

Blocking integrin $\beta 1$ decreases adhesion in chemoresistant urothelial cancer cell lines

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Abstract. Treatment failure in metastatic bladder cancer is commonly caused by acquisition of resistance to chemotherapy in association with tumor progression. Since alterations of integrins can influence the adhesive and invasive behaviors of urothelial bladder cancer cell lines, the present study aimed to evaluate the role of integrins in bladder cancer cells with acquired resistance to standard first-line chemotherapy with gemcitabine, and cisplatin. Therefore, four gemcitabine- and four cisplatin-resistant sublines out of a panel of four parental urothelial bladder cancer cell lines (TCC-SUP, HT1376, T24, and 5637) were used. Expression of integrin subunits $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, and $\beta 4$ was detected using flow cytometry. Adhesion and chemotaxis were analyzed. For functional assays, integrin $\beta 1$ was attenuated with a blocking antibody. In untreated cells, chemotaxis was upregulated in 3/4 gemcitabine-resistant sublines. In cisplatin-resistant cells, chemotaxis was enhanced in 2/4 cell lines. Acquired chemoresistance induced the upregulation of integrin $\beta 1$ in all four tested gemcitabine-resistant sublines, as well as an upregulation in 3/4 cisplatin-resistant sublines compared with parental cell lines. Following the inhibition of integrin $\beta 1$, adhesion to extracellular matrix components was downregulated in 3/4 gemcitabine-resistant sublines and in all four tested cisplatin-resistant sublines. Since integrin $\beta 1$ is frequently upregulated in chemoresistant urothelial cancer cell lines and inhibition of integrin $\beta 1$ may influence adhesion, further studies are warranted to evaluate integrin $\beta 1$ as a potential therapeutic target for bladder cancer *in vivo*.

Introduction

Urothelial cancer of the bladder is the 4th most commonly diagnosed cancer in men worldwide (1). Patients with metastatic disease are often treated with a combination chemotherapy containing gemcitabine and cisplatin as a standard of care (2,3). However, the treatment success is limited, resulting in a median survival of 12-15 months. Treatment failure is commonly caused by acquired resistance after primary response (2,3). Therefore, efficient second line chemotherapies are urgently needed.

Integrins have been identified to play an important role in the development of resistance to chemotherapy in bladder cancer (4). These molecules are transmembrane receptors with two different chains, an α (alpha) and a β (beta) subunit. Integrins are bridges for cell-cell and cell-extracellular matrix (ECM) interactions. Cell-matrix contact plays a fundamental role in the metastatic potential of tumors (5). Alterations of integrin expression may result in an enhanced adhesive behavior in bladder cancer (6). Moreover, the expression patterns of integrin subtypes are known to be important mediators of tumor cell de-differentiation and tumor proliferation (7). Furthermore, it was shown that integrins were involved in the development of metastasis and recurrence of urothelial cancer (6,8,9).

Drug-adapted cancer cell lines have been successfully used to study cancer cell resistance mechanisms (10,11). To reflect the heterogeneity of individual bladder cancer patients and to enable a systematic evaluation of the role of integrins concerning resistance acquisition, we used a panel of 12 urothelial cancer cell lines consisting of 4 parental chemosensitive cell lines and their sublines with acquired resistance to gemcitabine or cisplatin (12,13).

Materials and methods

Drugs. Cisplatin was purchased from Gry-Pharma (Kirchzarten, Germany), gemcitabine from Lilly (Bad Homburg, Germany).

Cell lines. The cell lines 5637, T24, HT1376, and TCC-SUP were obtained from the American Type Culture Collection

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(Manassas, VA, USA). The following drug-resistant sublines were established by continuous exposure to increasing drug concentrations as described previously (12,14) and are part of the Resistant Cancer Cell Line (RCCL) collection (www.kent.ac.uk/stms/cmp/RCCL/RCCLabout.html): 5637^rCDDP¹⁰⁰⁰ (cisplatin-resistant, 1,000 ng cisplatin/ml), 5637^rGEMCI²⁰ (gemcitabine-resistant, 20 ng gemcitabine/ml), T24^rCDDP¹⁰⁰⁰, T24^rGEMCI²⁰, HT1376^rCDDP¹⁰⁰⁰, HT1376^rGEMCI²⁰, TCC-SUP^rCDDP¹⁰⁰⁰, and TCC-SUP^rGEMCI²⁰. All cell lines were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS; Gibco, Karlsruhe, Germany), 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C. Cell line authentication was performed by short tandem repeat profiling.

Cell adhesion to extracellular matrix components. 24-well plates were coated with extracellular matrix components (Matrigel; Corning, Amsterdam, The Netherlands) overnight. Plates were washed with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) to block nonspecific cell adhesion. Thereafter, 0.5×10^6 tumor cells were added to each well for 60 min. Subsequently, non-adherent tumor cells were washed off. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields using a microscope (20x objective) to calculate the mean cellular adhesion rate.

Chemotaxis. Serum induced cell migration was examined using 6-well transwell chambers (Greiner, Frickenhausen, Germany) with 8 μ m pores. To evaluate cell migration, cells were placed in the upper chamber for 20 h in serum-free medium. The lower chamber contained 10% serum. After incubation, the upper surface of the transwell membrane was wiped gently with a cotton swab to remove non-migrating cells. Cells migrating to the lower surface of the membrane were stained using hematoxylin and counted. Cells migrating into the lower chamber were counted separately under the microscope.

Blocking study. Cells were preincubated for 60 min with a function-blocking anti-integrin $\beta 1$ monoclonal antibody (20 mg/l) (MAB 2253Z; MerckMillipore, Darmstadt, Germany). Controls remained untreated. Adhesion and chemotaxis was tested as indicated above.

Flow cytometry. Cells were washed in blocking solution (PBS, 0.5% BSA) and then incubated for 60 min at 4°C with phycoerythrin (PE)-conjugated monoclonal antibodies directed against the following integrin subtypes: Anti- $\alpha 3$ (IgG1; clone C3II.1), anti- $\alpha 5$ (IgG1; clone IIA1), anti- $\alpha 6$ (IgG2b, clone MP 4F10), anti- $\beta 1$ (IgG1; clone MAR4), anti- $\beta 3$ (IgG1; clone VI-PL2) or anti- $\beta 4$ (IgG2a; clone 439-9B; all: BD Biosciences, Heidelberg, Germany). Integrin expression was measured by flow cytometry (FACSCalibur; BD Biosciences, Heidelberg, Germany). Mouse IgG1-PE (MOPC-21) or mouse IgG2a-PE (G155-178; all: BD Biosciences) antibodies were used as isotype control.

Immunohistochemistry. 33 cases of invasive and non-invasive bladder cancers as well as corresponding normal urothelium were taken from the archive of the Dr. Senckenberg Institute of Pathology in Frankfurt. Tissue sections were stained for Integrin $\beta 1$, (D2E5) Rabbit mAb, Cell Signaling Technology

(Waltham, MA, USA), dilution 1:100. In brief, 4 μ m sections were cut and pretreated with TrilogyTM, Cell Marcque (Rocklin, CA, USA), incubated with the antibody, antigen retrieval was performed at pH 6 in a microwave oven using the Peroxidase-FLEX EnVision kit (Dako, Jena, Germany).

A pathologist, who was blinded to clinical history and therapeutic response, scored the immunohistochemical staining using a five-stage staining score: 0=negative; 1=weak; 2=moderate; 3=strong; 4=very strong.

Images were acquired using a digital slide scanner (ScanScope XT; Aperio, Vista, CA, USA).

Statistical analysis. Results are expressed as mean \pm SD of at least three independent experiments. For statistical analysis student's t-test, analysis of variance (ANOVA), and Student-Newman-Keuls-Test were performed whenever applicable. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Influence of acquired resistance on adhesion to extracellular matrix components. In untreated cells, adhesion to extracellular matrix components was decreased in 2 of 4 gemcitabine-resistant sublines (HT1376^rGEMCI²⁰ and TCC-SUP^rGEMCI²⁰) and upregulated in 2 of 4 cell lines (T24^rGEMCI²⁰ and 5637^rGEMCI²⁰) compared to parental cells. In cisplatin-resistant sublines, adhesion was decreased in 1 of 4 cisplatin-resistant sublines (TCC-SUP^rCDDP¹⁰⁰⁰) and enhanced in 2 cell lines (HT1376^rCDDP¹⁰⁰⁰ and 5637^rCDDP¹⁰⁰⁰) (Fig. 1).

Influence of acquired chemoresistance on chemotaxis. Chemotaxis was enhanced in 3 of 4 gemcitabine-resistant urothelial cancer cell lines (gemcitabine-resistant sublines of TCC-SUP, HT1376 and T24). In cisplatin-resistant sublines, chemotaxis was enhanced in HT1376^rCDDP¹⁰⁰⁰ and T24^rCDDP¹⁰⁰⁰ compared to parental cell lines (Fig. 2).

Differential expression of cell surface integrins. Expression of integrins on the cell surface was analyzed by flow cytometry (Fig. 3). In gemcitabine-resistant sublines, the expression of integrin $\alpha 3$ was enhanced in 3 sublines (gemcitabine-resistant sublines of T24, 5637, and TCC-SUP) and diminished in HT1376^rGEMCI²⁰ compared to parental cell lines. Integrin $\beta 1$ expression was upregulated in all gemcitabine-resistant sublines compared to parental cells. Integrin $\beta 4$ expression was enhanced in TCC-SUP^rGEMCI²⁰ and diminished in T24^rGEMCI²⁰ and in 5637^rGEMCI²⁰.

Comparing cisplatin-resistant cell lines, Integrin $\alpha 3$ expression was upregulated in 3 of 4 sublines (cisplatin-resistant sublines of T24, 5637, and TCC-SUP) and downregulated in HT1376^rCDDP¹⁰⁰⁰. Integrin $\alpha 5$ was upregulated in 3 of 4 sublines (cisplatin-resistant sublines of 5637, TCC-SUP, and HT1376). Integrin $\beta 1$ expression was upregulated in 3 of 4 sublines (cisplatin-resistant sublines of HT1376, T24, and 5637) and downregulated in TCC-SUP^rCDDP¹⁰⁰⁰. Integrin $\beta 4$ was upregulated in HT1376^rCDDP¹⁰⁰⁰ and downregulated in all other tested sublines (cisplatin-resistant sublines of T24, 5637, and TCC-SUP).

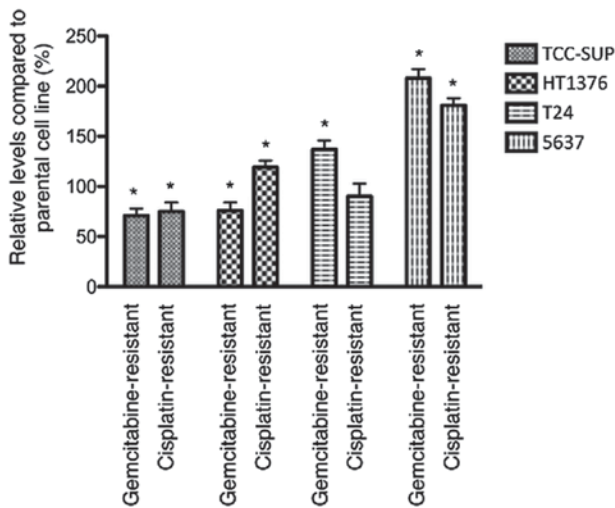


Figure 1. Evaluation of cell adhesion. Comparison of adhesive behavior of parental bladder cancer cell lines as well as of their gemcitabine- and cisplatin-resistant sublines. The plates were coated with extracellular matrix components overnight. Plates were washed with 1% BSA in PBS to block nonspecific cell adhesion. Thereafter, 0.5×10^6 tumor cells were added to each well for 60 min. Subsequently, non-adherent tumor cells were washed off. The adherent cells were fixed with 1% glutaraldehyde and counted in five different fields using a microscope (20x objective) to calculate the mean cellular adhesion rate. Adhesion of parental cell lines was set as 100%. * $P \leq 0.05$ vs. controls.

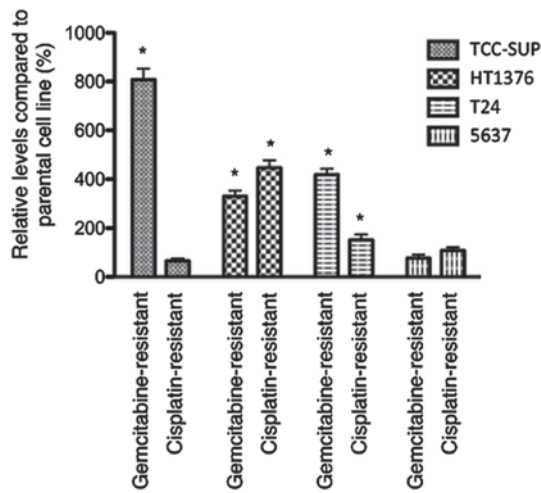


Figure 2. Evaluation of chemotaxis. Serum-induced chemotactic movement was examined using six-well transwell chambers with $8\text{-}\mu\text{m}$ pores. A total of 0.5×10^6 tumor cells per ml were placed in the upper chamber in serum-free medium. The lower chamber contained 10% serum. After 20 h incubation, the upper surface of the transwell membrane was gently wiped with a cotton swab to remove nonmigrating cells. Cells which had moved to the lower surface of the membrane were stained using haematoxylin and counted with a microscope (20x objective). The mean chemotaxis rate was calculated from five different fields. Chemotaxis of parental cell lines was set as 100%. * $P \leq 0.05$ vs. controls.

Immunohistochemical staining of integrin $\beta 1$. In non-malignant tissue samples, integrin $\beta 1$ was only visible in the basal layer of the urothelium. The medium staining score for normal urothelium was between ‘negative’ to ‘weak’ (0.88 ± 0.33). In bladder cancer samples, the mean integrin $\beta 1$ staining score was between ‘moderate’ and ‘strong’ (2.48 ± 1.42). There was no significant difference of integrin $\beta 1$ staining between low

grade tumors or high grade tumors and no significant difference between non-muscle invasive tumors and muscle invasive tumors (Fig. 4).

Influence of blocking integrin $\beta 1$ on adhesion and chemotaxis. Functional blocking of integrin $\beta 1$ resulted in a reduced adhesion in 2 of 4 parental urothelial cancer cell lines (HT1376 and T24). In gemcitabine-resistant cells, adhesion was downregulated in 3 of 4 cell lines (gemcitabine-resistant sublines of HT1376, T24 and TCC-SUP). In cisplatin-resistant cells, adhesion was downregulated in all 4 tested cell lines (Fig. 5). We could not detect an influence on chemotaxis after blocking integrin $\beta 1$ (Fig. 6).

Discussion

In the present study, we used a well-established panel of urothelial cancer cell lines with acquired resistance to gemcitabine or cisplatin, the standard therapeutics for metastatic urothelial cancer of the bladder (2,3). Cell line panels seem to be necessary to reflect the heterogeneity of different patient-derived cancer cell lines. Although the complex scenario of metastatic colonization is not fully understood, there is strong evidence that alterations of tumor-matrix contact are necessary to allow motile crawling into the surrounding tissue (15).

In several cancer cells, resistance to gemcitabine seems to be connected with integrins and associated proteins (16-18). In addition, resistance to cytotoxic drugs and proliferation regulation was shown to be dependent on extracellular matrix proteins (19). In this study, acquisition of resistance to gemcitabine or cisplatin showed a changed adhesive behavior with some resistant sublines showing an enhanced adhesive behavior and other resistant sublines being less adhesive (Fig. 1). In contrast, the influence on chemotaxis was more uniform, with 5 of 8 sublines showing an enhanced chemotaxis and no subline with a significantly diminished chemotaxis after acquisition of resistance (Fig. 2). This is in line with Ploenes *et al* who reported about an enhanced chemotaxis in lung cancer cell lines with an increased chemoresistance (20).

Since integrins seem to be involved in the development of resistance to chemotherapy in bladder cancer (4) and alterations of integrin expression change adhesive and invasive behavior of bladder cancer cells (6), we aimed to elucidate the role of integrins in this context.

Integrin $\alpha 3$ might be involved in resistance acquisition, since it was upregulated in 3 of 4 gemcitabine-resistant and also in 3 of 4 cisplatin-resistant sublines in this study (Fig. 3). Litynska *et al* (21) tried to analyze the role of integrin $\alpha 3$ in bladder cancer by blocking its function. They described that adhesion was up- or downregulated after blocking integrin $\alpha 3$ depending on the tested cell line. The cell line specific effects that can be triggered after acquisition of resistance show the heterogeneity between independent cell lines and underline the importance of using a panel of cell lines for a better interpretation.

It was reported that integrin $\alpha 5$ contributes to a more malignant phenotype in urothelial bladder cancer (22). In our study, this integrin subunit was overexpressed in most of the tested chemoresistant sublines what might underline the more malignant phenotype of the chemoresistant sublines (Fig. 3).

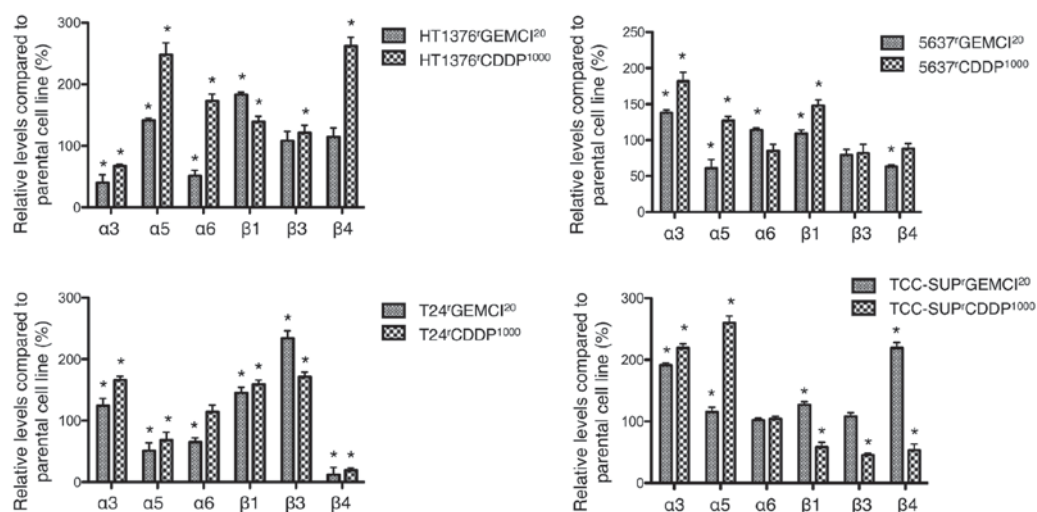


Figure 3. Flow cytometry analysis of integrin surface expression. Cells were washed in blocking solution and stained with specific monoclonal antibodies as listed in materials and methods. Mouse IgG1-PE or mouse IgG2a-PE antibodies were used as isotype controls. Fluorescence was analyzed using a FACScan flow cytometer. The relative fluorescence unit values are given in percentage difference to the parental cell lines. Parental cell lines were set as 100%. One of three independent experiments is shown here. * $P \leq 0.05$ vs. controls.

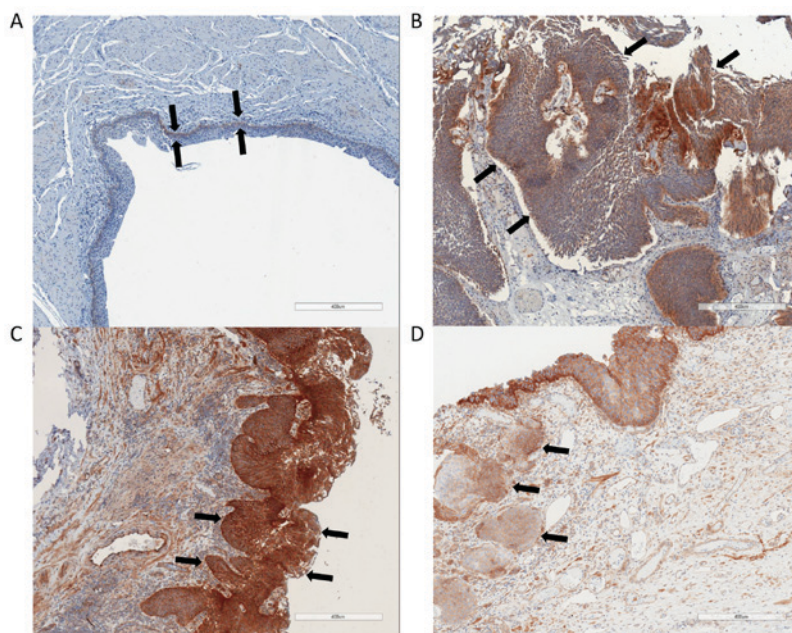


Figure 4. Expression of integrin $\beta 1$ in non-malignant urothelium and urothelial cancer. (A) Normal urothelium showed a negative or a weak expression of integrin $\beta 1$ only in the basal cell layer. A higher integrin $\beta 1$ expression was visible in samples of (B) non-invasive low grade urothelial cancer, (C) non-invasive high grade cancer, and (D) muscle-invasive high grade cancer. Immunostaining of 4 representative tissue samples.

We observed that most chemoresistant sublines showed a diminished expression of integrin $\beta 4$ compared to their parental counterparts (Fig. 3). Therefore, a downregulation of integrin $\beta 4$ could be connected with a more malignant behavior. This is in line with reports that an overexpression of integrin $\beta 4$ inhibits growth and migration in bladder cancer cell lines and plays an anti-tumoral role (23,24).

In all gemcitabine-resistant and in 3 of 4 cisplatin-resistant sublines, surface expressed integrin $\beta 1$ was upregulated compared to parental cell lines (Fig. 3). Since chemotaxis was frequently enhanced after acquisition of resistance, these results might support the conclusions of Chakraborty *et al* (25)

who postulated that blockade of $\beta 1$ -integrin with a specific antibody could result in alteration of multiple signaling pathways related to adhesion and migration. Interestingly, Zhang and coworkers showed that they could reverse chemoresistance to mitomycin *c* by blocking integrin $\beta 1$ (4). Integrin $\beta 1$ was overexpressed in most of the tested sublines and it was reported to contribute to a more malignant phenotype in urothelial bladder cancer (4,25). We could confirm in this study that overexpression of integrin $\beta 1$ is associated with a malignant phenotype since we detected a stronger expression in malignant tissue samples compared to normal urothelium. Nevertheless, there was no different expression comparing low

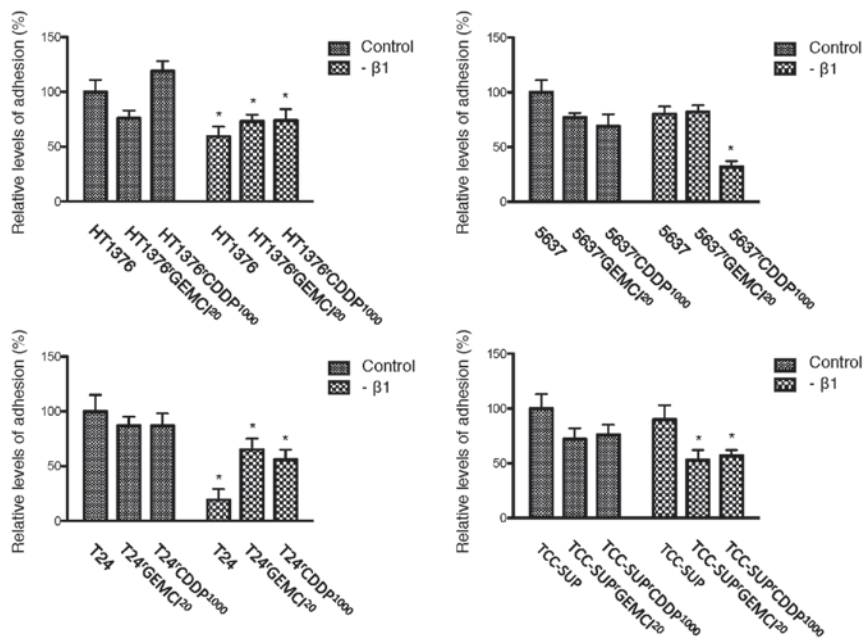


Figure 5. Influence on adhesion after blocking integrin $\beta 1$. Bladder cancer cell lines were preincubated for 60 min with a monoclonal anti-integrin $\beta 1$ blocking antibody (20 mg/l). Controls remained untreated. Cells were then subjected to the adhesion assay as indicated. Untreated parental cells were set as 100%. * $P \leq 0.05$ vs. controls.

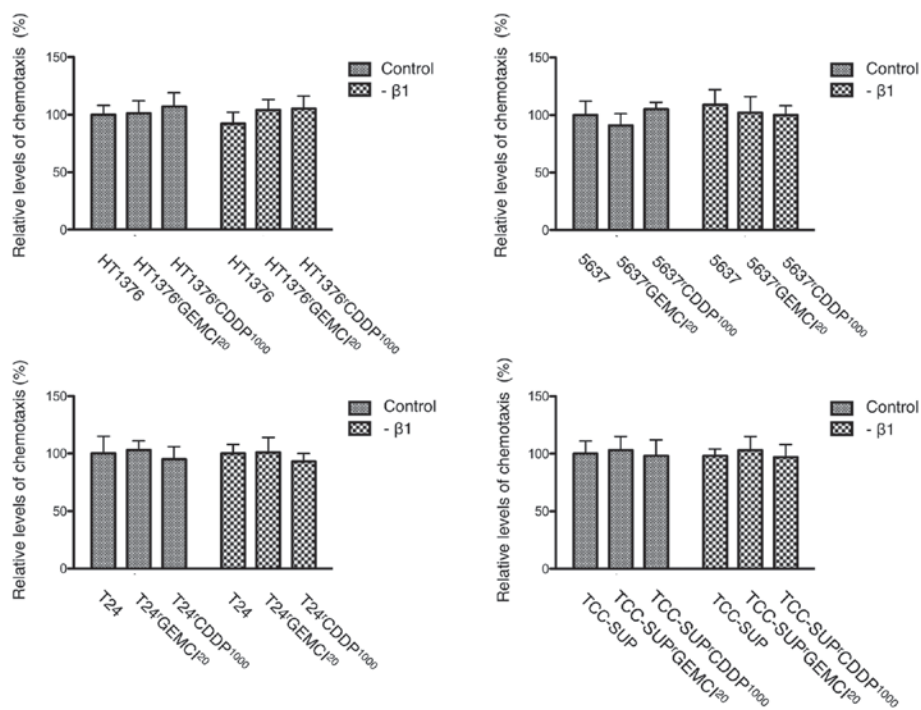


Figure 6. Influence on chemotaxis after blocking integrin $\beta 1$. Bladder cancer cell lines were preincubated for 60 min with a monoclonal anti-integrin $\beta 1$ blocking antibody (20 mg/l). Controls remained untreated. Cells were then subjected to the chemotaxis assay as indicated. Untreated parental cells were set as 100%. * $P \leq 0.05$ vs. controls.

grade with high grade tumors or between non-muscle invasive bladder cancer and muscle invasive bladder cancer (Fig. 4).

To further analyze the role of integrin $\beta 1$, we suppressed the function of integrin $\beta 1$ and measured adhesion and chemotaxis afterwards. There was an influence on adhesion after blocking integrin $\beta 1$ with a reduced adhesion in 2 of 4 parental and 3 of 4 gemcitabine-resistant sublines. In cisplatin-resistant

cells, adhesion was even downregulated in all 4 tested cell lines (Fig. 5).

We could not show an influence on chemotaxis after blocking integrin $\beta 1$ (Fig. 6). If there is no influence on chemotaxis or if the used transwell migration assay is not able to reflect the impact on chemotaxis is not clear. An explanation for the latter might be that effects that influence invasion after

blocking integrin $\beta 1$ are delayed and therefore not detectable with the used transwell migration assay.

Discussing the role of different integrins and the influence on adhesive and invasive behavior, we should be aware that differentially guided adhesive behavior of different tumor cell lines has been previously observed (21). Blocking integrin $\beta 1$ inhibited cell-matrix interactions in HCV29 and BC3726 cell lines, whereas binding of the bladder cancer cell lines T24 and Hu456 was enhanced (21). Each cell line therefore may possess a characteristic receptor set and long-term treatment with chemotherapy may influence integrin subfamilies differently. Therefore systematic analysis of cell line panels is fundamental (6).

We did not investigate the relevance of each integrin member in detail that was used here. To provide a complete picture of the role of integrin subtypes in gemcitabine- and cisplatin-resistant bladder cancer ongoing studies are necessary. Particularly, blocking experiments using integrins $\alpha 3$, $\alpha 5$, and $\beta 4$ would be of interest. In addition, these findings are limited to bladder cancer cell lines and bladder tissue. The role of integrins in chemoresistant bladder cancer should be further evaluated *in vivo* in an animal model.

Overall, evidence is presented here that acquired resistance to gemcitabine or cisplatin frequently enhances chemotaxis, what might be a surrogate for an increased invasive behavior in chemoresistant bladder cancer cell lines. Since overexpression of integrin $\beta 1$ seems to be frequently upregulated in chemoresistant urothelial bladder cancer cell lines, further *in vivo* studies should evaluate downregulation of integrin $\beta 1$ as a potential therapeutic target especially in chemotherapy refractory cases.

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