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Biological function and regulation mechanism of MTA2 expression in bladder cancer

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Bladder cancer that occurs on the bladder mucosa is the most common malignant tumor in the urinary system. Exposure to aromatic amine chemicals and cigarette smoking are the two risk factors which causes bladder cancer. The mechanism of complex molecular signaling during its progress is still not understood clearly. High expression of metastasis associated gene 2 (MTA2) in malignant tumors such as ovarian cancer and hepatocellular carcinoma indicates its possible relationship with migration and invasion. However, expression level and biological function of MTA2 in bladder cancer tissues have not been studied. Here, we investigated the biological function, regulation mechanism and clinical application of metastasis-related genes 2 (MTA2). The expression level of MTA2 in various bladder tumor cells was determined by Western Blot. T24 and EJ cells were selected for *in vitro* study. T24 cells and EJ cells were transfected with viral vector and SiMTA2, respectively, to induce MTA2 over- or low- expression. MTS colorimetric assay was used to explore the proliferative ability of bladder cancer cells *in vitro* under MTA2 over- or low-expression. Matrigel invasion assay and Transwell migration assay were used to detect the invasion and migration of cancer cells *in vitro* under different MTA2 expression conditions. The results of Western Blot assay on bladder cancer cell lines have shown that the MTA2expression level to be significantly lower in T24 cells compared to that of EJ and J82 cells. MTA2 level was significantly higher in MTA2 overexpressed T24 cells than those in the non-transfected and CD511B transfected bladder cancer cells, while the level of MTA2 was much lower in the MTA2-knockdown EJ cells than that in the non-transfected and si-NC transfected. The MTS colorimetric assay significant increase in proliferation of T24 cells with MTA2 overexpression, while the proliferation of EJ cells with MTA2 knockdown was decreased. Results of Transwell migration assay showed that MTA2 overexpression could significantly increase the migration ability of T24 cells, and MTA2 knockdown could significantly reduce the migration ability of EJ cells. Matrigel invasion assay showed that MTA2 overexpression could enhance the invasive ability of T24 cells significantly, while MTA2 knockdown weaken the invasive ability of EJ cells. The expression level of E-cadherin was lower in the T24 cells with MTA2 overexpression than that in the non-transfected group and CD511B transfected group, but the expression level of N-cadherin protein was significantly higher in the T24 cells with MTA2 overexpression compared to the other two groups. The expression level of E-cadherin protein was significantly higher in the EJ cells with MTA2 knockdown than that of the non-transfected group and si-NC transfected group, while the expression level of N-cadherin protein was significantly lower than that in the non-transfected group and si-NC transfected group.

Keywords: Migration, Overexpression, Proliferation, Tumor

Bladder cancer is the most common malignant tumor in the urinary system which occurs on the bladder mucosa, and it is also one of the ten most common tumors in the body[1]. It can occur in almost any age, even in children, and its incidence increases with age. In recent years, with the wide application of chemical products, the increase of tobacco consumption and aged society advancement, the incidence of bladder cancer is rising year by year[2]. According to the recent global cancer statistics 2020, incidence of bladder cancer is 0.573 million, and the mortality 0.213 million[3]. At present, active surgery and adjuvant treatments can improve the symptoms of bladder cancer patients, but it is confirmed that there is a high local relapse rate in patients with bladder cancer underwent surgeries, and distant metastasis and poor prognosis are common[4].

The causes of bladder cancer are complex. Currently, aromatic amine chemicals exposure and cigarette smoking are two risk factors, but people know few about the unpredictable biological behaviour inside the tumor and the mechanism of complex molecular signaling during the process of its progress[5]. Therefore, it is urgent to find a new molecular marker assessing its prognosis, so as to

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diagnose and treat bladder cancer early and effectively. Studies have found that Metastasis-associated gene 2 (MTA2), a member of the MTA family, is highly expressed in malignant tumors such as ovarian cancer and hepatocellular carcinoma, and it is closely related to migration and invasion[6]. There are only few studies available on the expression level and biological function of MTA2 in bladder cancer tissues. Therefore, in this study, we analyzed the expression of MTA2 in bladder cancer tissues and the effect of MTA2 overexpression or knockdown on proliferation, invasion and migration of cancer cells. This investigation has clinical significance of MTA2 expression in bladder cancer tissues, as understanding the mechanism of regulation of the progression of bladder cancer would be useful for early diagnosis of bladder cancer.

Materials and Methods

Materials

T24, EJ and J82 cells, MTA2 interference lentivirus, and plasmids were purchased from Chongqing Youbao Biotechnology Co., Ltd. Embedding machine, electrophoresis tank, paraffin slicer, centrifuge, pipette, optical microscope, gel imaging system, etc., were purchased from Shenyang LongShou Electronic Instrument Co. Ltd. Major reagents *viz*. ethanol, penicillin, hydrogen peroxide, PBS solution, fetal bovine serum, immunohistochemistry kit 4% paraformaldehyde, eosin solution, hematoxylin solution, etc., were purchased from Chongqing Youbao Biotechnology Co., Ltd.

Methods

The EJ, T24, and J82 cell lines were cultured in 10% FBS RPMI-1640 medium and MEM medium respectively, and those in logarithmic growth phase were taken for subsequent experiments[7]. The T24 cells were packaged according to the instructions of the lentiviral packaging kit. Then the T24 cells were infected with bovine clear medium and virus solution (1:1), and the infection was observed. The plasmid DNA was transformed into *E. coli*, cultured overnight at 37°C, and the plasmid was extracted using a plasmid extraction kit. The EJ cells in logarithmic growth phase were transfected with MTA2 gene[8] following the instructions for transfection reagents. MTS colorimetry, Matrigel invasion assay and Transwell migration assay were used to investigate the proliferative, invasive and migration ability of bladder cancer cells *in vitro* under the condition of MTA2 overexpression or knockdown. The expression of MTA2-and EMT-related proteins in bladder cancer cells was detected: Total protein of bladder cancer cells was extracted, and the expression of MTA2 and epithelial transformation (EMT)-related proteins was analyzed by the Western Blot.

Statistical analysis

The data were analyzed using software SPSS 20.0. The count data were expressed by [n(%)], and the comparison between groups was performed by χ^2 test. And the measurement data were expressed by ($\bar{x}\pm s$). Independent sample t-test was used to compare the relevant paired data between groups, and rank sum test was used to compare the non-normal distribution data between groups. The differences were statistically significant when $P<0.05$.

Results

Expression of MTA2 in various cell lines of bladder cancer

The results of Western Blot analysis showed that the expression level of MTA2 was much lower in T24 cells than that in EJ and J82 cells ($P<0.05$), and the expression level was much higher in EJ cells than that of the T24 snf J82 bladder cancer cells ($P<0.05$). T24 and EJ cell lines were selected for subsequent experiments as shown in Fig. 1.

Expression of MTA2 in T24 and EJ cells after MTA2 transfection detected by Western Blot assay

In this experiment, MTA2 gene was transfected into T24 cells. The expression level of MTA2 in T24 cells was much higher in the MTA2-transfacted group than that in the non-transfected group and the CD511B transfected group, and the difference was statistically significant ($P<0.01$). However, there was

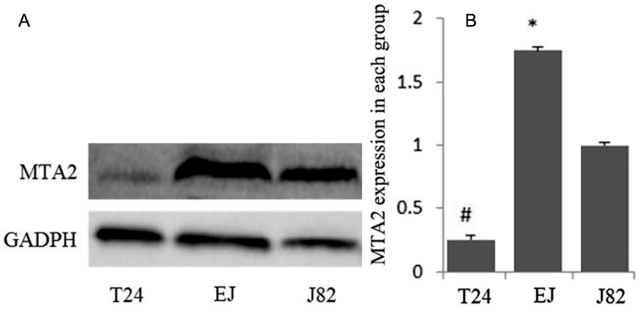

Fig. 1 — (A) Expression of MTA2 in the three cell lines (T24, EJ and J82) of bladder cancer; and (B) the comparison between them. [#compared with EJ and J82 cells, $P<0.05$; and *compared with T24 and J82 cells, $P<0.05$]

no significant difference in the expression level of MTA2 between the non-transfected group and the CD511B transfected group (P >0.05) as shown in Fig. 2.

MTA2 gene of EJ cells was successfully knocked down. The expression level of MTA2 in EJ cells was much lower in the si MTA2 transfected group than that in the non-transfected group and the si-NC transfected group with statistically significant (P <0.01) difference. However, there was no significant difference in the expression level of MTA2 between the non-transfected group and the si-NC transfected group (P >0.05) as shown in Fig. 3.

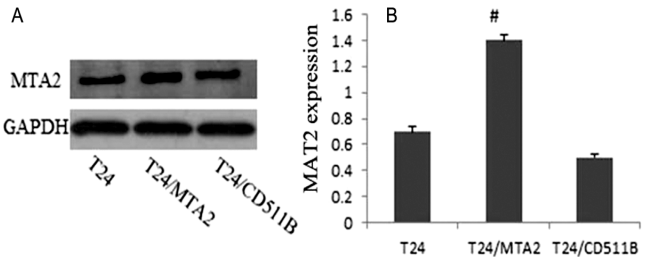

Fig. 2 — (A) Expression of MTA2 in T24 cells after MTA2 transfection detection by Western Blot assay; and (B) the comparison between them. [#compared with the non-transfected group and the CD511B transfected group, P <0.01]

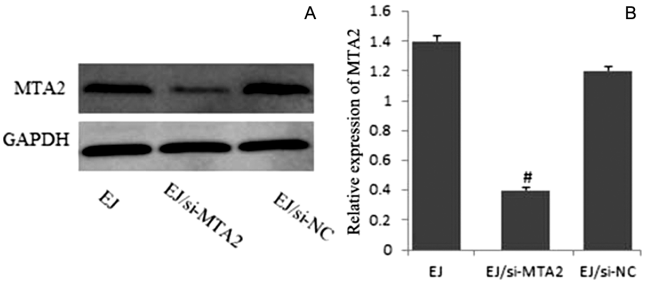

Fig. 3 — (A) Expression of MTA2 in EJ cells after knockdown detected by Western Blot assay; and (B) comparison between the non-transfected group and the si-NC transfected group. [#compared with the non-transfected group and the si-NC transfected group, P <0.01]

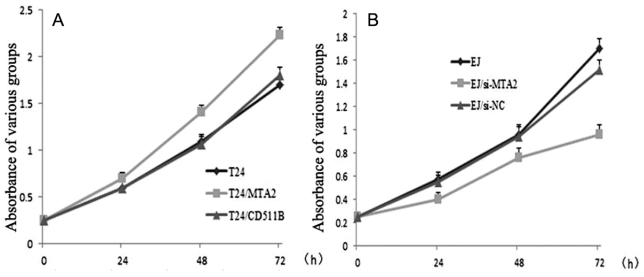

Fig. 4 — Absorbance of over- and low- expressed MTA2 groups; Growth curve of T234 cells (A) with MTA2 overexpression; and (B) with MTA2 knockdown

***In vitro* proliferation of over- and low-expressed MTA2 cells detected by MTS method**

The absorbance values of different groups at various times were analyzed. It was found that the absorbance of T24 cells was much higher in the MTA2 transfected group than that in the non-transfected group and the CD511B transfected group (P <0.01), and the difference was statistically significant Fig 4. There was no significant difference in the absorbance value between the non-transfected group and the CD511B transfected group (P >0.05). The absorbance of EJ cells was much lower in the Si-MTA2 transfection group than that of the non-transfected group and the si-NC transfected group (P <0.01). There was no significant difference in the absorbance values between the non-transfected group and the si-NC transfected group (P >0.05), as shown in Table 1.

***In vitro* migration of over- and low- expressed MTA2 cells**

The results of *in vitro* migration experiments revealed that the number of T24 cells passing through the basement membrane was much more in the MTA2 transfected group than that in the non-transfected group and the CD511B group, and the difference was statistically significant (P <0.01). There was no significant difference in the expression of MTA2 between the non-transfected group and the CD511B transfected group (P >0.05). The number of EJ cells passing through the basement membrane was much less in the Si MTA2 transfected group than that in the non-transfected group and the si-NC transfected group, and the difference was statistically significant (P <0.01). There was no significant difference in the expression of MTA2 between the non-transfected group and the si NC transfected group (P >0.05), as shown in Table 2.

***In vitro* invasion of over- and low- expressed MTA2 cells**

The findings of *in vitro* invasion experiment suggested that the number of T24 cells passing through the basement membrane was significantly higher in the MTA2 transfected group than that in the non-transfected group and the

Table 1 — *In vitro* proliferation of over- and low-expressed MTA2 cells ($\bar{x}$±s)

Group	1d	2d	3d
T24 non-transfected group	0.59±0.41	1.09±0.10	1.70±0.10
T24/MTA2 transfected group	0.70±0.10	1.40±0.11	2.23±0.09
T24/CD511B transfected group	0.59±0.04	1.06±0.14	1.80±0.0.17
EJ non-transfected group	0.57±0.06	0.96±0.01	1.70±0.04
EJ/si-MTA2 transfected group	0.40±0.03	0.76±0.05	0.96±0.06
EJ/si-NC transfected group	0.55±0.02	0.94±0.03	1.52±0.05

Table 2 — *In vitro* migration of over- and low- expressed MTA2 cells ($\bar{x}\pm s$)

Group	T24
T24 non-transfected group	0.59±0.41
T24/MTA2 transfected group	0.70±0.10#
T24/CD511B transfected group	0.59±0.04
Group	EJ
EJnon-transfected group	60.59±1.39
EJ/si-MTA2 transfected group	27.98±3.63#
EJ/si-NC transfected group	57.02±1.68

Notes:# indicates that it was compared with the other two groups, $P<0.01$

Table 3 — *In vitro* invasion of over- and low- expressed MTA2 cells ($\bar{x}\pm s$)

Group	T24
T24 non-transfected group	30.59±6.41
T24/MTA2 transfected group	51.70±3.70#
T24/CD511B transfected group	31.79±4.01
Group	EJ
EJ non-transfected group	63.59±6.39
EJ/si-MTA2 transfected group	23.08±3.63#
EJ/si-NC transfected group	57.82±2.68

Notes:# indicates that it was compared with the other two groups, $P<0.01$

CD511B transfected group, and the difference was statistically significant (P <0.01). However, there was no significant difference in the expression of MTA2 between the non-transfected group and the CD511B transfected group (P >0.05). The number of EJ cells passing through the basement membrane was significantly lower in the Si MTA2 transfected group than that in the non-transfected group, and the difference was statistically significant (P <0.01). There was no significant difference in the expression of MTA2 between the non-transfected group and the si NC transfected group (P >0.05), as shown in Table 3.

Expression of EMT-related proteins in over- and low-expressed MTA2 cells

The expression of E-cadherin in T24 cells was much lower in the MTA2 transfected group than that in the non-transfected group and the CD511B transfected group, while the expression level of N-cadherin was significantly higher in the MTA2 transfected group than that in the non-transfected group and the CD511B transfection group, and the difference was statistically significant (P <0.01).There was no significant difference in the expression levels of the two proteins between the non-transfected group and the CD511B transfected group (P >0.05), as shown in Fig. 5.

The expression level of E-cadherin in EJ cells was much higher in the si MTA2 transfected group than that in the non-transfected group and the si-NC

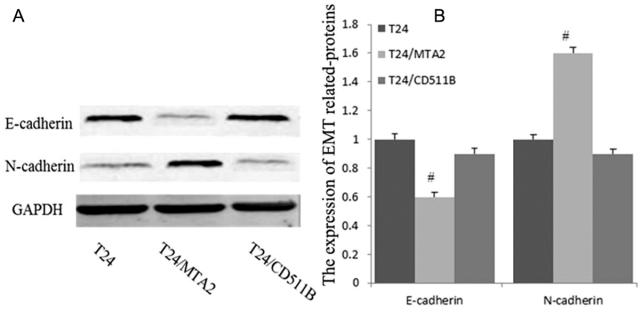

Fig. 5 — (A) Expression of EMT related-proteins in the MTA2 overexpressed group and (B) comparison between them. [#compared with the other two groups, P <0.01]

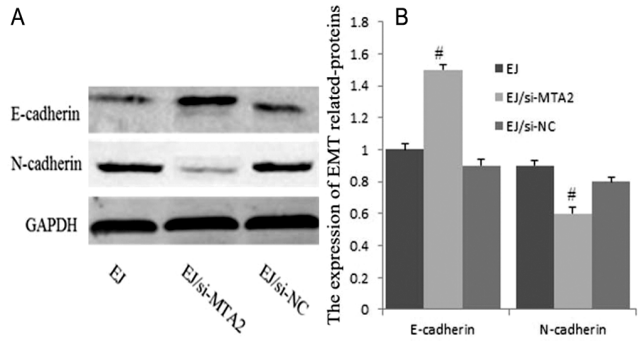

Fig. 6 — (A) Expression of EMT related-proteins in the MTA2 knocked down group and (B) comparison between them. [#compared with the other two groups, P <0.01]

transfected group, while the expression level of N-cadherin was significantly lower in the si MTA2 transfected group than that in the non-transfected group and the si-NC transfected group, and the difference was statistically significant (P <0.01). There was no significant difference in the expression levels of the two proteins between the non-transfected group and the CD511B transfected group (P >0.05), as shown in Fig. 6.

Discussion

As the most common disease that threatens people's lives, malignant tumors, with a persisting growth in incidence and mortality worldwide, have become the first death cause for residents in China[9]. It is currently believed that the main reason that the majority of cancer patients get a poor prognosis is the metastasis and invasiveness of malignant tumors[10]. Therefore, it is urgent to investigate the mechanism of metastasis and invasion of malignant tumor cells. As the most common malignant disease in urinary system diseases, bladder cancer has strong metastatic and invasive abilities, but its specific regulation mechanism is still unclear[11].

MTA2, a compound with 68 amino acids and nuclear small body plastic activity, is a member of MTA family involved in tumor metastasis. It has been found that MTA2 is highly expressed in some malignant tumor tissues, such as colon cancer and esophageal cancer, etc., and is closely related to TNM staging, differentiation of tumor tissues, lymph node metastasis, etc.[12]. Therefore, MTA2 may be involved in the development of bladder cancer. To further explore the relationship between MTA2 and the development of bladder cancer, this study used lentiviral overexpression vector to construct MTA2 overexpressed plasmid to induce T24 cancer cells to overexpress MTA2, and siMTA2 was used transfect EJ cells to knock down MTA2 gene. The findings of MTS method, Colorimetry, Matrigel invasion assay and Transwell migration assay showed that the proliferation, invasion and migration abilities of T24 cells with MTA2 overexpression were much stronger than those of the non-transfected group and the CD511B transfected group, while those of EJ cells with MTA2 gene knock down were inhibited. It is indicated that the expression of MTA2 may be involved in the progression of bladder cancer by affecting the invasion and proliferation of the cancer cells.

Studies have found that decreased adhesion between cells and EMT transition may be an essential cause of cancer metastasis[13]. Hereby, the mechanism that MTA2 regulates the invasion and migration of bladder cancer cells may be associated with EMT transformation. E-cadherin is a calcium-dependent adhesion molecule that aggregates between medicated cells in epithelial tissues[14]. It can maintain the intact structure of epithelial cells, but also plays an important role in keeping tight junctions between cells, so it is an important protein preventing cell proliferation and invasion[15,16]. N-cadherin is mostly located in interstitial cells with few connections and loose structure. Increase of E-cadherin expression and decrease of N-cadherin was used to be important indicators for assessing EMT transition in some studies[17,18]. In order to further explore the relationship between MTA2 and EMT transition. EMT-related proteins were detected after MTA2 overexpression or knockdown in bladder cancer cells in this study. The findings revealed that the expression of E-cadherin expression was elevated after MTA2 knockdown, but the expression of N-cadherin was reduced. In contrast, MTA2 overexpression in bladder cancer cells could reduce E-cadherin and N-cadherin, suggesting that MTA2 promoting the invasion and metastasis of bladder cancer cells may be related to the occurrence of EMT transition.

Conclusion

The observations in this study indicate that MTA2 overexpression could significantly enhance the proliferation, migration and invasiveness of bladder cancer cells, while MTA2 knockdown could reduce their proliferations, migrations and invasion. MTA2 knocked down bladder cancer cells can control the proliferation, migration and invasion of bladder cancer cells, which may be related to the inhibition of EMT. The reduced expression of E-cadherin and N-cadherin suggests EMT transition could possibly influence the MTA2 promoting the invasion and metastasis of bladder cancer cells. However, it requires further studies to understand the whole mechanism behind progression of the bladder cancer so as to develop tools for early diagnosis and better treatment.

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