Variation of M₃ muscarinic receptor expression in different prostate tissues and its significance

Wei Song, PhD, Mingzhen Yuan, MD, Shengtian Zhao, MD.

ABSTRACT

The 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 M₁, M₂, and M₃ receptors in each group were determined by Western-blotting. The gene expressions of the M₁ receptor and vascular endothelial growth factors (VEGF) in each group were determined by reverse transcriptase-polymerase chain reaction (RT-PCR).

Results: The protein and gene expressions of the M₃ 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 M₁ receptor and VEGF showed positive straight-line correlations of gene expressions with the 3 groups (r=0.4999, p=0.0001).

Conclusion: The M₃ receptor may have a close relationship with prostatic oncogenesis.


From the Department of Urology, The Second Hospital of Shandong University, Jinan, China.

Address correspondence and reprint request to: Dr. Mingzhen Yuan, Department of Urology, The Second Affiliated Hospital of Shandong University, No 247 Beiyuan Road, Jinan 250033, China. Tel. +86 (531) 85875401. Fax. +86 (531) 85875401. E-mail: yuanmingzhen2005@126.com

Normal human prostatic tissue contains many types of M receptors; however, the M₁, M₂, and M₃ 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 M₁, M₂, and M₃ 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<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> 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<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> 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<sub>3</sub> 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 β-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<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> 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<sub>3</sub> muscarinic receptors and VEGF, we used the Pearson’s correlation analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
<th>Length of objective fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF primer</td>
<td>upstream sequence: 5’-CGAAACCATGAACCTTTCTGC-3’</td>
<td>303 bp</td>
</tr>
<tr>
<td></td>
<td>downstream sequence: 5’-CCTCAGTGGCCACGACTCC-3’</td>
<td></td>
</tr>
<tr>
<td>M&lt;sub&gt;3&lt;/sub&gt; primer</td>
<td>upstream sequence: 5’-ACCCAGCTCCAGCAGATGGAC-3’</td>
<td>339 bp</td>
</tr>
<tr>
<td></td>
<td>downstream sequence: 5’-CGGCTGACTCTAGCTGGATGGG-3’</td>
<td></td>
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<tr>
<td>Human β-actin</td>
<td>upstream sequence: 5’-GTGGGGGGCCCCAGGCAACCA-3’</td>
<td>539 bp</td>
</tr>
<tr>
<td></td>
<td>downstream sequence: 5’-CTCCTTAATGTCACGCACGTTC-3’</td>
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</table>

**Table 1** - Length of primer sequences and fragments.

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 ($M_1$ versus $M_2$, $p=0.0006$; $M_2$ versus $M_1$, $p=0.0001$). The expression of $M_1$, $M_2$, $M_3$ in benign prostatic hyperplasia tissues was $M_1$, $>M_2$, $>M_3$ ($p=0.0001$). 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 $M_1$ and $M_2$ receptors expression ($p>0.05$). In prostatic carcinoma tissues, the expression was $M_3$, $>M_2$, $>M_1$ ($\chi^2=11.69$, $p=0.0002$), both $M_3$ and $M_2$ receptors express higher than $M_1$, and the difference was statistically significant ($M_3$ versus $M_1$, $\chi^2=9.80$, $p=0.0001$). 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 $M_1$ 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: $0.8354 \pm 0.1897$ in the prostatic carcinoma group, $0.6735 \pm 0.1603$ in the benign prostatic hyperplasia group, and $0.5425 \pm 0.1629$ 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.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Normal prostate</th>
<th>Benign prostatic hyperplasia</th>
<th>Prostatic carcinoma</th>
<th>R-square</th>
<th>F value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_1$ receptor</td>
<td>0.1802 ± 0.0839</td>
<td>0.1819 ± 0.0745</td>
<td>0.2036 ± 0.0485</td>
<td>0.0072</td>
<td>0.28</td>
<td>&gt;0.05</td>
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<tr>
<td>$M_2$ receptor</td>
<td>0.3348 ± 0.1515</td>
<td>0.3553 ± 0.1451</td>
<td>0.4131 ± 0.1209</td>
<td>0.0223</td>
<td>0.86</td>
<td>&gt;0.05</td>
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<tr>
<td>$M_3$ receptor</td>
<td>0.2659 ± 0.1076</td>
<td>0.3655 ± 0.1474</td>
<td>0.4777 ± 0.1638</td>
<td>0.2069</td>
<td>9.91</td>
<td>0.0001</td>
</tr>
<tr>
<td>R-Square</td>
<td>0.2289</td>
<td>0.3116</td>
<td></td>
<td></td>
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<tr>
<td>F value</td>
<td>15.59</td>
<td>23.77</td>
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<td></td>
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<tr>
<td>P-value</td>
<td>0.0001</td>
<td>0.0001</td>
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</table>

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 $M_3$ muscarinic receptor in normal prostate tissues, benign prostatic hyperplasia (BPH) tissues, and prostatic carcinoma (PCa) tissues.
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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 M1, M2, M3 are the 3 main subtypes. 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 M3, M5 receptors in benign prostatic hyperplasia tissue and prostatic carcinoma tissue were higher than that of normal prostate tissue. The expression of M3 was higher than M2 and M1 in prostatic carcinoma tissue, which shows that the M receptor subtype indeed has a certain relationship with the pathological changes of the prostate. The M1 receptor especially, has been considered to have relationship with several kinds of tumor. This experiment further confirms that the M3 receptor has a correlation with prostatic carcinoma. We also found that the M3 receptor is the most highly expressed in the normal prostatic tissue. The M2 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 M3 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 M3 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 M3 receptor can be expressed abundantly not only in the normal tissue, but also on the surface of many tumor cells. The M3 receptor can stimulate the proliferation of tumor cells through different signal pathways. Rayford et al found that M3 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. In vitro experiments also showed that the M receptor is an important index for prostatic epithelial differentiation; the expression of the M3 receptor is closely related to the classification of tumor. 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, including tumor cells, can secrete acetylcholine. Acetylcholine can bind the M3 receptor located on the cell surface through the self-secretion or paracrine tumor cell to stimulate the growth of tumors. The M3 receptor supplies a new target for the treatment of prostatic carcinoma. At present, M3 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 LMM1, we found that the main cholinergic receptor subtype is M3, and the amount of its expression is 40 times higher than that of LMM1 cell carbachol. Carbachol can stimulate the growth of LMM1 cell and neovascularization, which can be inhibited by selective M3 receptor antagonist p-Fluoro-hexahydrosila-difenidol (pf-HHSiD). Carbachol can stimulate the expression of VEGF-A, which can be also inhibited by the selective M3 receptor antagonist, pf-HHSiD. These data indicate that the M3 receptor plays an important role in the growth of human mammary tumor cells and vascularization.

We detected the gene expression of M3 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 M3 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 M3 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. Vascular endothelial growth factors can be regulated by other factors. Therefore, the results also show that the expression of the M3 receptor has a positive linear correlation with that of VEGF, further indicates that the M3 receptor participates in the pathological process.
of prostatic carcinoma. The function of the M₃ 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 M₃ receptor is expressed abundantly on the surface of lymphocytes. It not only participates in the apoptosis process of lymphocytes but also regulates many kinds of immune cells by complex mechanisms. Another study confirmed that the M receptor agonist carpine 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, 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 M₃ 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 M₃ receptor located on the lymphocyte.

References


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