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Original Article

Expression and importance of serum MicroRNAs in prostate cancer

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Abstract: The study was to investigate the expression and clinical significance of serum microRNAs in prostate cancer. Illumina Human miRNA high-throughput chip was used to detect the serum microRNA expression in prostate tissues from 10 healthy subjects, 30 benign prostatic hyperplasia patients and 75 prostate cancer patients. Moreover, differentially expressed microRNAs were selected for further validation in 10 benign prostatic hyperplasia patients and 30 prostate cancer patients with real time fluorescent quantitative PCR. At last, the correlation between Gleason score of microRNA expression and clinical stages was evaluated. A total of 130 differentially expressed microRNAs were identified, of which 101 showed reduced expression and 29 displayed elevated expression. 22 microRNAs were chosen for validation by PCR, in which miR-29a, miR-625, miR-1323 and let-7d showed significantly down-regulated expression. Let-7d expression was related to Gleason score of prostate cancer ($P<0.05$), and the higher the Gleason score, the lower the let-7d expression was. microRNAs with their reduced expression, including miR-29a, miR-625, miR-1323 and let-7d, may act as anti-tumor genes in the occurrence and development of prostate cancer. Furthermore, let-7d may become a new marker for the early diagnosis and prognostic evaluation of prostate cancer.

Keywords: MicroRNA, prostate cancer, biomarker, real time fluorescent quantitative PCR

Introduction

MicroRNAs (miRNAs) are a critical class of small, non-coding, endogenous RNAs with ~22 nucleotides and can control the post-transcriptional gene expression in eukaryotes [1, 2]. miRNAs are transcribed in the nucleus of cells from DNA. The enzyme RNA polymerase II (RNase II) transcribes DNA into a primary RNA (pri-miRNA) within the nucleus. The pri-miRNA is recognized by the nuclear protein DiGeorge syndrome critical region 8 (DGCR8) that associates with Drosha, a RNase III, which cleaves the pri-miRNA generating a precursor miR-RNA (pre-miRNA) [1, 2]. The pre-miRNA then exits the nucleus via a nuclear pore with the assistance of Exportin 5. Finally, the pre-miRNA is cleaved by the enzyme Dicer, resulting in the mature miRNA. To date, approximately 1,872 precursors and 2,578 mature miRs have been identified in Homo sapiens. These small RNAs participate in the regulation of expressions of 1/3 of human genes, and play important roles in cell growth, differentiation and apoptosis [3]. Growing evidence indicates that miRs play critical roles in cancer pathogenesis, chemo/radiotherapy resistance, tumor relapse, and metastasis [4, 5]. In recent years, the number of reports regarding cancer-related miRs has increased dramatically.

Recent studies have reported that there are differentially expressed microRNAs between prostate cancer (PCa) and normal prostate tissues [6, 7], and increasing studies reveal that microRNAs are of great significance in the development, diagnosis, treatment and prognosis of PCa. The available studies about the relationship between microRNAs and tumor mostly focus on tumor tissues. However, the examination of specimens is difficult because the collection of tumor tissues is invasive, which limits its use in clinical practice. Serum is easy to collect and shows long-time stability [8]. Thus, detection of serum microRNAs is more practicable for the clinical diagnosis and prognosis of tumors. In blood, microRNAs form complexes with proteins, and thus they are stable and resistant to RNA hydrolase [9]. The majority of microRNAs is evolutionarily conserved among

Table 1. 22 microRNAs selected in this study

	Target ID	Change
1	hsa-let-7c	down
2	hsa-let-7e	down
3	hsa-miR-1224-3p	down
4	hsa-miR-1323	down
5	hsa-miR-190	down
6	hsa-miR-216b	down
7	hsa-miR-218-1	down
8	hsa-miR-369-5p	down
9	hsa-miR-889	down
10	hsa-miR-1305	down
11	hsa-miR-625	down
12	hsa-let-7d	down
13	hsa-miR-200c	down
14	hsa-miR-181b	down
15	hsa-miR-29a	down
16	hsa-miR-768-3p:11.0	up
17	hsa-miR-544	up
18	hsa-miR-193a-5p	up
19	hsa-miR-7-1	up
20	hsa-miR-720	up
21	hsa-miR-589	up
22	hsa-miR-550	up

Table 2. CT ratios of miR-29a, miR-625, miR-1323 and let-7d

		MiR-625	Let-7d	MiR-29a
BPH	0.0722	17.6913	2.2812	6.5220
PCa	0.0368	6.1783	0.3973	2.0516
P	0.0145	0.0042	0.0126	0.0094

distantly related organisms and may be expressed in a tissue-specific or developmental stage-specific manner. Moreover, microRNAs expression in tumors is also tissue specific [10], and the tumor tissues can secrete microRNAs into blood. Though the content of microRNAs in the blood is lower than that in tissues, the trend of changes in these microRNAs is consistent [11, 12]. In the present study, serum microRNAs were detected as new molecular markers for the early diagnosis of PCa.

Materials and methods

Sample collection

For the detection of microRNAs with the Illumina Human miRNA high throughput chip, blood sample was collected from 10 subjects with normal prostate, 30 patients with benign prostatic hyperplasia and 75 patients with PCa from the Department of Urology of Affiliated Tongji Hospital of Wuhan University between 2010 and 2011. For fluorescence quantitative PCR, blood sample was collected was from 10 patients with benign prostatic hyperplasia and 30 patients with PCa from the Department of Urology of Affiliated Tongji Hospital of Tongji University between 2011 and 2012. Serum was harvested after centrifugation and stored at -80°C.

Clinical characteristics

The mean age of PCa patients was 77.1±6.82 years; According to the Gleason grading system, 19 patients were graded at score 2-7 PCa and 11 patients were at score 8-10 PCa; According to the clinical staging system, 19 patients were classified at stage A+B PCa, and 11 patients were at stage C+D PCa; According to serum PSA expression before surgery, PSA in 7 patients had mild increase (4 ng<PSA≤10 ng/L), 16 patients had moderate increase (10 ng<PSA≤135 ng/L), and 7 patients had significant increase (PSA>135 ng/L).

Reagents and microRNA chip assay

Human miRNA high throughput chip was purchased from YuBo Biological Co. LTD, and a total of 130 differentially expressed microRNAs were identified through the microRNA high-throughput chip assay. Images were captured with Bead studio software, normalization was performed with Quantile function and then the differentially expressed genes were screened. 22 primers and reagents used for fluorescence quantitative PCR were designed and provided by the Qiagen Company.

RNA extraction and qRT-PCR

RNA was extracted according to the manufacturer's instruction (miRNeasy Mini Kit; 50; 217004). First-strand cDNA was synthesized from 1 μg of total RNA. Amplification was tested using the miScript Reverse Transcription Kit. Detection was performed using the Bio-Rad MyiQ™2 Two-Color Real-Time PCR Detection System. The comparative cycle threshold (Ct) method was employed to analyze the relative expressions of microRNAs.

Table 3. Clinical characteristics and CT ratio of 4 microRNAs

		MiR-29a		Let-7d		MiR-625		MiR-1323	
	n	CT ratio	P	CT ratio	P	CT ratio	P	CT ratio	P
Age									
<73	8	0.032	0.93	3.935	0.96	4.152	0.23	1.764	0.87
≥73	22	0.033		3.863		6.879		1.909	
Clinical stage									
A+B	19	0.029	0.47	3.191	0.73	5.888	0.67	1.676	0.49
C+D	11	0.040		3.346		6.774		2.239	
PSA									
4<PSA≤10	7	0.026	0.66	3.127	0.27	6.812	0.16	1.553	0.43
10<PSA≤135	16	0.039		5.019		7.457		1.594	
PSA>135	7	0.033		2.358		3.066		2.751	
Gleason score									
2-7	15	0.031	0.75	5.049	0.02	4.684	0.12	2.410	0.16
4-10	15	0.035		1.712		7.713		1.336	

Statistical analysis

SPSS version 13.0 was used for statistical analysis. The CT value from real time fluorescence quantitative PCR reflects the quantity of microRNAs. The CT value of each microRNA after conversion with a specific formula was normalized to that of internal reference, and the resultant ratio was used for T test. The relationship between microRNAs and clinical pathological types was analyzed by one dimensional analysis of variance, and a value of $P<0.05$ was considered statistically significant.

Results

A total of 130 differentially expressed microRNAs were identified by miRNA chip assay. Results showed that the expression of 101 microRNAs was down-regulated and 29 was up-regulated. Of them, 19 microRNAs with most significant changes in their expressions were selected, and another 3 microRNAs were selected after reviewing literatures. Finally, 22 microRNAs (**Table 1**) were selected for further experiments.

Results of fluorescence real-time quantitative PCR showed 4 microRNAs (miR-29a, miR-625, miR-1323 and let-7d) had significant changes in their expression (**Table 2**). The CT ratios were 0.0722, 17.6913, 2.2812 and 6.5220, respectively in prostatic hyperplasia patients, and 0.0368, 6.1783, 0.3973 and 2.0516, respectively in PCa patients. The expressions of these 4 microRNAs were decreased in PCa patients ($P<0.05$), suggesting that 4 microRNAs have the potential to inhibit the occurrence and development of PCa.

Of 4 microRNAs, the let-7d expression was correlated with Gleason score in PCa patients ($P<0.05$). In patients with Gleason score at 2-7, the CT ratios were significantly lower than in those with Gleason score at 8-10. It suggests that the higher the Gleason score, the lower the let-7d expression was. The expression of rest microRNAs showed no obvious relationship with Gleason score. Moreover, the expression of these 4 microRNAs had no association with the age, clinical stage or average blood PSA ($P>0.05$) (**Table 3**).

Discussions

In 1993, Lee et al. [13] identified the first 22-nt small RNA (Lin-4) in a study on the caenorhabditis elegans. The 22-nt smallRNA-Lin-4 cannot encode the protein, but may inhibit the protein translation by interacting with the specific area of the 3'UTR of target mRNA in an incomplete complementary manner, leading to the inhibition of protein synthesis. Currently, microRNAs have attracted increasing attention infield of oncology. Studies on the microRNA expression profiles demonstrate that microRNAs are not only tumor-specific, but also have specificity in the pathological type of a specific cancer [14]. Therefore, microRNAs have the potential to act as early tumor markers. Studies have indicated that microRNAs are also relevant to the cell differentiation. Silber et al. [9] found that miR-124 and miR-137 were able to promote the differentiation of rat neural stem cells and CD133 positive human polymorphism glioma stem cells. MicroRNAs are important molecules in the regulation of proliferation and apoptosis in a wide variety of tumor cells, and play an important role in the progression of tumor.

PCa is one of the most common cancers threatening the health of males [15]. Statistics reveal that the incidence of PCa increases from 1.6

per 100,000 men to 7.7 per 100,000 men from 1973 to 2000, and PCa has become the most common malignancy of the urinary system in males [16, 17]. Clinical studies have shown that about 30% of PCa patients is diagnosed with clinically processive PCa at initial, which predicts a poor prognosis. Moreover, about 80% androgen independent PCa will transform into castration resistant PCa after 18-24 months of androgen deprivation therapy. To date, no effective therapy has not been developed for the castration resistant PCa [18], and the pathogenesis of PCa is still poorly understood. Recently, castration resistant PCa has drawn much attention. Therefore, the early prediction of prognosis of PCa and the identification of new targets are beneficial for the effective therapy of castration resistant PCa.

In recent years, increasing studies confirm that microRNAs are closely related to the pathogenesis of cancers [5], including PCa [6]. Spahn et al. [19] found that miR-221 expression gradually reduced in the invasive PCa and in the metastasis of cancers, suggesting that miR-221 is a marker of cancer metastasis. Therefore, miR-221 may be of great significance in the identification of patients having increased risk for aggressive PCa. Moreover, the microRNAs are also related to the drug resistance and prognosis of PCa [7, 20] as well as the progression to antiandrogen therapy resistance [21]. In addition, microRNAs are also involved in the regulation of the stemness of PCa stem cells with different mechanisms, which proposes the potential roles of microRNAs in PCa therapy [22].

To date, studies have shown that microRNAs exist in both blood and other body fluids, including urine, tear, semen and amniotic fluid [23, 24]. Hessels et al. [25] reported that in the urine of patients with PCa, the expression of DD3PCA3 was 66 times higher than in healthy subjects. The sensitivity and specificity of urine DD3PCA3 were 67% and 83%, respectively, in the diagnosis of PCa. Chen et al. [9] also found microRNAs existed in animal serum. Moreover, the expressions of serum microRNAs are consistent in different individuals of the same species of animals and humans. The expression of serum microRNAs reflects the tumor size to a certain extent, and can be used as new biological targets in the diagnosis and treatment of cancers [11].

In this study, a total of 130 differentially expressed microRNAs were identified through the microRNA high-throughput chip assay, of which 101 showed down-regulated expression and 29 had up-regulated expression. However, the false-positive rate of microRNA chip assay is relatively high, and the discrimination of mature microRNAs from precursor microRNAs is difficult. Thus, fluorescence quantitative PCR was applied in this study to verify the results from microRNA chip assay. In the present study, serum was collected for the measurement of microRNAs, which is more practical in clinical practice. Our results showed that the expression of miR-29a, miR-625, miR-1323 and let-7d in the serum of PCa patients decreased significantly compared to other groups, suggesting that these 4 microRNAs may act as tumor suppressor genes in the occurrence and development of PCa. Moreover, they may serve as molecular markers for the early diagnosis of PCa.

microRNAs encoded by let-7 microRNA family are the first microRNA found to regulate oncogene ras [26]. Let-7 microRNA family is universally expressed in mammals, and the missing of let-7 in cancers may cause reverse embryonal differentiation and dedifferentiation of cells. Let-7a is one of the members in let-7 microRNA family, which is related to the pathogenesis of human lung and colon cancer [27, 28]. This study further analyzed the relationship between microRNAs and clinical pathological characteristics. Results showed that let-7d was related to the Gleason scores, indicating that let-7d may reflect the prognosis of PCa to a certain extent.

Although great progresses have been achieved in studies about microRNAs in cancers, the specific mechanism underlying the regulation of microRNAs is still unclear. It has a long way to go before the application of serum microRNAs as early tumor markers. More studies are required to further interpret the regulation of microRNAs, which may provide new clues for the cancer treatment.

Disclosure of conflict of interest

None.

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