize circulating IgG, but they were not specific for the non-B-cell-derived IgG (6). RP215 was originally generated by Lee et al (20) using the cell lysate of the OC-3-VGH ovarian cancer cell line as an immunogen. In our previous studies, it was determined that the RP215 antibody specifically recognizes a glycosylated epitope of a non-B-cell-expressed IgG (RP215-recognized IgG) (21-23).

Liang et al (24) found that IgG was expressed in bladder cancer cells using a commercial anti-human IgG, but its significance remains unclear. In the present study, IgG and its transcripts were shown to be expressed in bladder cancer cells using RP215 and reverse transcription-polymerase chain reaction (RT-PCR), respectively. Notably, functional IgG transcripts with unique VDJ rearrangements were found in these cancer cells. The knockdown of IgG in bladder cancer cell lines resulted in the significant inhibition of cell proliferation, migration and invasion. Furthermore, it was demonstrated that high IgG expression was significantly correlated with histological grade and recurrence.

Materials and methods

Ethics statement. This study was approved by the ethics committee of Peking University People's Hospital (Beijing, China). All patients provided written informed consent.

Patients and clinical samples. The clinical samples, including 77 bladder cancer specimens, 3 cystitis glandularis tissues and 4 normal tissues, were obtained from patients who underwent surgical resection of primary tumors at Peking University People's Hospital between April 2011 and August 2012. Patients who received preoperative radiotherapy or adjuvant chemotherapy were excluded from this study. The biopsy samples for immunohistochemical staining were fixed immediately in 10% buffered formalin and, 24 h later, were dehydrated in increasing concentrations of alcohol, coagulated and embedded in paraffin.

Cell culture. The bladder cancer cell line 5637 was obtained from American Type Culture Collection (Manassas, VA, USA). The bladder cancer cell lines BIU87 and EJ were obtained from the urology department of Peking University First Hospital (Beijing, China). The cells were cultured in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences) and incubated at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}.

Immunofluorescence. RP215 was provided by Professor Gregory Lee of the University of British Columbia in Vancouver, Canada. The 5637, BIU87 and EJ cells were seeded into 12-well plates upon reaching 60-70% confluence and were maintained in an incubator at 37°C containing 5% CO\textsubscript{2}. The cells were fixed in acetone for 5 min at room temperature, after which they were blocked with 10% normal goat serum (HyClone; GE Healthcare Life Sciences) for 30 min and incubated with 12.5 µg/ml purified RP215 as a primary antibody at 4°C for 45 min. The cells were then incubated with the fluorescein isothiocyanate-conjugated goat anti-mouse polyclonal secondary antibody (1:400; cat. no. ab97022; Abcam, Cambridge, UK) at 4°C for 30 min. Images were captured using an inverted fluorescence microscope subsequent to mounting with 50% glycerin.

Immunohistochemical analysis. Tissue sections (4-µm) from the clinical samples were deparaffinized, rehydrated and then heated in 10 mmol/l citrate buffer (pH 6.0) for antigen retrieval. Subsequently, the sections were washed in PBS, blocked with 10% normal goat serum for 30 min and incubated with 7.5 µg/ml purified RP215 in a humidified chamber overnight at 4°C. Immunodetection was performed using the Envision\textsuperscript{TM} ABC kit (GeneTech Co., Ltd., Shanghai, China). After staining with hematoxylin, the tissues were dehydrated and mounted. A pathologist independently evaluated the extent and intensity of RP215 staining and was blinded with respect to the clinical data.

The relative number of positive cells and the intensity of staining were assessed in five random 200x microscopic fields. The percentage of stained cells per field was scored as follows: 0, 0% (negative); 1, 1-25%; 2, 26-50%; and 3, 51-100%. The staining intensity was scored on a 4-tiered scale, as follows: 0, absence of signal; 1, low-intensity signal (light brown); 2, moderate-intensity signal (brown); and 3, high-intensity signal (dark brown). The percentage score and intensity score were multiplied to obtain the score for each field, and the final score for each case was the average score of the five fields. The score for RP215 staining was described as follows: negative (-) when the score was 0-1; ‘low expression’ (+) when the score was 2-3; and ‘high expression’ when the score was 4-6 and 7-9 (++ and +++, respectively). All evaluations were conducted using a Leica DM4000B/M microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Western blot analysis. Protein was extracted using lysis buffer containing radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA), 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 0.15 M NaCl and 10 mM Tris (pH 7.2). The protein concentration was assessed using a Pierce Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Inc.). Protein samples (50 µg) were separated by 12.5% SDS-PAGE and transferred onto a nitrocellulose membrane (EMD Millipore, Bedford, MA, USA). The membrane was blocked with 5% non-fat milk for 1 h and then incubated with the RP215 and anti-GAPDH (cat. no. TA-08; OriGene Technologies, Inc., Rockville, MD, USA) primary antibodies (7.5 µg/ml) overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:3,000; cat. no. ab97040; Abcam) for 1 h at room temperature. Immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.).
RT-PCR. Total RNA was extracted from the bladder cancer cell lines EJ, 5637 and BIU87 using TRIzol reagent (Thermo Fisher Scientific, Inc.), treated with DNase (Magen, Guangzhou, China), and then reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. The primers for the constant region of the IgGH were as follows: Forward, 5'-CAGGACTGCTGAGCAGAAGC-3' and reverse, 5'-GGCGTG GTCTTTGGATTTT-3'. The primers for GAPDH were: Forward, 5'-CAAGGTCACTCCATGAACTTTT-3' and reverse, 5'-GTCACACCTGTTGCTTAG-3'.

In order to amplify the human IgVH gene by nested PCR, the first round of PCR was performed using upstream primers to VH1 (5'-GAGGTGACACAGTCAGAGATCGGGG-3'), VH2 (5'-CAGGCTAGCTGAGCAGTGGG-3'), VH3 (5'-CAGGTAAGCTGAGCAGTGGG-3') and VH4 (5'-CAGGGTCAGCTGAGCAGTGGG-3'), coupled with a CH1 region primer (5'-ACACGTCAACCGTTGCG-3'). PCR was performed using 2X Taq PCR Master Mix kit (Biomed, Beijing, China). The reaction conditions for the first round of PCR were as follows: Pre-denaturation at 94°C for 5 min; followed by denaturation at 94°C for 30 sec, annealing at 59-47°C for 30 sec for the first 18 cycles (with decreases of 2°C increments at each step); then 20 cycles of denaturation at 94°C for 30 sec, annealing at 47°C for 30 sec and polymerization at 72°C for 30 sec with a final elongation step for 7 min at 72°C. A total of 38 cycles were performed. For the second round of PCR, an upstream primer targeting the framework 2 region [5'-TGG(A/G)TCCG(A/C/G) CAG(G/C)C(T/C)CC(A/C/G/T) GG-3'], coupled with a JH primer (5'-AAGTGCAGAGGAGACGGTGACC-3'), was used. The reaction conditions for the second round of PCR were as follows: Pre-incubation at 95°C for 15 min, followed by 38 cycles of denaturation at 94°C for 1.5 min, annealing at 57°C for 1.5 min and polymerization at 72°C for 3 min, with a final elongation step for 5 min at 72°C. PCR products were subjected to electrophoresis on 1.5% agarose gels containing 0.5 µg/ml ethidium bromide.

Sequencing and analysis of rearranged genes. PCR products were cloned into a pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA) and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The VγDJH sequences were compared with those found in the Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the International Immunogenetics information system (http://www.imgt.org/) databases in order to identify the best matched germline gene segments and V(D)J junctions. V gene sequences belonging to a set of VγDJH recombinants were defined on the basis of identical VH, DH and JH gene usage and V-D and D-J junction sequences. The repertoire of the cancer-derived IgV genes was compared with that of published B-cell-derived IgV genes (17).

Cell transfection with small interfering (si)RNA. siRNAs against the constant region of the Ig γ-chain (siRNA1, 5'-GUUGAAGACAGAGAGUGG-3'; and siRNA2, 5'-AGUGCAAGGGUCUCACCAAA-3') and the non-silencing control RNA (NC, 5'-UUCCUGACAGUGUCAGCU-3') were produced by Shanghai GenePharma Co. Ltd. (Shanghai, China). The siRNAs and NC were transfected into the 5637 and BIU87 cell lines using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The knockdown efficiency of IgG was verified by western blot analysis.

Cell proliferation assay. The 5637 and BIU87 cells were transfected with siRNA (50 µg/ml) in 96-well plates and incubated at 37°C. Cell proliferation was analyzed using the Cell Counting kit-8 (CCK8) Cell Proliferation Assay (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, CCK8 reagent (5 µl) was added to each well for 2 h, after which the number of viable cells was calculated by measuring the absorbance at 450 nm. Each condition was assessed in triplicate.

Cell migration assay. Migration assays were performed in 24-well Transwell plates (Corning Incorporated, Corning, NY, USA) containing a polycarbonate filter (8-µm pore size). In total, 10⁴ 5637 and BIU87 cells from each group were added to the Transwell chamber. Conditioned medium supplemented with 10% FBS was added to the bottom of the chamber. The cells were incubated in an incubator containing 5% CO₂ at 37°C for 24 h. Following incubation, the cells in the upper chamber that were attached and had not migrated were removed, and the cells that had migrated to the bottom of the filter were fixed in methanol and stained with hematoxylin. The number of cells was counted in at least 6 randomized fields under a light microscope. The results were obtained from at least three individual experiments.

Cell invasion assay. Invasion assays were performed in 24-well invasion chambers (Corning Incorporated) containing a polycarbonate filter (8-µm pore size). In total, 10⁴ 5637 and BIU87 cells from each group were added to the invasion chamber. Conditioned medium supplemented with 10% FBS was added to the bottom portion of the chamber. The cells were incubated in an incubator with 5% CO₂ at 37°C for 24 h. Following incubation, the cells in the upper chamber that were attached and had not migrated were removed, and the cells that had migrated to the bottom of the filter were fixed in methanol and stained with hematoxylin. The number of cells was counted in at least 6 randomized fields under a light microscope. The results were obtained from at least three individual experiments.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using the χ² test and Student’s t-test. P<0.05 was considered statistically significant. All statistical evaluations were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

IgG is found in transitional epithelial cancer cells. In the present study, IgG expression was first detected in bladder cancer cell lines (EJ, 5637 and BIU87) by immunofluorescence. IgG staining was observed in the cell cytoplasm, filopodia-like structures and the extracellular area (Fig. 1A). Furthermore, IgG expression was also detected in these bladder cancer cell lines by western blotting (Fig. 1B). Subsequently, immunohistochemistry demonstrated that IgG immunoreactivity...
was recurrently localized to the cytoplasm of tumor cells in bladder cancer tissues (58/77, 75.3%) and to the cytoplasm of cells in cystitis glandularis tissues (3/3, 100%), but not to the transitional epithelial cells of normal tissues (0/4) (Fig. 1C).

IgG transcripts with unique patterns of V_HDJ_H rearrangements are found in bladder cancer cells. In order to determine whether IgG was produced by the cancer cells themselves and was not the result of uptake from the extracellular environment, the transcription of the IgGH in EJ, 5637 and BIU87 cells was examined by RT-PCR using primers targeting both the constant and V regions. The results showed that the transcript of the IgGH was expressed in these three cancer cell lines (Fig. 2A).

Subsequently, the sequences of these V_HDJ_H rearrangements were analyzed by a comparison with the best matched functional germline IgVH, IgDH and IgJH genes. The results demonstrated that, similar to the B-cell-Igs, all of the bladder cancer-IgG transcripts displayed classical and functional V_HDJ_H rearrangement patterns. However, unlike B-cell-derived IgVH, which has great diversity, several sets of V_HDJ_H rearrangements were frequently shared among these cell lines, and even among different cell lines (VH3-30/D6-6/JH4 was shown in 3/6 5637 cells, VH3-30/D1-26/JH5 was shown in 2/6 5637 cells and VH3-30/D5-5/JH4 was shared between 3/4 BIU87 cells and 3/5 EJ cells). The bladder cancer-V_HDJ_H rearrangements showed restricted VH, DH and JH usage, and a unique V_HDJ_H pattern, including the VH3 region, which was dominantly used (14/16 V_HDJ_H rearrangements analyzed in this study). In particular, the VH3-30 region was dominantly expressed in 5637 (8/9), BIU87 (3/4) and EJ (3/5) cells. Furthermore, among the germ-line IgHJ1-6 genes, only IgHJ4 (11/16) and IgHJ5 (5/16) were frequently used. Conversely, IgDH showed diversity within each cell line, as follows: DH6-6, DH6-19 and DH1-26 were shown in 5637 cells, DH5-5 and DH6-19 were shown in BIU87 cells, and DH5-5, DH3-16 and DH3-9 were shown in EJ cells. However, DH6-19 was shown in 5637 cells and in BIU87 cells, whereas DH5-5 was shown in BIU87 and EJ cells (Table I, Fig. 2B).

To enhance IgG affinity, the B-cell-derived IgVH of IgG is usually hypermutated (25). Therefore, the present study analyzed the mutation pattern in bladder cancer-derived IgVH and compared the sequence homology among V_HDJ_H rearrangements from three cancer cell lines. It was demonstrated that the bladder cancer-derived IgVH showed only a low frequency of mutation (Table I). Furthermore, the same mutations were frequently present among different V_HDJ_H rearrangements, which resulted in a high homology among V_HDJ_H rearrangements in IgVH (Fig. 2). These results suggested that the conservative domain of IgVH, as well as IgHJ4 and IgHJ5, may exert a similar framework effect in different V_HDJ_H rearrangements in bladder cancer cells, but that IgDH determines the unique biological activity of each V_HDJ_H rearrangement.

Knockdown of IgG significantly reduces the proliferation of 5637 and BIU87 cells. In order to investigate whether IgG...
has a role in the proliferation of bladder cells, two siRNAs to knockdown IgG expression in bladder cancer cell lines were designed and the proliferation of these cells was analyzed following transfection. The results showed that the ability of these cells to proliferate was significantly downregulated following the knockdown of IgG heavy chain expression in 5637 and BIU87 cells (P<0.05; Fig. 3).

Knockdown of IgG significantly reduces the migration and invasion of 5637 and BIU87 cells. The present study investigated whether IgG is involved in the migration and invasiveness of bladder cancer cells. Migration and invasion assays were performed in 24-well invasion chambers using the same number of 5637 and BIU87 cells in each group. It was demonstrated that the migration and invasion of 5637 and BIU87 cells was significantly decreased following knockdown of IgG expression (P<0.05; Figs. 4 and 5).

IgG expression significantly correlates with a high histological grade and recurrence. Next, the present study aimed to determine whether the expression of IgG was correlated with a high histological grade, the size of the tumor and/or the recurrence of bladder cancer. A high positivity of IgG staining was found in patients with high-grade bladder cancer (32/36, 88.9%) compared with patients with low-grade bladder cancer (26/41, 63.4%), and this difference was statistically significant (P=0.045; Table II). Following a comparison of the frequency of positivity between larger tumors (>3 cm) and smaller tumors (≤3 cm), it was found that the rate of IgG positivity
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in patients with large tumors was 94.1% (16/17), which was significantly higher than patients with smaller tumors (42/60, 70.0%) (P=0.003; Table II). Furthermore, IgG expression was significantly associated with the recurrence of bladder cancer (P=0.012). The rate of positivity (++ and ++++) in patients who experienced bladder cancer recurrence was 62.9% (17/27), whereas the rate of positivity (++ and ++++) in patients with non-recurrence was 22% (11/50) (P=0.012; Table II).

Discussion

The present study demonstrated that IgG, with a unique $V_{H}DJ_{H}$ pattern, was expressed in bladder cancer cells. Furthermore, it was determined that IgG was involved in the proliferation, migration and invasiveness of bladder cancer cells. Importantly, IgG expression was correlated with histologically high-grade cancer, a large tumor size and recurrence.

To date, IgG has been considered to be produced solely by B-cells and to function strictly as an antibody (26). Since Qiu et al (6) first reported that IgG was expressed in non-B-cell-derived cancer cells, evidence has accumulated in support of Ig expression in non-B-cells (7,10,27). Igs have been shown to be overexpressed in numerous cancer cells, including many types of cancer cells derived from epithelial and mesenchymal tissues (12,13). In addition, in our previous study, it was demonstrated that IgG expression was strongly correlated with poor-differentiation, local invasion, metastasis and a poor prognosis in patients with lung adenocarcinoma, which suggested that IgG may serve as a novel prognostic biomarker for lung adenocarcinoma (15). In the future, the authors of the present study will investigate the role of IgG in the prognosis of patients with bladder cancer. The present study further confirmed that IgG is expressed in primary bladder cancer cells and in cancer cell lines at both the protein and mRNA levels. Furthermore,

Table I. Assignment of likely matching germline variable region genes to the $V_{H}DJ_{H}$ recombinants from different bladder cancer cell lines and analysis of the $V$ gene somatic mutation rate.

| Cell lines | No. of patterns | $V_{H}DJ_{H}$ | No. of clones | Mutation rate (%) |
|------------|----------------|---------------|--------------|------------------|
| 5637       | 4              | IgHV3-30/IgHD6-6/IgHJ4 | 3            | 2.7              |
|            |                | IgHV3-30/IgHD1-26/IgHJ5 | 2            | 1.6              |
|            |                | IgHV3-30/IgHD6-19/IgHJ4 | 1            | 3.7              |
|            |                | IgHV3-15/IgHD4-17/IgHJ4 | 1            | 3.1              |
| BIU87      | 2              | IgHV3-30/IgHD5-5/IgHJ4 | 3            | 1.0              |
|            |                | IgHV4-61/IgHD6-19/IgHJ5 | 1            | 3.2              |
| EJ         | 3              | IgHV3-30/IgHD5-5/IgHJ4 | 3            | 2.1              |
|            |                | IgHV3-15/IgHD3-9/IgHJ5 | 1            | 6.6              |
|            |                | IgHVL-18/IgHD3-9/IgHJ5 | 1            | 5.8              |

IgHV, immunoglobulin heavy chain variable region; IgHD, immunoglobulin heavy chain diversity region; IgHJ, immunoglobulin heavy chain joining region.

Figure 4. Knockdown of IgG significantly reduces the migration of bladder cancer cells. Knockdown of the expression of non-B-IgG was shown to inhibit the migration ability of 5637 and BIU87 cells. The relative migration values of 5637 and BIU87 cells, as assessed by Transwell assays, are shown above. The number of migrated cells per field was quantified (scale bar=50 µm). Data are presented as the mean ± standard error of the mean of the cell counts in at least three random fields. The experiment was repeated three times. **P<0.01 and ***P<0.001. IgG, immunoglobulin G; siNC, normal control siRNA; siRNA, small interfering RNA.
immunohistochemical analysis detected IgG expression in transitional epithelial cells of cystitis glandularis tissues. However, IgG staining was either weak or negative in normal transitional epithelial cells. These results suggested that overexpression of IgG may be induced by various pathological factors, including factors that promote cancer or proinflammatory factors. However, it cannot be determined whether IgG is expressed in normal human transitional epithelial cells based on the few cases used in the present study because of the small sample size.

The $\text{V}_\text{H}\text{DJ}_\text{H}$ rearrangement is the best evidence for IgG expression, which, for a long time, has been considered to occur only in B-cells (28). In the present study, whether the $\text{V}_\text{H}\text{DJ}_\text{H}$ rearrangement could be identified in bladder cancer cell lines without B-cell contamination was investigated. The results

| Variables                        | No. of patients | (-) | (+)  | (++) | (++++) | P-value |
|----------------------------------|-----------------|-----|------|------|-------|---------|
| Age (years)                      |                 |     |      |      |       |         |
| >60                              | 53              | 12  | 21   | 7    | 13    | 0.4933  |
| ≤60                              | 24              | 7   | 9    | 4    | 4     |         |
| Gender                           |                 |     |      |      |       | 0.9635  |
| Male                             | 66              | 15  | 28   | 9    | 14    |         |
| Female                           | 11              | 4   | 2    | 2    | 3     |         |
| Histological grade               |                 |     |      |      |       | 0.0451  |
| High-grade                       | 36              | 4   | 17   | 5    | 10    |         |
| Low-grade                        | 41              | 15  | 13   | 6    | 7     |         |
| Tumor size, cm                   |                 |     |      |      |       | 0.0028  |
| >3                               | 17              | 1   | 4    | 3    | 9     |         |
| ≤3                               | 60              | 18  | 26   | 8    | 8     |         |
| Tumor number                     |                 |     |      |      |       | 0.9815  |
| <8                               | 62              | 16  | 23   | 9    | 14    |         |
| ≥8                               | 11              | 3   | 4    | 2    | 2     |         |
| Recurrence                       |                 |     |      |      |       | 0.0120  |
| Yes                              | 28              | 4   | 7    | 5    | 12    |         |
| No                               | 37              | 3   | 23   | 6    | 5     |         |

IgG, immunoglobulin G.

Figure 5. Knockdown of IgG significantly reduces the invasion ability of bladder cancer cells. Knockdown of the expression of non-B-IgG was shown to inhibit the invasion ability of 5637 and BIU87 cells. The relative invasion values of 5637 and BIU87 cells, as assessed using Matrigel assays, are shown above. The number of migrated cells per field was quantified (scale bars=50 µm). Data are presented as the mean ± standard error of the mean of cell counts in at least three random fields. The experiment was repeated three times. "*"P<0.01 and "**"P<0.001. IgG, immunoglobulin G; siNC, normal control siRNA; siRNA, small interfering RNA.
clearly showed that the germline IgVH, IgDH and IgJH gene segments were selected and rearranged to form V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements with a classical junction pattern in these bladder cancer cell lines. Furthermore, the bladder cancer-V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements had a distinct usage and features that differed from those of B-cell-derived IgG. It has been reported that each B-cell expresses a unique V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> recombination, including a random N region sequence (16). Therefore, the frequency of identical junction sequences from two independent B-cell clones present in an individual should be <1 in 4 million. However, the bladder cancer-V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements showed conserved IgVH and IgJH, but not IgDH, among the different bladder cancer cell lines. The usage of VH3 and IgJH4 was frequent in the cancer cells, although the primers used for the PCR were designed to be applicable to almost all V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements. In addition, the sequence differences among the V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements were primarily in the D region and not in the V and J regions. These results suggested an unknown mechanism that allows bladder cancer cells to express several dominant V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> sequences.

It has previously been reported that the expression of IgG in cancer cells is associated with growth factor-like activity, which can promote the proliferation and survival of cancer cells (6), and is involved in the carcinogenesis of breast cancer (29). The present study demonstrated that bladder cancer cell-derived IgG was able to promote cell proliferation. Tumor metastasis is the most important cause of cancer-associated mortality worldwide, and the migration and invasion of cancer cells is a prerequisite for metastasis (30). In the present study, an important finding was that the bladder cancer cell-derived IgG showed significant effects in the promotion of cell migration and invasion, which suggested that IgG may be involved in the metastasis and prognosis of bladder cancer.

A high histological grade has been associated with a poor prognosis (31). However, to date, no independent prognostic factor has been shown to be useful for the prediction of bladder cancer prognosis. The present study further analyzed the relationship between IgG expression and the histological grade of bladder cancer. It was found that IgG expression was significantly correlated with a high histological grade. Furthermore, recurrence and tumor size are indicators for survival of patients with bladder cancer (32,33). The present study also found that IgG expression was related to tumor size and the recurrence of bladder cancer, which suggests that IgG has the potential to be a novel independent predictor of bladder cancer prognosis.

In conclusion, the present study demonstrated that IgG was overexpressed in bladder cancer and was involved in the proliferation, migration and invasion of bladder cancer cells. Furthermore, IgG transcripts with unique patterns of V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> rearrangements were found in bladder cancer cells, although the underlying mechanism remains unclear. The results of the present study suggested that IgG expression may serve as a potential target in cancer therapies and might be used as a prognostic marker.

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