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RESEARCH ARTICLE

CHANGES IN THE EXPRESSION OF NOTCH AND HIF-1A SIGNALING MOLECULES IN PROSTATE CANCER PROGRESSION

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Abstract

Objective: To explore the expression and clinical significance of Notch and HIF-1 α signaling molecules in the progression of prostate cancer.

Method: A total of 68 cases with prostate cancer were enrolled to detecting the expression level of Notch1, HIF-1 α and Hes1 mRNA by qRT-PCR, in which the protein expression was measured by western blotting and immunohistochemistry staining, respectively. Correlation between molecules above and clinical pathological parameters were further analyzed.

Results: Both Notch signaling and HIF-1 α signaling molecules were overexpressed in prostate cancer on the protein level as well as mRNA level when compared to the para-cancer tissues ($P < 0.05$). The expression of these molecules were positive related to local lymphatic metastasis ($P < 0.05$). However, this difference was not significant both in tumor volume and patient's age.

Conclusion: Both Notch signaling and HIF-1 α signaling pathway related molecules were overexpressed in prostate cancer, which might play an important role in the progression of prostate cancer.

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Introduction:-

With the development of modern society, prostate cancer (PCa) has become the most common malignancy tumor for male, which affects the reproductive systems even leading to the death of males^[1]. In recent years, many studies found that the incidence of PCa had gradually increased in developing countries and regions such as Asia. In order to enhance the survival rate of PCa patients, new therapeutic targets are required to be further studied. Variety factors including family history, genetics, diet, obesity and signaling pathways are considered as potential or independent risk factors for PCa^[2, 3]. Abnormal expression of hypoxia inducible factor 1 alpha (HIF-1 α) could promote the cancer cell proliferation through high oxidation state caused by local hypoxia environment^[4, 5]. It has been confirmed that multiple signaling molecules in the Notch signaling pathway are involved in the occurrence and development of various tumors such as ovarian cancer, cholangiocarcinoma, lung cancer and breast cancer^[6]. Nevertheless, there are few studies focus on the expression of HIF-1 α , Notch1 and the target Hes family in prostate

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cancer based on clinical sample [7]. The present study aims to explore the expression and clinical significance of Notch and HIF-1 α signaling pathway in the progression of prostate cancer.

Materials and Methods:-

Patient and sample:

A total of 68 cases with prostate cancer were included during January 2016 to April 2020 in Zigong Fourth People's Hospital. The inclusion criteria is the patient's prostate cancer was confirmed by pathology detection. Moreover, only the patient without any pre-treatment of drug could be finally included. Those patients with chronic infected diseases and other major mental disorder could be excluded from this study. The control group was defined as the para-cancer tissue in the same patient. The sampling requirements for the control group need to be at least 2cm away from the cancer tissue. The basic characteristics of patients were listed in Table 1. This research has been proved by Ethics Committee of Zigong Fourth People's Hospital. The patients received the information on this study and gave their consent.

Median age of 68 cases was 57 years old (51 to 86 years old). The average diameter of cancer was 4.58 \pm 2.47cm (2.15~9.72cm).

Table 1:- Basic characteristics of patients.

Indicator	Value
Age (years old)	57 (51 ~ 86)
tPSA (ng/ml)	23.64 \pm 6.38
Tumor volume (cm)	4.58 \pm 2.47(2.15~9.72cm)
Gleason score	6.92 \pm 0.84
Local lymphatic metastasis	24(35.29%)

Quantitative-reverse transcription PCR (qRT-PCR):

For quantifying the expression of Notch1, HIF-1 α and Hes1, total RNA was prior isolated from different prostate tissues by TRIzol reagent (Invitrogen, USA) in the light of manufacturer's specification. Total RNAs were reverse transcribed to cDNA applying cDNA synthesis kit (Sigma, USA). The reaction was carried out under the following conditions according to previous studies: pre-degeneration at 95 centigrade continued for 10 min, denaturation at 95 centigrade continued for 30s, re-naturation at 55 centigrade continued for 30s, and extension at 72 centigrade lasted 2 min, for a total of 35 cycles. The relative expression of mRNAs was calculated by the 2^{- $\Delta\Delta$ Ct} method. All experiments were repeated three times. The corresponding primer sequences of Notch1, HIF-1 α and Hes1 were listed in Table 2.

Table 2:- Primer sequences for Notch1, HIF-1 α , Hes1 and GAPDH.

Gene	Forward	Reverse
Notch1	CATGGATCAGAACTCAGCAAAGTC	CATGCCATGCCTTGTCTTCA
HIF-1 α	GATCTCGAGGCTTTTTCTTAATTTTCATT CC	GATGCGGCCGCGCCTGGTCCACAGAAGATGTT TA
Hes1	GAACGATAACCTTTGCAGGC	TTTCGATTCCGCTATGTGTG
GAPDH	CCTAGTTCGTCATGGGTGTGAACCA	GCCAGTAGAGGCAGGGATGATGTTC

Western blotting:

A total of 50 μ g protein was separated by 10% SDS-PAGE and then transferred onto PVDF membranes (Thermo Fisher Scientific, Inc.) for 2h. The membranes were then blocked by 5% bovine serum at room temperature for 1h, followed by incubation at 4 $^{\circ}$ C overnight with the following primary antibodies: anti-Notch1 (Epitomics, Inc.; 1:1,000), anti-HIF-1 α (Abcam; 1:500), anti-Hes1 (Abcam; 1:500) and anti-GAPDH (Abcam; 1:1,000). Subsequently, the membrane was washed three times with PBS and Tween-20 (TBST) and then incubated with the corresponding secondary antibody for 1h. The antibody staining intensities were detected using ECL chemiluminescence reagent (Thermo Fisher Scientific, Inc.) after the final wash with PBST was completed. The relative grey level was calculated by ImageJ 1.52a (Wayne Rasband, National Institute of Health, USA) and plotted by GraphPad Prism 8.0 (GraphPad Software, Inc).

Immunohistochemistry staining:

Conventional method was applied to immunohistochemical staining with 5 μ m-thick paraffin sections. All antigen-antibody reactions are performed in accordance with manufacturer's instructions. Notch1, HIF-1 α and Hes1 primary monoclonal antibodies were obtained from Abcam Co. Ltd. (Cambridge, UK).

Statistical analysis:

Statistical procedure in this study was performed in STATA software version 12.0 STATA, College Station, TX, USA). Continuous data were described by mean and corresponding standard error. The difference between two groups was analyzed by two independent student's t-test; all countable data were described by percentage. The difference between groups was analyzed by Chi-square test. If the P value was below 0.05, the difference of results was supposed to be significant.

Results:-**Expression of Notch1, HIF-1 α and Hes1 mRNA in PCa:**

To investigate the expression of Notch1, HIF-1 α and Hes1 in PCa, the corresponding mRNA was detected. The qRT-PCR result showed that the relative mRNA expression of Notch1, HIF-1 α and Hes1 were significant improved (Figure 1).

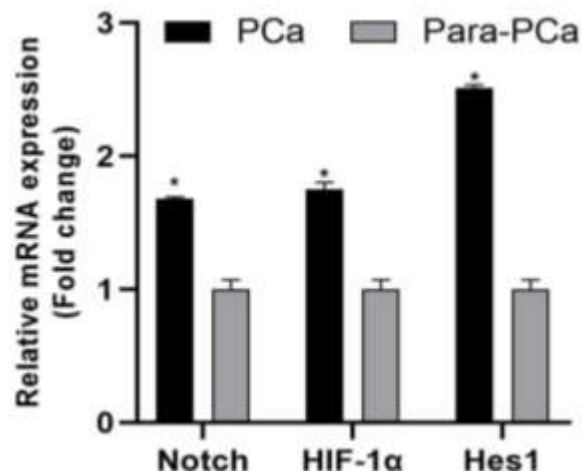


Figure 1:- Quantitative-reverse transcription PCR analysis of Notch1, HIF-1 α and Hes1 mRNA in PCa.

Expression of Notch1, HIF-1 α and Hes1 protein in PCa:

To verify whether Notch1, HIF-1 α and Hes1 protein were overexpressed in PCa, the western blot and immunohistochemistry methods were used to confirm the expression. Western blot results proved that the expression of Notch1, HIF-1 α and Hes1 protein in PCa were significant improved (Figure 2). The result of immunohistochemistry stain was listed in Table 1. The result revealed that the positive expression of Notch1, HIF-1 α and Hes1 were significant in PCa higher than that in Para-PCa (Notch1 60.29% vs 19.12%; HIF-1 α 64.71% vs 22.06%; Hes1 69.12% vs 23.53%).

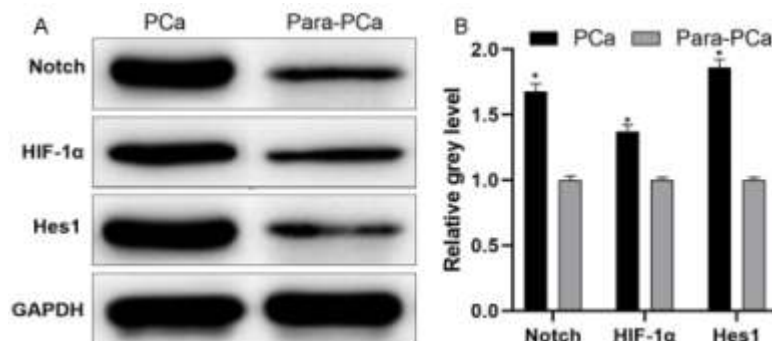


Figure 2:- Western blot analysis of Notch1, HIF-1 α and Hes1 mRNA in PCa in PCa (A) and corresponding gray value statistics (B).

Table 1:- The rate of positive expression of Notch1, HIF-1 α and Hes1 in PCa n(%).

Group	Notch1+(%)	HIF-1 α +(%)	Hes1+(%)
PCa	41(60.29)*	44(64.71)*	47(69.12)*
Para-PCa	13(19.12)	15(22.06)	16(23.53)

* P<0.05 when compared PCa to Para-PCa.

Clinicopathological characteristics of Notch1, HIF-1 α and Hes1 in PCa:

To investigate the relationship between the proteins above and clinicopathological characteristics, the factors of age, tumor volume and local lymphatic metastasis were analyzed. Results identified that the expression of Notch1, HIF-1 α and Hes1 were significantly related to local lymphatic metastasis (P<0.05), whereas this difference were not significant both in the factor of age and tumor volume (P>0.05).

Table 3:- The Clinicopathological characteristics of Notch1, HIF-1 α and Hes1 in PCa n(%).

Factor	Number n=68	Notch1+ n=41	χ^2	P	HIF-1 α + n=44	χ^2	P	Hes1+ n=47	χ^2	P
Age (years old)			0.12	0.73		1.29	0.26		0.65	0.42
≤60	42	26			25			25		
>60	26	15			19			18		
Tumor volume			2.53	0.11		0.10	0.76		2.95	0.09
≤5cm	62	41			45			56		
>5cm	6	2			4			4		
LLM*			8.05	0.005		15.98	<0.01		5.28	0.02
Yes	24	9			8			12		
No	44	32			36			34		

* LLM: Local lymphatic metastasis

Discussion:-

Prostate cancer is one of the most common cancers of males. Multiple molecules have participated in the progression of prostate cancer^[8-12]. Notch signaling pathway is involved in the occurrence and development of a variety of tumors, and it can regulate a variety of cell processes, including cell differentiation, proliferation, apoptosis and migration^[13, 14]. When the Notch ligand specifically binds to the receptor, the Notch signaling pathway is subsequently activated, which in turn activates the transcription of the downstream gene Hes family. During this process, Hes1 would be also activated, which plays as the major member of Hes family in the Notch signaling pathway^[10, 11]. The Notch signaling pathway has the dual effects of promoting cancer and anti-cancer^[15, 16]. Numerous research results show that it could exhibit the cancer progression in ovarian cancer, cholangiocarcinoma, lung cancer and breast cancer^[17, 18], while this effect would be totally reversed in skin cancer and neuroendocrine tumors^[9].

In the present study, we found that both the expression of Notch signaling molecules Notch1 and Hes1 were significantly higher in prostate cancer tissue than that in para-cancer tissue. Our result initially demonstrated Notch signaling could play an important role in the progression, invasion and metastasis of prostate cancer. Moreover, both Notch1 and Hes1 were significant improved in the patients with local lymphatic metastasis. It could be potentially biomarker in the diagnosis of advanced prostate cancer^[19, 20]. However, we cannot analyze the sensitivity and specificity of this diagnostic ability since the limitation of cases.

The other important finding of our study is focusing on the relationship between hypoxia environment and prostate cancer progression. Just like Notch signaling, our research on HIF-1 α has shown similar effects^[21]. The change of hypoxic environment is an important factor that promotes the abnormal regulation of cancer cell proliferation. As a member of the hypoxia-inducible factor family, HIF-1 α is responsible for the damage of mitochondria, ATP energy utilization obstacles or transmission obstacles of oxidative free radicals under hypoxic environment in local environment^[22]. HIF-1 α could also affect the amplification rate of tumor cell DNA, as well as promote the increase of cancer cell nuclear atypia and the activation of multiple signaling pathways such as AKT and MAPK in cancer

cells^[12, 23]. Our study found that abnormally overexpression of HIF-1 α could be detected in metastatic prostate cancer. This trend suggests that HIF-1 α may be involved in the progression of prostate cancer. The high expression of HIF-1 α could promote the inflammation or oxidative response via inducing local overactive interleukin-6 (IL-6) or interleukin-10 (IL-10)^[24, 25].

Studies have shown that the positive expression rate of Notch1 or HIF-1 α in people with higher mortality or poor clinical treatment sensitivity can be increased significantly in the process of clinical prognosis of prostate cancer patients^[26, 27]. In the study of the relationship between relevant indicators and clinical characteristics of prostate cancer patients, it was found that HIF-1 α is related to elevated PSA levels, increased Gleason scores, advanced clinical stages and bone metastasis. This suggests that elevated expression of HIF-1 α can affect tumors metastasis and histological deterioration. HIF-1 α and Notch1 proteins are closely related to the occurrence of prostate cancer bone metastasis, which suggesting that the positive expression may play as important evidence for predicting bones metastasis. The mechanism may relate to the abnormal expression of both HIF-1 α and Notch1 protein could increase the infiltration and adhesion of spine bone cells and bone interstitial cells.

In summary, both HIF-1 α and Notch signaling pathway related proteins are overexpressed in prostate cancer tissues. The activation of Notch and HIF-1 α signaling pathways may play an important role in the progression of prostate cancer. This study requires further research to reveal the specific mechanisms of Notch and HIF-1 α signaling pathways on the anti-apoptosis and tumor metastasis prostate cancer.

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Disclosure of Potential Conflicts of Interest:

No potential conflicts of interest were disclosed.

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