Variation of M_3 muscarinic receptor expression in different prostate tissues and its significance

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ABSTRACT

الأهداف: تحديد ظهور عامل استقبال (M) في أنسجة البروستاتا المختلفة وتحليل دورها الفرعي في مكونات الورم البروستاتية.

الطريقة: تم استعمال عدد 36 حالة من تضخم البروستات الطبيعي والخبيث على التوالي و8 حالات من ورم البروستات في هذه الدراسة لدينا في جامعة شاندونغ، شاندونغ، الصين خلال الفترة ما بين عام 2003 إلى 2006م. تم تحديد ظهور البروتين في مستقبلات (M_1) و (M_2) و (M_3) في كل مجموعة بواسطة طريقة اللطخة الغربية. تم تحديد ظهور الجين لمستقبل (M_3) و (VEGF) لكل مجموعة بواسطة تفاعل سلسلة الخمائر الناقلة (RT–PCR).

النتائج: كان البروتين وظهور الجين لعامل استقبال (M_3) في مجموعة الورم الغدي السرطاني البروستاتي أعلى من المجموعة التي تعاني من فرط التنسج الحميد (p=0.0001) ومجموعة البروستات الطبيعية (p=0.0001). أظهر مستقبل (M_3) و (VEGF) علاقات إيجابية ذات خط مستقيم لتعرضات الجين في المجوعات الثلاثة (r=0.4999, p=0.0001).

خاتمة: قد يكون لدى مستقبل (M_3) علاقة قريبة مع مكونات الورم البروستاتية.

Objectives: To detect the expression of the muscarinic receptor (M receptor) in different prostate tissues and analyze the role of its subtype in prostatic oncogenesis.

Methods: Thirty-six cases of normal prostate and benign prostatic hyperplasia, and 8 cases of prostatic tumor, were used in this study from the Shandong University, Shandong, China, between 2003-2006. The protein expressions of M_1, M_2, and M_3 receptors in each group were determined by Western-blotting. The gene expressions of the M_3 receptor and vascular endothelial growth factors (VEGF) in each group were determined by reverse transcriptase-polymerase chain reaction.

Results: The protein and gene expressions of the M_3 receptor in the prostatic carcinoma group were higher than that of benign prostatic hyperplasia group (p=0.0001) and normal prostate group (p=0.0001). The M_3 receptor and VEGF showed positive straight-line correlations of gene expressions with the 3 groups (r=0.4999, p=0.0001).

Conclusion: The M_3 receptor may have a close relationship with prostatic oncogenesis.

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Normal human prostatic tissue contains many types of M receptors; however, the M_1, M_2, and M_3 receptors appear to have a close relationship with prostatic oncogenesis.[1-3] Benign prostatic hyperplasia is the most common tumor found and is seriously harmful to the health and life of middle- and old-aged men. Data shows that over 50% of men >60 years suffer from varying degrees of prostatic hyperplasia.[4] Prostatic carcinoma is the most common male malignancy in Europe and America.[5] We detected the expression of M_1, M_2, and M_3 receptors at the messenger ribonucleic acid (mRNA) and protein levels in normal prostatic, hyperplastic and carcinoma specimens by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western-blotting, Therefore in this paper, we aimed to analyze the expression patterns of the subtype of M receptor in different prostatic tissues at the mRNA and protein levels, and explore the role of its subtype in prostatic oncogenesis.

Methods. ***Patients and tissues.*** Ethical approval for this study, and informed consent of all patients was obtained from Shandong University, Shandong, China. Each subject signed an agreement of participation in this study that was approved by Shandong University, Shandong, China. The protocol of the study adhered to the tenets of the Declaration of Helsinki and was approved by the local ethics committee. Thirty-six cases of fresh prostatic hyperplasia (BPH) specimens and 8 cases of fresh prostatic carcinoma specimens were taken from patients after informed consent in our hospital between 2003-2006. Fresh normal prostatic specimens were available from 36 healthy adults, due to accidental death. The median age in the prostatic hyperplasia group was 71.5 years (range 59-78 years); in the prostatic carcinoma group it was 56 years (range 52-65 years) and in the normal prostatic hyperplasia group it was 33 years (range 23-45 years). All specimens were frozen with liquid nitrogen after being taken from the patient and stored at -80°C until used.

Reagent. A membrane protein extraction kit was purchased from the Calbiochem Company (La Kolla, USA). Primary antibodies for vascular endothelial growth factors (VEGF), M_1, M_2 and M_3 receptors were purchased from Santa Cruz Biotechnology Company (Sta. Cruz, USA) and secondary antibodies were purchased from Beijing Zhongshan Biotechnology Company (Beijing, China) Primers and the RT-PCR kit were purchased from Shanghai Biotechnology Co., Ltd (Shanghai, China).

Protein expressions of M_1, M_2, and M_3 receptor were detected by Western-blotting. 1) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were dissolved in sample buffer. Samples were fractionated by SDS-PAGE using a 5% stacking gel and 12% separation gel. Molecular weight markers were run simultaneously.

2) Western blotting. Proteins were subjected to SDS-PAGE and then transferred at 100 V for 60 minutes to Hybond membranes. The membranes were subsequently blocked for one-hour at room temperature with phosphate buffered saline (PBS) solution containing 7% (w/v) skimmed milk powder. After washing with PBS, the membranes were incubated with the primary antibody overnight. The primary antibodies were diluted at 1/1000. The membranes were washed twice and then incubated with secondary antibody (anti-rabbit immunoglobulin) at a dilution of 1/1000, for 2-hours. After washing, the membranes were exposed to diaminobenzidine (DAB). The densities of the protein bands were measured by a transmittance/reflectance densitometer.

Detection of the gene expression of M_3 and VEGF by RT-PCR. The total cellular RNA was extracted from fresh tissue according to the manufacturer's instructions using Trizol (Applied Biosystems Inc. (ABI), Foster City, CA, USA). Complementary DNA (cDNA) was synthesized using First Choice RLM-race kit (Ambion Company, Austin, USA). The RT-PCR primers are listed in **Table 1**. Polymerase chain reaction was performed by running 35 cycles of 94°C x 50 seconds, 55°C x 50 seconds and 72°C x 60 seconds. The products of PCR were scanned by optical densitometry after electrophoresis on a 1.5% agarose gel. The ratio of the optical density of objective products to that of internal reference ß-actin was used as the parameter of comparison.

Statistical analysis. The SPSS 10.0 statistical package was used. Data were described in the application of mean ± SD. In analyzing the different expression between the 3 prostate groups, we used analysis of variance and post hoc test to compare and determine where exactly the significance is. In analyzing the different expression of M_1, M_2, and M_3 receptors in the group of prostate carcinoma, we used Kruskal-Wallis one-way analysis of variance with appropriate post hoc. In analyzing the relationship between M_3 muscarinic receptors and VEGF, we used the Pearson's correlation analysis.

Table 1 - Length of primer sequences and fragments.

Primer	Primer sequence	Length of objective fragment
VEGF primer	upstream sequence:5'-CGAAACCATGAACTTTCTGC-3'	303 bp
	downstream sequence:5'-CCTCAGTGGGCACACACTCC-3'	
M_3 primer	upstream sequence:5'-ACCCAGCTCCGAGCAGATGGAC-3'	339 bp
	downstream sequence:5'-CGGCTGACTCTAGCTGGATGGG-3'	
Human ß-actin	upstream sequence:5'-GTGGGGCGCCCCAGGCACCA-3'	539bp
	downstream sequence:5'-CTCCTTAATGTCACGCACGATTTC-3'	

VEGF - vascular endothelial growth factors

Results. There were different protein expressions of M_1, M_2 and M_3 receptors in the groups of normal prostate, benign prostatic hyperplasia, and prostatic carcinoma (**Table 2**). The expressions of M_1, M_2, and M_3 in normal prostate tissues was M_2, >M_3, and >M_1 and there was statistical significant among the 3 groups (p=0.0001). Multiple comparison showed a significant difference among the 3 M receptors in normal prostate tissues (M_2 versus M_3, p=0.0006; M_3 versus M1, p=0.0001). The expression of M_1, M_2, M3 in benign prostatic hyperplasia tissues was M_3, >M_2, >M_1 (p=0.0001). Both the expressions of M_2 and M_3 receptor were higher than M_1, and the difference was statistically significant (M_2 versus M_1, p=0.0009; M_3 versus M_1, p=0.0007); there was no statistical significance between M_3 and M_2 receptors expression (p>0.05). In prostatic carcinoma tissues, the expression was M_3, >M_2, >M_1 (χ^2=11.69, p=0.0029), both M_3 and M_2 receptors express higher than M_1 and the difference was of statistically significant (M_3 versus M_1, χ^2 =9.80, p=0.00017; M_2 versus M_1, χ^2 =6.86, p=0.008). However, there was no statistical significance between M_3 and M_2 expression (χ^2 = 0.4939, p>0.05). Variance of protein expressions of M_1, M_2, and M_3 receptors in different prostate tissues are shown in **Figure 1**. There was no statistically significant difference in the expression of the M_1 receptor among the 3 groups by multiple comparison (p>0.05) (**Figure 1**). There was no statistically significant difference in expression of the M_2 receptor among the 3 groups by multiple comparison (p>0.05) (**Figure 2**). The expression of the M_3 receptor in prostatic carcinoma tissue was higher than that in the benign prostate hyperplasia tissue. The M_3 expression in normal prostate tissue was the lowest. There were statistically significant differences among the 3 groups using multiple comparisons. (p=0.0001) (prostatic carcinoma tissue versus benign prostate hyperplasia tissue [p=0.0002], benign prostate hyperplasia tissue versus normal prostate tissue [p=0.0002]) (**Figure 3**). The expressions of M_3 receptor with the expected fragment lengths of 339 bp and VEGF of 303 bp could both be detected in normal prostate tissues, benign prostatic hyperplasia tissues, and prostatic carcinoma tissues. There was a variance in gene expression of the M_3 receptor in different prostate tissues. The expression sequences were as follows: 0.8354 ± 0.1897 in the prostatic carcinoma group, 0.6735 ± 0.1603 in the benign prostatic hyperplasia group, and 0.5425 ± 0.1629 in the normal prostate group. The differences among

Table 2 - Protein expression comparison of M_1, M_2, and M_3 receptors in different prostate tissues.

Receptor	Normal prostate	Benign prostatic hyperplasia	Prostatic carcinoma	R-square	F value	*P*-value
M_1 receptor	0.1802 ± 0.0839	0.1819 ± 0.0745	0.2036 ± 0.0485	0.0072	0.28	>0.05
M_2 receptor	0.3348 ± 0.1515	0.3553 ± 0.1451	0.4131 ± 0.1209	0.0223	0.86	>0.05
M_3 receptor	0.2659 ± 0.1076	0.3655 ± 0.1474	0.4777 ± 0.1638	0.2069	9.91	0.0001
R-Square	0.2289	0.3116				
F value	15.59	23.77				
P-value	0.0001	0.0001				

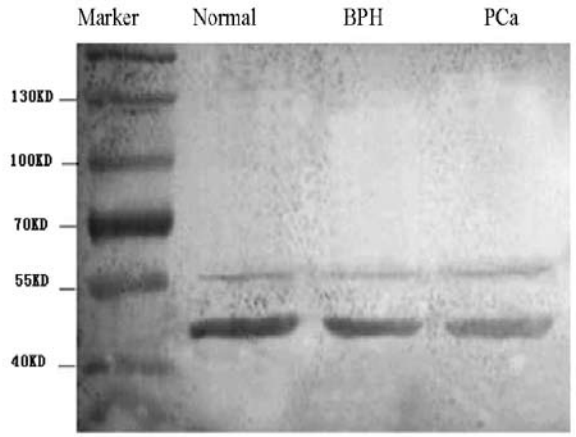

Figure 1 - Western-blot results of the M_1 muscarinic receptor in normal prostate tissues, benign prostatic hyperplasia (BPH) tissues, and prostatic carcinoma (PCa) tissues.

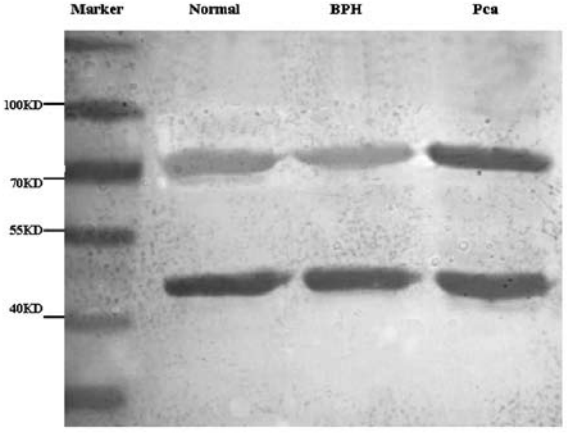

Figure 2 - Western-blot results of the M_2 muscarinic receptor in normal prostate tissues, benign prostatic hyperplasia (BPH) tissues, and prostatic carcinoma (PCa) tissues.

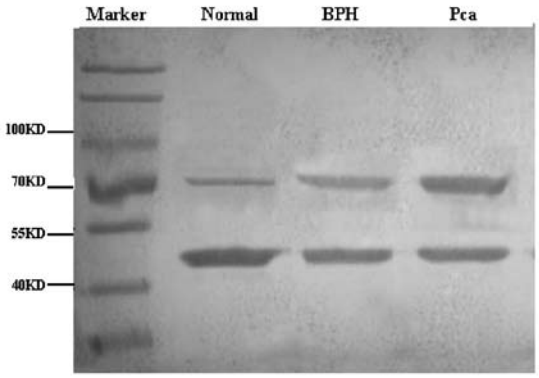

Figure 3 - Western-blot results of the M_3 muscarinic receptor in normal prostate tissues, benign prostatic hyperplasia (BPH) tissues and prostatic carcinoma (PCa) tissues.

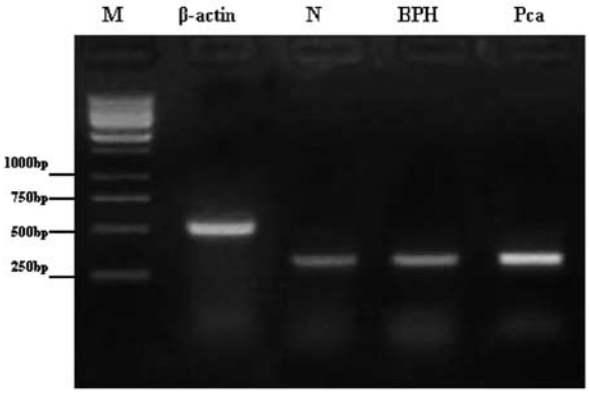

Figure 5 - Reverse transcription polymerase chain reaction results of vascular endothelial growth factors Messenger ribonucleic acid (mRNA) in normal prostate tissue, benign prostatic hyperplasia tissue and prostatic carcinoma tissue. M - marker, N - normal prostate tissues, BPH - benign prostatic hyperplasia tissues, PCa - prostatic carcinoma tissues

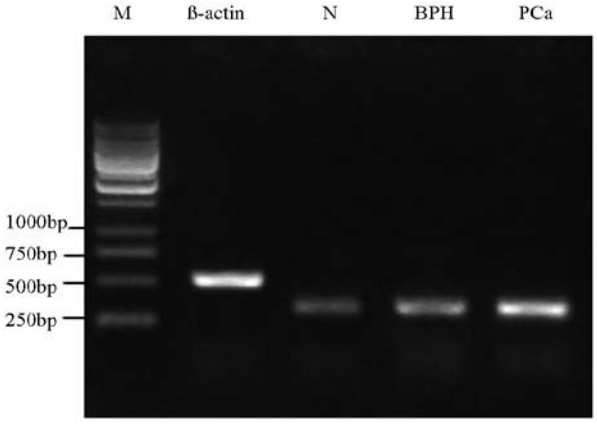

Figure 4 - Reverse transcription polymerase chain reaction results of M_3 muscarinic receptor messenger ribonucleic acid in normal prostate tissue, benign prostatic hyperplasia tissue and prostatic carcinoma tissue. M - marker, N - normal prostate tissues, BPH - benign prostatic hyperplasia tissues, PCa - prostatic carcinoma tissues

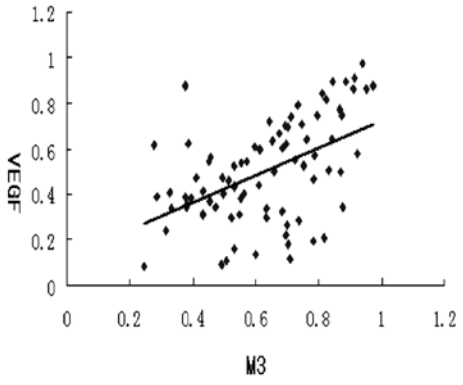

Figure 6 - Correlation of the expression of M_3 muscarinic receptor and the expression of vascular endothelial growth factors (VEGF) (r=0.4999, p=0.001)

them were statistically significant (p=0.0001) (prostatic carcinoma tissue versus benign prostate hyperplasia tissue [p=0.0001], benign prostate hyperplasia tissue versus normal prostate tissue [p=0.0003]) (**Figure 4**). There was a variance of expression of VEGF in different prostate tissues. The expression in the prostatic carcinoma group was 0.7824 ± 0.2047, in the benign prostatic hyperplasia group was 0.6021 ± 0.1637, and in the normal prostate group was 0.3436 ± 0.1581. The differences among them were statistically significant (p=0.0001) (prostatic carcinoma tissue versus benign prostate hyperplasia tissue [p=0.0007], benign prostate hyperplasia tissue versus normal prostate tissue [p=0.0008]) (**Figure 5**). There was a positive linear relationship between the expression of M_3 receptor and VEGF (r=0.4999, p=0.0001) (**Figure 6**).

Discussion. The prostate is an organ of great importance for adult males; it secretes prostatic fluid, assists ejaculation, and urination. The physiological and functional disorders of the prostate can lead to many diseases, especially prostatic hyperplasia and prostate carcinoma, which seriously influence the health of middle and old-aged males. The prostate tissue is regulated by androgens, noradrenergic nerves, and cholinergic receptors. The occurrence and the progression of prostatic hyperplasia and prostatic carcinoma are influenced by many factors.[6]

Research has shown that the M-cholinergic receptor has a relationship with the occurrence of many neoplastic diseases. The M receptor is expressed in normal human prostatic tissue and might participate in the pathological process of prostatic diseases. The M receptor includes 5 subtypes (M1-M5), of which M_1, M_2, M_3 are the 3 main subtypes.[7] We detected the expression of the 3 M receptor subtypes in normal prostate tissue, benign prostatic hyperplasia tissue, and prostatic carcinoma tissue using Western blotting. The expression of the M_2, M_3 receptors in benign prostatic hyperplasia tissue and prostatic carcinoma tissue were higher than that of normal prostate tissue. The expression of M_3 was higher than M_2 and M_1 in prostatic carcinoma tissue, which shows that the M receptor subtype indeed has a certain relationship with the pathological changes of the prostate. The M_3 receptor especially, has been considered to have relationship with several kinds of tumor.[8,9] This experiment further confirms that the M_3 receptor has a correlation with prostatic carcinoma. We also found that the M_2 receptor is the most highly expressed in the normal prostatic tissue. The M_2 receptor is considered to be most closely related to the normal physiological function of the prostate, and plays an important role in the secretion and contractile function of the prostate. Western blotting showed that the protein expressions of the M_2 receptor in benign prostatic hyperplasia tissue and prostatic carcinoma tissue are higher than that in normal prostate tissue, but the expressions in benign prostatic hyperplasia tissue and prostatic carcinoma tissue are the same, which indicates that the M_2 receptor might be related to the occurrence of prostatic carcinoma. However, there are no reports in the literature relating to this and the mechanism needs to be further explored.

The M_3 receptor can be expressed abundantly not only in the normal tissue, but also on the surface of many tumor cells.[10,11] The M_3 receptor can stimulate the proliferation of tumor cells through different signal pathways.[12,13] Rayford et al[1] found that M_3 receptor agonists can facilitate the division and proliferation of human prostate adenocarcinoma cell line of prostatic carcinoma and specimens of benign prostatic hyperplasia, and that the effect in prostatic carcinoma cells was 10-times that in benign prostatic hyperplasia cells.[1] In vitro experiments also showed that the M receptor is an important index for prostatic epithelial differentiation; the expression of the M_3 receptor is closely related to the classification of tumor.[14] We had very few fresh specimens of prostatic carcinoma tissue, therefore, we could not analyze the relationship of its expression with the clinical stage and pathological grade of prostatic carcinoma. A large number of studies have shown many non-nervous tissue cells,[15-20] including tumor cells, can secrete acetylcholine.[21-25] Acetylcholine can bind the M_3 receptor located on the cell surface through the self-secretion or paracrine tumor cell to stimulate the growth of tumors.[22,24,25] The M_3 receptor supplies a new target for the treatment of prostatic carcinoma. At present, M_3 receptor blockers are widely used for the treatment of overactive bladder and chronic obstructive pulmonary diseases, and have good tolerance. The proliferation of tumor cells and the formation of new blood vessels are important elements in the establishment of tumors. In the process of tumor angiogenesis, VEGF is an important factor in facilitating vascularization and accelerating the development of the tumor. Vascular endothelial growth factors can be synthesized by endotheliocytes of the normal catheter, adenocarcinoma cells, and infiltrative lymphocytes in the prostate. The expression of VEGF increases with the rise of transfer characteristics of human prostatic tumor cells and it has a relationship with the expression of regulatory proteins of the cell cycle. Vascular endothelial growth factors synthesized and secreted by malignant cells have a high efficacy in stimulating angiogenesis. By studying the breast cancer cell line LMM_3 we found that the main cholinergic receptor subtype is M_3, and the amount of its expression is 40 times higher than that of LMM_3 cell carbachol. Carbachol can stimulate the growth of LMM_3 cell and neovascularization, which can be inhibited by selective M_3 receptor antagonist p-Fluoro-hexahydrosila-difenidol (pf-HHSiD). Carbachol can stimulate the expression of VEGF-A, which can be also inhibited by the selective M_3 receptor antagonist, pf-HHSiD. These data indicate that the M_3 receptor plays an important role in the growth of human mammary tumor cells and vascularization.[26]

We detected the gene expression of M_3 receptor and VEGF, and showed that both are most highly expressed in prostatic carcinoma tissue and are least expressed in the normal prostate tissue. There is a variation of expression among the normal prostatic tissue, prostatic hyperplasia tissue, and prostatic carcinoma tissue. There is a positive linear correlation between the expression of the M_3 receptor and that of VEGF in the 3 tissues. The result confirms the relationship of VEGF with prostatic oncogenesis. It also indicates that acetylcholine cannot only directly stimulate the growth and proliferation of the prostate, but also increases the formation of new microvasculature through strengthening the function of M_3 indirectly, thus promoting the occurrence of prostatic carcinoma. At present, inhibiting the occurrence of vascularization of tumor tissue is a hotspot in the research of anti-prostatic carcinoma agents.[27] Vascular endothelial growth factors can be regulated by other factors.[28] Therefore, the results also show that the expression of the M_3 receptor has a positive linear correlation with that of VEGF, further indicates that the M_3 receptor participates in the pathological process

of prostatic carcinoma. The function of the M_3 receptor influences the expression of VEGF and can be used as a novel target in the treatment of prostatic carcinoma.

Immune tissue has abundant parasympathetic innervations. The M_3 receptor is expressed abundantly on the surface of lymphocytes. It not only participates in the apoptosis process of lymphocytes[29] but also regulates many kinds of immune cells by complex mechanisms.[30] Another study confirmed that the M receptor agonist carpiline can imitate the role of acetylcholine and inhibit the natural killer cell, while the M receptor antagonist atropine can fully block the inhibitory role of acetylcholine,[31] which indicates that the M receptor participates in the process of acetylcholine inhibition of the activity of natural killer cells. In human prostate tissue, the varying expression of M_3 receptor possibly influences the stability of the immune system, which causes the proliferation of prostate cells and the secretion of VEGF, leading to the occurrence of prostatic carcinoma. This supplies us with new treatment options for prostatic carcinoma, namely, we can regulate the immune system to treat diseases by regulating the M_3 receptor located on the lymphocyte.[32]

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