An Inhibitory Effect of MAD1 on Bladder Tumor Cellular Proliferation in Vivo

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OBJECTIVE To observe the inhibitory effect on bladder tumor proliveration after transfection with the expression plasmid pcDNA3.1(+)/Mad1.
METHODS Bladder tumors were induced in SD rats by intravesical instillation of MNU . The tumor-bearing rats were randomly divided into 3 groups: group A, transfected with pcDNA3.1 (+)/Mad1, group B, transfected with an empty vector and group C, transfected with saline. Rat body weight (RBW), bladder absolute weight (BAW) and bladder relative weight (BRW) were measured and expression levels of Mad1 and TERT were assayed. Flow cytometer analysis was used to observe the effect of Mad1 on the bladder tumors.
RESULTS Comparions of RBW among the 3 groups showed there were no differences (P>0.05). But the BAW and BRW for group A were significantly decreased (P<0.01, P<0.05, respectively) comparded to groups B and C. In group A, the Mad1 mRNA expression level was markedly improved, while the TERT mRNA expression level was decreased. Flow cytometry showed an increase in G_0/G_1-phase cells and a decrease of S-phase cells after transfection with Mad1.
CONCLUSION Over expression of Mad1 can inhibit the cellular proliferation of bladder tumors.

KEYWORDS: bladder tumor, animal model, cell cycle, transfection.

Bladder tumors are one of the most common urogenital system malignant tumors in China. Human telomerase reverse transcriptase (hTERT) messenger RNA has been detected in 95% of bladder tumors using RT-PCR, but it is not found in normal bladder tissue.[1,2] Therefore, TERT-targeted bladder tumor therapeutic approaches are worth exploring.

Mad1(mitotic arrest deficiency-1) is a basic-helix-loop-helix-zipper protein that heterodimerizes with Max in vitro and in vivo.[3,4] The Mad1 protein is synthesized in mammal cells as a 35-kD nuclear phosphoprotein with an extremely short half-life.[5] It is a negative regulatory factor of cell growth that is expressed in differentiating, terminally differentiated and resting cells, and moreover it can inhibit cell cycle progression.[6] Using a model of bladder tumor formation in rats we examined the effect of transfection with a Mad1 expression plasmid on cellular proliferation. Our study has shown that over expression of Mad1 can inhibit proliferatory activity of bladder tumor cells in this rat tumor model.[7]

MATERIALS AND METHODS

Materials

The materials were obtained from the following sources: a. Female

Sprague-Dawley (SD) rats (The Experimental Animal Center of the Third Military Medical University, Chongqing); b. N-methyl-N-nitrosourea (MNU)(Sigma Co., USA); c. Lipofectamine (Invitrogen Co., USA); d. restriction enzyme and enzyme marker determinator (TaKaRa Co., Dalian); e. Propyl pyridine iodide staining solution and RNase A (Dingguo Co., Beijing); f. plasmid extraction reagent kit(V-gene Co., Hangzhou); g. RNA extraction reagent kit (Shanghai Watson Co.); h. Flow cytometer (Becton Dickinson Co., USA); i. Gel analysis system (Bio-Rad Co., USA).

Methods

Construction of animal model

MNU-induced rat bladder tumor model has been previously reported.[8] Brietly, the rats (140~160 g, n =56) were anesthetized using an I.P. injection of 30 mg/kg pentobarbital sodium. Bladder tumors were induced in the rats by intravesical instillation of MNU. The carcinogen (2.5 mg) was instilled into the experimental animals once every three weeks over a 12-week period. Fresh rat urine samples were collected at 12 weeks after the fisrt MNU infusion and centrifuged for 15 min at 1,500 rev/min. Urinary sediment slides were prepared and fixed in 95% methanol for 10 min. The slides were stained with 0.01% acridine orange in 0.1 M phosphate buffer (pH 6.0), followed by phosphate buffer rinses. Finally slides were rinsed in 0.1 M $CaCl_2$ for 30 s and in phosphate buffer 3 times for 15 s. Using fluorescence microscopy the cells were classified into three types: malignant tumor cells (positive), cells suspicious for malignancy and normal cells using. The latter 2 types were categorized as negative. Rats producing positive cells were identified as tumor-bearing animals (n=33) and were randomly divided into 3 groups: transfected with pcDNA3.1(+)/Mad1(group A) and transfected with an empty vector (group B) or injected with saline (group C).

Preparation and purification of plasmids

Recombinant plasmid pcDNA3.1 (+)/Mad1 and an empty vector pcDNA3.1(+) were prepared on a miniscale by an alkaline splitting method and identified with digesting with restriction enzymes Hind III and Xba I. A large quantity of plasmids were extracted according to the reagent kit manual. Concentration of the plasmids was determined by UV and stored at -20℃.

Gene transfecation and preparation of samples

Transfection of the animals with plasmids was performed using Lipofectamine according to the manufacturer instructions. The dosages of plasmids and Lipofectamine were 20 μg and 60 μg respectively. The infusion method of the liposome complex was similar to MNU instillation. Transfections were repeated on the 3rd and 5th day after the first liposome complex infusion. On the 7th day all of the rats were anesthetized , weighed and sacrificed. The whole bladders were resected and weighed and the tumor tissue prepared for further study.

Reverse transcriptase-polymerase chain reaction

RNA was extracted from frozen neoplastic tissues according to the kit protocol and the quality of the isolated RNA verified by agarose gel electrophoresis. A RT-PCR kit was used for gene expression analysis. The cDNA was amplified by PCR using specific oligonucleotide primers. The primer sequences and reaction parameters for different genes are shown in Table 1. PCR fragments were separated by gel electrophoresis (2%) and stained with ethidium bromide. Gel analysis and relative expression levels of Mad 1 and TERT were performed using the Bio-Rad analysis system and Quantity1 software.

Flow cytometric analysis

Single cell suspensions were made from frozen neoplastic tissues and the cell concentration adjusted to 1×

Table 1. Primer Sequence and reaction parameters for different genes

Gene Name	Primer Sequence	Product Length	Reaction Parameters
Mad1	sense: 5'-TGTGAGCGACTCTGACGA-3' antisense: 5'-GTGGGACACTGAAGTTTACG -3'	210bp	94℃, 30 s; 54℃, 30 s; 72℃, 1 min
β-actin	sense: 5'-TTGTAACCTTCTGGGACGATATGG- 3' antisense: 5'-TCATAGGCCCCGGTCACATC -3'	650bp	94℃, 30 s; 55℃, 30 s; 72℃, 1 min
TERT	sense: 5'-AGGTCATTCTTTTACATCAC-3' antisense: 5'-TCATAGGCCCCGGTCACATC -3'	482bp	94℃, 30 s; 53℃, 30 s; 72℃, 1 min

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10^6/L. The cells were fixed in 70% ice-cold ethanol overnight, and then treated with RNase (1mg/ml) for 30 min at 37℃. The pellets were resuspended in PBS containing 50 mg/L of propidium iodide. The staining was performed at 4℃ for at least 30 min and the cell cycle of each sample analysed 3 times by flow cytometry. The results are shown as mean value.

Statistical analysis

Results are presented as mean±SD. Experimental data were analyzed by one-way ANOVA using SAS software. A *P* value <0.05 was considered significant, and a *P* value <0.01 was considered highly significant.

RESULTS

Changes of RBW, BAW and BRW

Values of the RBW, BAW and BRW for the different groups are shown in Table 2. Analysis of the data showed that the RBW of group A was not statistically different from that of groups B and C ($P>0.05$). However the BAW and BRW of group A was significantly lower compared to groups B and C ($P<0.01$ and $P<0.05$, respectively). Our results indicated that over expression of Mad1 inhibited proliferation of bladder tumor cells in group A.

Table 2. Values of RBW, BAW and BRW for the different groups (n=11, $\bar{x}\pm s$)

Group	RBW(g)	BAW(mg)	BRW (mg/g)
A	309 ± 14^a	302 ± 17^b	0.98 ± 0.07^c
B	307 ± 16^d	327 ± 15^d	1.07 ± 0.08^d
C	306 ± 14	323 ± 16	1.06 ± 0.08

$^aP>0.05$ *vs.* group B and group C, $^bP<0.01$ *vs.* group B and group C, $^cP<0.05$ *vs.* group B and group C, $^dP>0.05$ *vs.* group C, BRW(mg/g)= BAW(mg)/RBW(g).

Flow cytometric analysis of the cell cycle

Using flow cytometry we analyzed the effect of Mad1 on bladder tumor cell cycle progression. Fig.1 shows shifts in the cell cycle in different groups. It was found that Mad1 arrested the cell cycle at the G_0/G_1 transition. Compared with groups B and C, the percentage of cells in the G_0/G_1 transition was incresed significantly in group A ($P<0.01$) and the percentage of cells in the S phase was decresed signifcantly ($P<0.01$). In addition, no significant changes in G_2/M transition were observed among the 3 groups ($P>0.05$). These results indicated that proliferation of cells in the bladder tumor was reduced in group A.

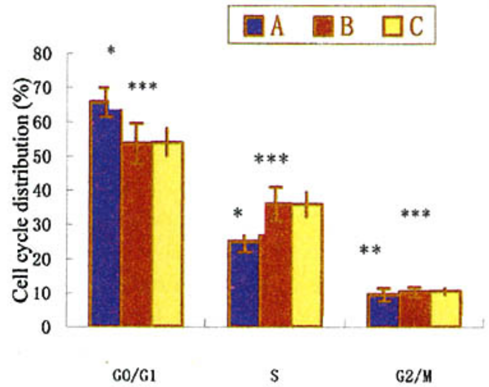

Fig.1. Comparsion cell cycles of different groups (%, $\bar{x}\pm s$). *$P<0.01$ *vs.* groups B and C, **$P>0.05$ *vs.* groups B and C, *** $P>0.05$ *vs.* group C.

Mad1 and TERT mRNA expression levels

As depicted in Figs.2A and 2B, RT-PCR showed that the relative expression level of Mad1 mRNA to β-actin mRNA in group A (83.1 ± 10.1)% was greatly increased compared to groups B (30.4 ± 8.1)% and C (33.6 ± 7.0)% ($P<0.01$), while the relative expression level of TERT mRNA to β-actin mRNA in group A (56.1 ± 4.1)% was greatly decreased compared to groups B (72.4 ± 4.8)% and C (69.6 ± 2.7)% ($P<0.01$). Our study suggested that the exogenous Mad1 gene had been successfully transfected into the bladder tumor cells of group A and effectively down-regulated the expression of the TERT gene.

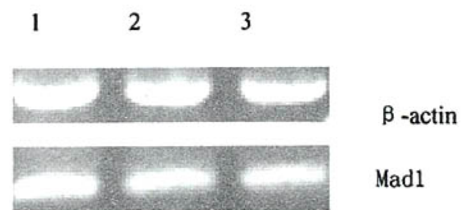

Fig.2A. Mad1 mRNA expression in different groups after transfection. 1: group A; 2: group B; 3: group C.

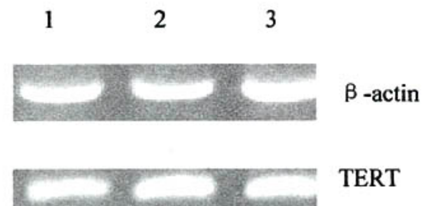

Fig.2B. TERT mRNA expression in different groups after transfection. 1: group A; 2: group B; 3: group C.

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DISCUSSION

Telomerase is a specialized reverse transcriptase responsible for synthesizing telomeric DNA at the ends of chromosomes. The subunits comprising the human telomerase complex have been identified: telomerase RNA (TR), telomerase-associated protein 1 (TEP1), and telomerase reverse transcriptase (TERT).[9] Recently, a marked correlation between expression of TERT mRNA and presence of telomerase activity has been documented, suggesting that the overall regulation of telomerase activity is achieved, at least in part, through rate limiting mechanisms governing the expression of the hTERT gene.[10] The expression of TERT is specifically associated with more than 90% of urothelial cancers.[11] Measurement of hTERT mRNA expression using a quantitative RT-PCR assay demonstrated that the levels of hTERT mRNA expression were significantly associated with bladder cancer grade and stage.[12] Inhibition of telomerase and TERT activity may open up a new strategy for anti-bladder cancer therapy.

The Myc/Max/Mad family of transcription factors plays a fundamental role in the regulation of cell proliferation, oncogenic transformation, and cell differentiation.[13] Mad family proteins are mainly expressed at distinct stages of development and differentiation, almost all of which are associated with growth arrest. In contrast, Myc proteins are expressed in all dividing cells and repressed under virtually all growth-arrest conditions. Histone acetylation has been implicated in Myc-mediated transactivation of target genes, whereas deacetylation has been shown to be important for Mad1 repression.[14] These alterations were consistent with changes in the levels of TERT expression. The Mad1/Max dimer recruits mSin3A, mSin3B, HDACs, and corepressors, which in turn results in increased deacetylation of histones and repression of TERT gene transcription. On the other hand the c-Myc/Max dimer recruits histone acetyltransferases, which in turn results in increased acetylation of histones and activation of TERT gene transcription.[15,16]

In our study, the inhibitory effcet on proliferation of bladder tumor cells in situ was accompanided with TERT down-regulation in the Mad1-transfected group, indicating that telomerase and TERT-targeted bladder tumor therapeutic approaches are feasible. The expression of the Mad1 protein is tightly regulated during cell cycle progression and in response to induced differentiation in various cell lines.[17,18] Our research showed that the percent of bladder tumor cells in the G_1 phase was increased and the percent of bladder tumor cells in the S phase was decreased after transfection with Mad1. In addition, the proliferative activity of bladder tumor cells transfected with the Mad1 gene was significantly inhibited when compared with the proliferative activity of bladder tumor cells treated with an empty vector or saline. It can be inferred that the main effect of the Mad1 gene is to specifically block the G_1 to S progrestion of the cell cycling, resulting in inhibition of growth of the bladder tumors.

On the whole, our reseach has demonstrated that over expression of the Mad1 gene can inhibit the proliferation of bladder tumor cells in situ, and it may be a suitable candidate for bladder tumor gene therapy. It has been demoustrated that inhibition of telomerase and TERT activity in malignant tumor cells can increase their sensitivity to chemotherapy.[19,20] Though over expression of Mad1 may not revert the malignant phenotype of bladder tumors completely, transfection with the Mad1 gene is a effective method for repressing the expression of the TERT gene. In other words, the significance of over-exprssion of Mad1 gene lies not only in its anti-bladder tumor effects, but also in overcoming resistance of bladder tumors to chemotherapy.

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