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Brief communication (Original)

Establishment and characterization of urine-resistance cell sub-strain of human bladder cancer

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Background: Metastasis of tumor implantation includes a series of processes from detachment from the primary tumor to formation of the implanted metastase. Tumor cells survival in urine is a necessary condition for metastasis. Adaptation to urine is essential for this.
Objective: Establish a urine-resistant cell sub-strain of human bladder cancer cell line (ET cell lines), and study different characteristics compared to parent cells.
Methods: EJ cell lines were cultured in nutrient medium. Urine-resistance cell sub-strain (EJ-U) was harvested after prolonged culture by gradually increasing the concentration of urine. Gen chip was used to detect the genome series of EJ and EJ-U and to analyze the difference of gene expression.
Results: EJ-U in urine had a higher survival rate after 24 hours in urine compared with EJ. The EJ-U had almost the same growth velocity with EJ, and they had the analogous growth curves. The time-duration for EJ-U to survive was longer than EJ in urine. In gene ontology analysis, 272 significant different genes were found.
Conclusion: EJ-U cell sub-strain was more adaptable than its parent cell lines EJ. The different genes may explain the reason why bladder cancer cells could survive for a long time in urine.

Keywords: Bladder cancer, cell sub-strain, gene chip, urine, urine-resistance

Bladder cancer is a common cancer [1]. The main pathological features are multiple recurrent implantation metastases in bladder [2]. The process of metastasis includes a series of processes. These are detachment from the primary tumor, adherence, and invasion at new sites. Another important process is survival of tumor cells in urine. Therefore, tumor cell survival in urine is a necessary condition for bladder cancer metastasis. In fact, Masaru et al. [3] found that fresh human urine could stimulate proliferation of human bladder cancer cell lines. Stein et al. [4] reported that urine could induce urinary bladder epithelial cells to be programmed into cell death in vitro.

According to Parris [5], various cell subsets have different biological and metastatic behaviors in the same tumor. Consequently, establishing bladder cancer cells with high adaptation in urine is one important process. Such established cells may become an ideal material for studies of implantation metastasis of bladder tumor. Human bladder cancer lines (EJ cell lines) were recognized by Lin CW et al. [6]. They may grow rapidly in culture with a doubling time of 16 hours and high migration activity [7]. In this study, we attempted to establish a urine-resistance cell sub-strain of ET cell lines, and to analyze their different characteristics compared with its parent cells. The analysis of the difference may reveal the physiology of bladder tumor cells prolonged survival in urine.

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Materials and methods

This study was approved by the Ethics Committee of Kunming Medical College. Normal male urine was collected, and filtered for sterilization in super-clean bench. We mixed medium with the urine, and prepared three concentrations (a quarter, half, and three-quarters) of the mixed media. We then placed three mixed media and urine into incubator with the temperature 37°C, CO_2 of 25% volume fraction and

95% of humidity content. Osmotic pressure of urine was measured two, eight, 12, 24, 48, and 72 hours later. Parent EJ cells were grown in medium supplemented with 10% fetal calf serum. The cells grew in culture as adherent cells. The cells were trypsin-digested at the logarithmic growth phase for the preparation of cell suspension. Cell concentration was adjusted to $1x10^6$/mL, and was inoculated in a 50 mL glass bottle. When cell growth was to 80% confluence, we added the mixed media where the proportion of urine was a quarter.

After 24 hours cultivation under normal condition, we discarded the mixed media and the death cells floating in the mixed medium. We then added the new medium. The medium was changed one to two days later. When the surviving cells resumed growth and proliferation, we adjusted the cell concentration to $1x10^6$/mL and cultured the cells in another 50 mL glass bottle. This was repeated until the parent EJ cells were no longer included or by a small amount death in the environment of the mixed media. Then, we added the mixed media from a quarter to three quarters in the proportion of urine. At last, we obtained a 100% urine concentration. When our induced EJ cells had no (or less than 0.1%) death cells after 24 hours in the 100% urine concentration, and the cell lines passed 16 generations stable, we called the cells urine-resistance cell sub-strain (EJ-U for shortness).

We then compared biological characteristics between EJ and EJ-U, and analyzed the difference of gene expression.

Statistical analysis

Groups were analyzed using Student t-test. A p-value of <0.05 was considered significantly different.

Results

The growth state and basic properties of EJ-U cells

The biochemical detection and osmotic pressure test showed that three different concentrations of mixed media, urine, and media were not statistically different. The urine-resistance EJ-U cells and the parent cells EJ had the same growth velocity, and they showed the similar growth curves.

The urine-resistance cell sub-strain EJ-U cultured in 100% urine could survive and return to normal proliferation after 24 hours and number increased to 0.001. **Figure 1** shows microscopic images to demonstrate the survival of the EJ cells and EJ-U cells after 24 and 48 hours in the urine.

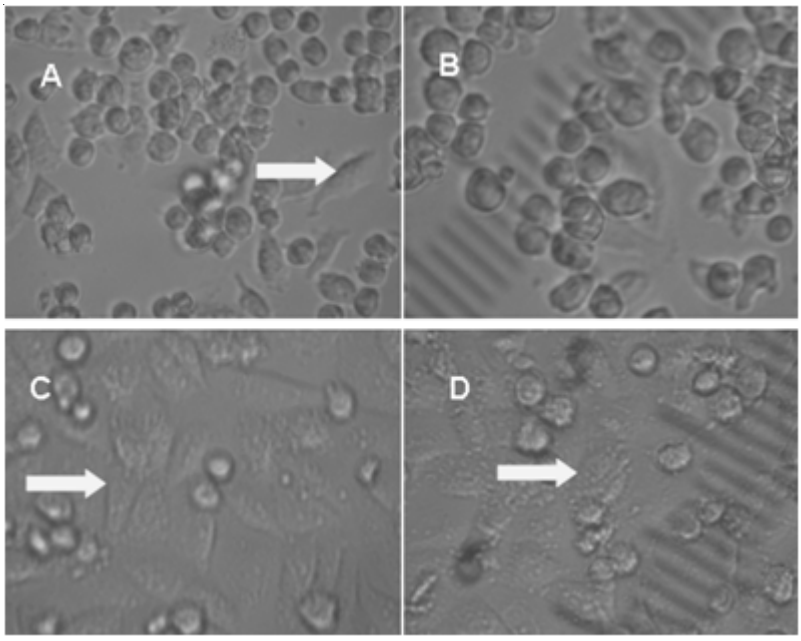

Figure 1. Microscopic images showing the survival of the EJ cells and EJ-U cells in the urine. **A:** After 24 hours in the urine, a small part of the EJ cells survived, as shown by the arrow (x200). **B:** After 48 hours in the urine, there was no EJ cells survival (x200). **C:** After 24 hours in the urine, most of the EJ-U cells survived, as shown by the arrow (x200). **D:** After 48 hours in the urine, still part of the EJ-U cells survived, as shown by the arrow (x200).

By using tetrazolium bromide (MTT), we compared the survival time in urine, 1640 serum-free liquid, and phosphate buffered solution (PBS) between EJ-U and EJ. The results showed that EJ-U survival time was much longer than EJ in urine (p <0.05).

There were no difference of the survival time between EJ-U cells and EJ cells in 1640 serum-free liquid (p >0.05) and PBS (p >0.05). **Figure 2** shows the cell cycle of EJ-U cells and EJ cells using flow cytometry. For the EJ-U cells, pre-synthetic gap 1 period (G1) was 54.5%, pre-synthetic gap 2 period (G2) was 17.5%, phase (S) was 28.0%, and proliferation index (PI) was 45.5% under the basic culture medium. For the EJ cells, G1 was 78.2%, G2 was 10.8%, S was 11.0%, and PI was 21.8%.

The difference of gene expression between EJ-U and EJ

By using the gene chip technology, we compared the genetic differences between EJ-U and EJ. **Table 1** shows different expression up-regulated genes between EJ and EJ-U cells. In gene ontology analysis, there were 151, 79, and 42 significantly different expressed genes at the views of biological process, cellular localization, and molecules, respectively.

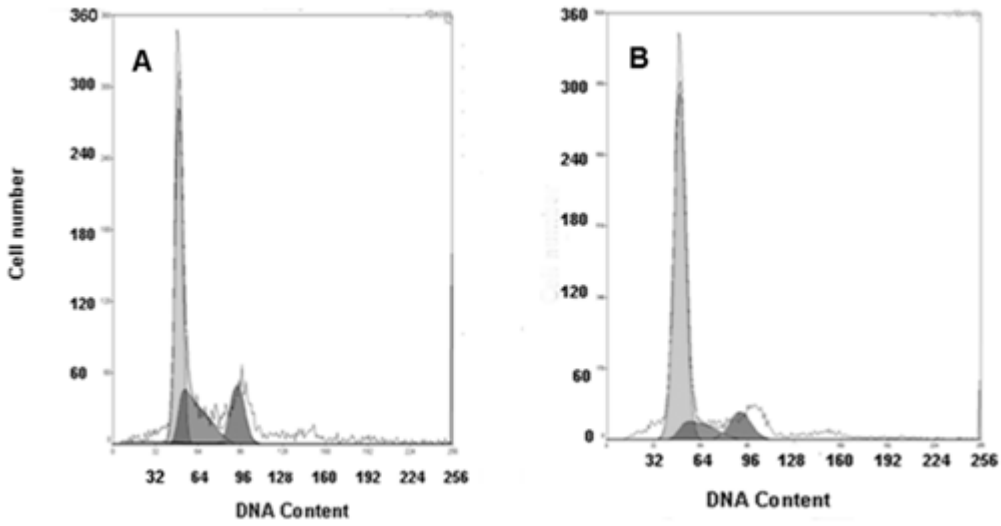

Figure 2. The cell cycle diagrams of the EJ cells (**A**) and EJ-U cells (**B**).

Table 1. Different expression of up-regulated genes between EJ and EJ-U cells (p <0.01).

Entrez gene ID	Official symbol	Official full name
241	ALOX5AP	arachidonate 5-lipoxygenase-activating protein
1293	COL6A3	collagen, type VI, alpha 3
1437	CSF2	colony stimulating factor 2 (granulocyte-macrophage)
2012	EMP1	epithelial membrane protein 1
8337	HIST2H2AA	histone cluster 2, H2aa3
3553	IL1B	interleukin 1, beta
3569	IL6	interleukin 6 (interferon, beta 2)
7805	LAPTM5	lysosomal protein transmembrane 5
283316	M160	CD163 molecule-like 1
11343	MGLL	monoglyceride lipase
9788	MTSS1	metastasis suppressor 1
8829	NRP1	neuropilin 1
6284	S100A13	S100 calcium binding protein A13
6447	SCG5	secretogranin V (7B2 protein)
6590	SLPI	secretory leukocyte peptidase inhibitor
8848	TSC22D1	TSC22 domain family, member 1

Table 2. Different expression down-regulated genes between EJ and EJ-U cells ($p < 0.01$).

Entrez gene ID	Official symbol	Official full name
1013	CDH15	cadherin 15, type 1, M-cadherin (myotubule)
123	ADFP	perilipin 2
1675	CFD	complement factor D (adipsin)
27075	TSPAN13	tetraspanin 13
23753	SDF2L1	stromal cell-derived factor 2-like 1
757	TMEM50B	tetraspanin 13
1000	CDH2	cadherin 2, type 1, N-cadherin (neuronal)
5569	PKIA	protein kinase (cAMP-dependent, catalytic) inhibitor alpha
5413	5-Sep	septin 5
10725	NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive
9601	PDIA4	protein disulfide isomerase family A, member 4
79174	CRELD2	cysteine-rich with EGF-like domains 2
811	CALR	calreticulin
2888	GRB14	growth factor receptor-bound protein 14

Discussion

Fidler and Kripke [8] proposed tumor cell population heterogeneity theoretically and experimentally. According to Bai et al. [9], the same tumor may be isolated with cell lines of different potentials. EJ cells are adherent growth cell and triangular in the light microscope, while the urine-resistance of EJ cells is inhomogeneous. Therefore, we started our experiment from a low urine concentration. By adding urine intermittently, we increased urine concentration and repeated screening. Finally, we obtained the cell sub-strains EJ-U. The EJ- sub-strains could survive for 24 hours or longer period in urine. When cultured in pure urine for 24 hours, very small number of the parent cells EJ survived, and only a few parent cells in the urine-resistant cells were primary. EJ-U survival-time was much longer than EJ in urine ($p < 0.05$). This suggests that in EJ cells, multiple higher urine-resistant cell sub-lines could be isolated after urine sensitization induction culture.

We found different biological characteristics between EJ-U and EJ. Our cell count method showed that urine-resistance EJ-U cells increased at 24 hours in pure urine, compared to EJ cells. The present MTT method showed that EJ-U survived longer than EJ cells in urine. We suggest that the cell morphological changes lead to decrease cell membrane surface area. These changes might bring the cells into less-contact with the urine. This may be one of the reasons why EJ-U cells can survive longer in the urine.

Gene chip is a high-throughput, rapid, efficient, and automated platform for genetic analysis [10]. Since gene chip can detect the expression of thousands of genes in parallel, it can be used to identify the target interventions to provide clues and to monitor gene expression after drug treatment. Our gene chip experiment showed that out of 151 significant differentially expressed genes at the biological process viewpoint, most were involved in metabolic and apoptosis. Out of 79 significant differentially expressed genes at the cellular localization viewpoint, most were genes involved in cell membrane. Out of 42 significant differentially expressed genes at the molecular viewpoint, most were genes involved in enzyme activity and nucleotide synthesis.

In conclusion, EJ-U cell sub-strain was more adaptable than its parent cell lines EJ. The different genes may provide a clue to explain the reason why bladder cancer cells could survive for a long period in urine.

The authors have no conflict of interest to report.

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