Original Article

Analysis of mRNA profile in different strategy of bladder cancer carcinoma by deep sequencing

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Abstract: Harboring gene mutations is an essential step that causes the onset and progression of tumors, and diverse phenotypes of tumors. Analyzing the bladder cells with different malignance is a significant method seeking possible genes that lead to tumor development, malignant transformation and migrating differentiation. This study aimed to find the key genes controlling the progression of bladder cancer. We performed a large-scale RNA sequencing on SV-HUV-1, RT4 (low grade bladder cancer), T24 (malignant bladder cancer), and 5637 (malignant bladder cancer) cells. We screened the differentially expressed genes (DEGs) in comparison pairwise between SV-HUV-1, RT4 and T24; as well as compared DEGs between SV-HUV-1, RT4 and 5637. Further, we performed trend analysis approach on DEGs with k-mean cluster. Gene ontology and pathway analysis were performed on DEGs to observe the biological functions. The molecular signature of SV-HUV-1-RT4-5637 was found to be distinct to that of SV-HUV-1-RT4-T24. In SV-HUV-1-RT4-5637, the majority downregulated DEGs were enriched in Notch pathway were greatly decreased. In the SV-HUV-1-RT4-T24, profile3 gene mainly enriched in the biological processes of fat metabolism, the gradual increased DEGs in SV-HUV-1-RT4-T24 enriched in the mTOR and HIF-a pathway. In summary, this study revealed that Notch pathway played pivotal roles in the formation process of bladder cancer and may be a potential target for the treatment of bladder cancer; reveal molecular mechanisms of different biological characteristics of various bladder cancers.

Keywords: Bladder cancer, RNA-seq, notch pathway, differentially expressed genes, fatty acid metabolic process

Introduction

Bladder cancer is one of the most common malignancies, which can threaten to human health and has raised the fourth position among malignancies in western countries [1, 2]. When the tumor developed clinically detectable, tumor genome would acquire numerous mutations [3], the genome mutation would lead to shift of the whole cell gene expression structure, and these abnormal expressions of genes often promoted the incidence and development, malignant transformation, invasion and differentiation of the tumor, as well as determined the different biological traits such as different malignancy, sensitivity to drugs, and clinical prognosis. Analysis of the changes of gene expression profiles in different stages of bladder cancer cells progress plays an important role in tumor biology. The existing researches mainly focus on the culture of tumor lines, to study the function of individual genes [4]. This study would analyze expression profiles of bladder cancer cells with diverse malignance through RNA sequencing, attempted to changes of gene expression as a whole in bladder cancer progression and find the key pathway of tumor formation.

Notch signaling pathway, is a novel tumor suppressor which has been firstly found in mouse skin cancer [4] and human keratinocytes [5]. Previous evidences showed that Notch pathway was involved in mediating signaling in myeloid [6], and was associated with the development of bladder cancer, lung epithelial adenocarci-
noma, and head and neck cancer [7-9]. Due to the diversity of Notch signaling pathway functions, the opinions of its function in bladder cancer was diverse, suggesting it might be a cancer-promoting gene while might also have anticancer activity [10]. Under different circumstance, it plays a distinct role, positive or negative impact on the proliferation and differentiation of cells and apoptosis [10]. Therefore, exploring its role in different states of bladder cancer is necessary.

Abnormal metabolic pathway is an important symbol of a tumor [11]. Oncogenes and tumor suppressor genes can normally regulate metabolic pathways, and different gene mutations of a tumor have different metabolic states [12, 13]. We analyzed the abnormal gene in SV-HUV-1-RT4-T24 progression and found several abnormal expressions involved in multiple metabolic pathways, especially a large number of mTOR and HIF-a pathway expression in advanced bladder cancer.

Our research addressed to various stages of differentially expressed genes (DEGs) in progress and development of bladder cancer cells, combining trend analysis with functional assays, to explain the role of genes differentially expressed in the development of tumorigenesis. Through large-scale sequencing, we first analyzed the trend of differential expression gene SV-HUV-1, RT4 and 5637. GO function and pathway analysis showed that there was little difference in expression of SV-HUV-1, RT4, but a steep increase of DEGs in 5637 which mainly concentrated in the Notch signaling pathway. The escalating SV-HUV-1-RT4-T24 gene mainly concentrated in mTOR and HIF-1 pathway. Finally, we demonstrated the variation of gene expression in related pathways by RT-PCR.

Materials and methods

Cell lines

Bladder cell lines SV-HUV-1, RT4, 5637, and T24 were all purchased from ATCC and cultured in RPMI-1640 medium containing 10% fetal serum bovine (FBS) at 37°C in an incubator with 5% CO2. When the mixture confluent reaches to 80%, 10^7 tumor cells were collected for the application of RNA sequencing.

RNA extraction and sequencing

Different bladder cancer cells were counted in 3 x 10^6 and cellular RNA was extracted by TRziol (Invitrogen Life Technologies, Carlsbad, CA, USA). The amount and integrity of RNA were detected using ND-1000 spectrophotometer (Nanodrop) and Agilent Bioanalyzer 2100. Illumina RNA-Seq chips were the platform for hybridization and sequencing.

Differentially expressed genes screening

The DEGs in different groups were selected using bioinformatics methods. The genes gradually reducing with the increase of malignancy were assorted as group 0; those RT4 reduced, 5637 or T24 had little difference to RT4 as group 1; those RT4 was lower than SV-HUV-1, 5637 or T24 had little difference to SV-HUV-1 as group 2; those RT4 had little difference to SV-HUV-1, 5637 or T24 decreased significantly as group 3; those RT4 had little difference to SV-HUV-1, 5637 or T24 significantly increase as group 4; those RT4 was higher than SV-HUV-1, 5637 or T24 had little difference to SV-HUV-1 as group 5; those RT4 increased, 5637 or T24 had little difference to RT4 as group 6; those RT4 expression was higher than SV-HUV-1, 5637 or T24 was higher than RT4 as group 7.

Gene ontology (GO) database and DAVID web tool v6.7 were employed to analyze the biological functions of selected DEGs [14, 15] in each group. False discovery rate (FDR) < 0.2 was considered as significantly different.

RT-PCR analysis

RT-PCR analysis was used to analyze the expressions of DEGs to confirm the changes of mRNA in different tumor cells. RNA in tumor cells was extracted, reverse transcribed, then quantified with specific primers PCR. RT-PCR was performed under Real-Time PCR System with cycling conditions of 94°C for 10 min, followed by 94°C for 15 s and 58°C for 30 s, 72°C for 20 s, 45 cycles in total. The relative expression levels of mRNA were calculated by 2^-ΔΔCT. Specific primer sequences are as follow.

Results

DEGs in bladder epithelial cells

We would first perform RNA sequencing on four different urothelial cells SV-HUV-1, RT4, 5637,
T24 to analyze the changes of their expression profiles. SV-HUV-1 was considered as normal bladder epithelial cell line, RT4 was low-grade urothelial or benign cell, 5637 and T24 were two different high-grade bladder cancer cell lines. Sequenced genes were comprehensive, including expression of non-coding RNA (Table S1).

Cluster analysis of DEGs

We compared SV-HUV-1-RT4-5637 DEGs pairwise (Tables S2-S4). DEGs were divided into 8 groups; grouping method is seen in ‘approach’. We obtained 336 DEGs in group 0, 1,165 DEGs in group 1, 640 DEGs in Group 2, 288 DEGs in group 3, 702 DEGs in group 4, 768 DEGs in group 5, 366 DEGs in group 6, 350 DEGs in group 7 (Figure 1A and Table S5).

DEGs in SV-HUV-1-RT4-T24 were clustered (Tables S6-S8), and DEGs were divided into 8 groups. We obtained 188 DEGs in group 0, 533 in group 1, 143 in Group 2, 124 in group 3, 345 in group 4, 369 in group 5, 111 in group 6, 131 in group 7 (Figure 1B and Table S9).

Functional analysis of DEGs

In order to further explore the function of each differential gene, we would carry out the GO data analysis on DEGs in each group, as shown in the results, DEGs in group 0 in SV-HUV-1-
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RT4-5637 were mainly concentrated in gene expression, DNA damage repair pathways, RNA metabolic pathways; group 1 mainly concentrated in the cell cycle and cell division; group 2 mainly concentrated in protein translocation and chromatin regulation; group 3 concentrated in the gene expression; Notch receptors in group 4 processed the highest score; group 5 concentrated in the apoptotic pathway; group 6 concentrated in JNKK activation and cell adhesion; group 7 was IL-1 receptor signal and ribosome biogenesis (Figure 2A).

Meanwhile, we conducted GO gene differential expression data analysis on each group of SV-HUV-1-RT4-T24, as shown in the results, DEGs in group 0 in SV-HUV-1-RT4-5637 mainly concentrated in mitochondrial ATP synthesis and respiratory oxidation of anion chain; group 1 mainly concentrated in mitosis; group 2 mainly concentrated in mitosis and cell differentiation; group 3 concentrated in fatty acid metabolism; group 4 concentrated in cell proliferation regulation; group 5 concentrated in lipid metabolism and interferon inducing cell signaling pathways; group 6 concentrated in polyamine catabolism; group 7 concentrated in platelet activation and insulin receptor signaling pathway (Figure 2B).

Pathway analysis of DEGs

In order to further understand the pathways of DEGs in each group, we performed gene pathway analysis for SV-HUV-1-RT4-5637 and SV-HUV-1-RT4-T24 (Figure 3A and 3B). Through analysis, we found that each group of genes were consistent with GO analysis pathway in profile 4 in SV-HUV-1-RT4-5637, and genes causing 5637 increase were mostly concentrated in the Notch signaling pathway. We found that those genes gradually increased in profile 7 of the SV-HUV-1-RT4-T24 were mainly concentrated in HIF-1 and mTOR, which all prompted that T24 and metabolism genes had some relevance.

RT-PCR analysis

RT-PCR was performed to verify the DEGs in RNA-seq. In order to further validate the results of our RNA-seq, we cultured the SV-HUV-1, RT4, 5637 and T24 cells, and extracted their RNA, and expression levels of these genes were validated in messenger RNA level. Results showed that Jagged 1 (JAG1), Histone Deacetylase 5 (HDAC5), JAG2, Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ) was significantly increased in Notch signaling pathway in SV-HUV-1-RT4-5637. In SV-HUV-1, RT4, 5637, we detected genes EIF4 and EBP1 in HIF-a pathway and VEGFB in mTOR pathway. With the increasing degree of malignancy of bladder cancer, the expression of these genes gradually increased.

Discussion

It has been widely known that genomic mutations leads to the expression structure mutations in bladder cancer, and heterogeneity is an important feature of bladder cancer [15]. mRNA and DNA chip technology [16] have been used to detect abnormal expression of genes in bladder cancer cells, related prognosis marks [17-19], and parsed the biological nature of bladder cancer [20, 21]. However, due to the self-limitation of chip technology such as narrow coverage [22], more and more studies tended to use second-generation sequencing technology for the changes of expression profiles in bladder cancer [23]. The next generation RNA sequencing technology not on lycan detects mRNA but also discover non-coding RNA. In this study, we first used the next generation sequence to analyze the gene expression profiles in bladder cancer cell lines of different degree of malignancy, attempted to discover the key cancer progression pathway and molecules through trend analysis and GO, pathway analysis and other functions.

In this study, DEGs in SV-HUV-1-RT4-5637 were initial elevated, and several DEGs were involved in the Notch signaling pathway. Notch played an important role in the development of bladder cancer and suppressed tumor progression [24, 25]. We found that the expression of JAG1, HDAC5, JAG2 and RBPJ were significantly increased, which were coincidence with former evidences of these genes of JAG1 [26], HDAC5 [27], JAG2 [28], RBPJ [29] in other tumors such as breast cancer, liver cancer, lung cancer. However, roles of these DEGs on effecting bladder cancer still remain unclear. HIF-a and mTOR played a crucial role in the process of occurring and development of bladder cancer [30], promoted energy metabolism and tumor angiogenesis [31]. We found EIF4EBP1 and VEGFB had
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A

GO-Analysis_BP

B

GO-Analysis_BP

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Figure 2. Gene ontology analysis of the screened DEGs in different group. A: The significant GO terms of DEGs in SV-HUV-1-RT4-T24 group; B: The significant GO terms of DEGs in SV-HUV-1-RT4-5637 group.
Figure 3. Pathway analysis of the screened DEGs in each group. A: The enriched significant pathways of DEGs in SV-HUV-1-RT4-T24 group; B: The enriched significant pathways of DEGs in SV-HUV-1-RT4-5637 group.
significantly increased expression in the SV-HUV-1-RT4-T24, suggesting that these developments might involve in the development of bladder cancer.

In future experiments, we will detect the changes of differential expression genes on genome and protein level, combining with genome sequencing and protein detection experiments. Then combined with tumor function experiments, such as apoptosis, proliferation, drug reactions, we will explain the functions of detected differentially expressed genes, provide appropriate therapeutic targets for personalized treatment of bladder cancer [32-34].

In conclusion, the data presented in this study revealed that Notch pathway plays pivotal roles in the formation process of bladder cancer and may be a potential target for the treatment of bladder cancer, reveal molecular mechanisms of different biological characteristics of various bladder cancers. Our study may provide theoretical basis for the future exploration of Notch pathway in bladder cancer.

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Disclosure of conflict of interest

None.

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