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Author(s)	Hara, Yasuo
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# 主論文

Neoplastic epithelial cells express  $\alpha$ -subunit of muscle nicotinic acetylcholine receptor in thymomas from patients with myasthenia gravis

Yasuo Hara, Satoshi Ueno, Tomoyuki Uemichi, Nobuyuki Takahashi, Shiro Yorifuji, Yoshitaka Fujii\*, Seiichiro Tarui

The Second Department of Internal Medicine and Department of Neurology,

\*The First Department of Surgery, Osaka University Medical School. Osaka 553, Japan.

We studied the expression of mRNAs coding for  $\alpha$ -subunit of muscle nicotinic acetylcholine receptor (AChR) in thymomas from patients with myasthenia gravis (MG). Northern blot analysis failed to detect the expression, but amplification of mRNAs derived from thymomas by reverse transcription and polymerase chain reaction produced the DNA fragments containing the nucleotide sequence coding for a part of the  $\alpha$ -subunit. We further revealed that the  $\alpha$ -subunit mRNA was derived from neoplastic epithelial cells of thymoma. Our results support the hypothesis that AChR expressed in thymoma is a candidate for the primary antigen which induces autoimmune responses to muscle AChR. The close relationship between MG and thymoma may be at least in part explained by this hypothesis.

Key words: Thymoma; Myasthenia gravis; Acetylcholine receptor; mRNA expression; Polymerase chain reaction; Epithelial cell.

Correspondence address: Y. Hara, The Second Department of Internal Medicine, Osaka University Medical School. 1-1-50 Fukushima, Fukushima-Ku Osaka 553, Japan.

Abbreviations: AChR, acetylcholine receptor; MG, myasthenia gravis; PCR, polymerase chain reaction; bp, base pairs

## 1. INTRODUCTION

Myasthenia gravis (MG) is classified as an autoimmune disease in which the autoantibodies accelerate the degradation of the nicotinic acetylcholine receptor (AChR) at the muscle endplates, impairing the neuromuscular transmission [1]. However, a fundamental question remains unanswered as to where the locus responsible for the initiation of the antibody production to muscle AChR is present. Clinical evidence implicating the thymus in MG includes a high incidence of thymoma in patients with MG and the effectiveness of thymectomy as a treatment for many of them [2]. At the cellular level, much attention has been paid to myoid cells in the thymus, because these cells are suggested to have AChR on their surfaces [3].

However, the myoid cell seems to be over emphasized as a candidate for an antigen presenting cell. There is no difference in the myoid cell frequency between the thymuses from MG patients and control subjects. Our working hypothesis is that the neoplastic changes of epithelial cells start to express proteins, which may trigger an autoimmune response against AChR at the muscle endplates in MG patients. In the present study, we have provided direct evidence for the expression of mRNA coding for a part of  $\alpha$ -subunit of muscle nicotinic AChR in neoplastic epithelial cells from thymomas.

## 2. MATERIALS AND METHODS

### 2.1. RNA preparation

Total RNAs were prepared from biopsied specimens of kidney and muscle, and autopsied brain tissues from non-MG patients. Test samples from patients with MG included 5 thymomas and 5 hyperplastic thymuses obtained at surgery. Peripheral leukocytes from MG patients, and neoplastic epithelial

cells isolated from 2 individual thymomas of MG patients were cultured under the conditions as described previously [4]. The specimens were homogenized in a buffer containing guanidine thiocyanate, and layered over a cushion of CsCl and centrifuged [5]. After extraction with phenol and ethanol-precipitation, the total RNAs were subjected to Northern blot analysis and amplified by polymerase chain reaction (PCR) experiments.

#### 2.2. Northern blot analysis

Thirty  $\mu$ g of total RNAs from control muscle and MG patients' thymomas and hyperplastic thymuses were fractionated by 1 % agarose gel electrophoresis, and transferred to nylon membranes (Amersham). cDNA clone for the  $\alpha$ -subunit of human muscle AChR was labeled with  $^{32}$ P-dCTP by the random primer method [6]. After incubation with a  $^{32}$ P-cDNA probe, the membranes were washed according to the method of Merlie et al [7].

#### 2.3. Oligonucleotide primers for amplification of $\alpha$ -subunit mRNA

Based on the published nucleotide sequence of the  $\alpha$ -subunit gene of nicotinic AChR of human muscle [8], two oligonucleotide primers encompassing the putative acetylcholine binding site were prepared on Applied Biosystems model 381 A DNA synthesizer (Bioapplied) and purified by NENSORB column (Dupont). The forward primer hybridizes to nucleotides 375-398 of the antisense strand of exon 5 of the  $\alpha$ -subunit gene, while the reverse primer anneals to nucleotides 596-619 of the sense strand of exon 6. The sequences of these primers are (5'-AAGCTACTGTGAGATCATCGTCAC-3') and (5'-TGACGAAGTGGTAGGTGATGTCCA-3'), respectively.

#### 2.4. Reverse transcription and PCR of RNA

The first strands of cDNA were synthesized from 3.0  $\mu$ g of total RNAs prepared from 5 thymomas, hyperplastic thymuses, and neoplastic epithelial cells from 2 thymomas using Amersham cDNA Synthesis System according to the manufacturer's direction. The first strands were added to the PCR

reaction buffer to a total volume of 100  $\mu$ l, and amplified in the presence of the two synthesized primers using Taq polymerase under the conditions as specified by Perkin Elmer Cetus. The PCR run was consisted of 30 cycles of denaturation at 94°C, annealing at 42°C and extension at 74°C, for 60 seconds for each step. Three  $\mu$ g of total RNAs obtained from brain, kidney, and leukocytes were amplified in the same manner for dot blot analysis.

15 ng of muscle RNA and 280 ng of thymoma and hyperplastic thymus RNAs were subjected to serial doubling dilutions and amplified in the same manner to estimate the relative abundance of  $\alpha$ -subunit mRNA expression in the tissues. PCR products from these RNAs were precipitated with ethanol, fractionated on 6% polyacrylamide gel, and visualized by staining with ethidium bromide.

#### 2.5. Hybridization and sequence analyses

One tenth of PCR products from RNAs of thymic tissues, and cultured neoplastic epithelial cells were fractionated on 6% polyacrylamide gel, and electrophoretically transferred to nylon membranes. And one tenth of PCR products of total RNAs from brain, muscle, thymoma, kidney, leukocytes, and neoplastic epithelial cells were dotted on the nylon membrane. The membranes were hybridized with  $\alpha$ -subunit cDNA probe, washed in 0.1 XSSC at 65°C and autoradiographed. The fragments of 245 base pairs(bp) amplified from RNAs of neoplastic epithelial cells were cloned into M13 vector and sequenced by the dideoxy chain termination method [9].

### 3. RESULTS AND DISCUSSION

The expression of mRNA coding for AChR  $\alpha$ -subunit was detected in control muscles but not in 5 thymomas and 5 hyperplastic thymuses from

patients with MG by Northern blot analysis (fig.1). Since sufficient amounts of intact mRNAs were not obtained from the surgical specimens, we must take into account of the possibility that the sensitivity of Northern blot analysis was not sufficiently high for detecting  $\alpha$ -subunit mRNA of limiting amount. We used a more sensitive method of PCR combined with reverse transcriptase reaction. PCR requires only that the average molecular weight of the template RNA is slightly greater than the largest fragment to be amplified, and is capable of amplifying reverse transcribed mRNA by several orders of magnitude.

We could detect the amplified band of 245 bp in 4 thymomas and in 3 hyperplastic thymuses. Hybridization of the band with  $\alpha$ -subunit cDNA probe indicated that the thymic tissues contained  $\alpha$ -subunit mRNA (fig.2). Dot blot analysis showed that PCR product of RNA from the brain contained no fragments that bind to the  $\alpha$ -subunit cDNA probe. Amplified RNA from kidney showed a faint signal on autoradiograph indicating a low abundance of  $\alpha$ -subunit mRNA expression in the tissue (fig.3). Minimal doses of template RNAs were 1.9 ng from muscle (fig.4) and 35 ng from thymoma and hyperplastic thymus (data not shown) to identify the bands by ethidium bromide staining on a polyacrylamide gel. From these results, the relative abundance of  $\alpha$ -subunit mRNA of muscle to thymic tissues was estimated about 35 to 1.9.

Next, we investigated the origin of the  $\alpha$ -subunit mRNA in the thymic constituents. Dot blot analysis of the amplified leukocyte RNA from MG patients was negative (fig.3). The amplified products using total RNAs from cultured epithelial cells obtained from 2 thymomas were confirmed as an authentic copy of  $\alpha$ -subunit by Southern blot (fig.2) and sequence analyses. The product of 245 bp contained the nucleotide sequence identical to that of  $\alpha$ -subunit cDNA (125-206) including the coding region for the

acetylcholine binding site (Cys-Cys) (fig.5) [10,11]. The sequence was distinct from those of  $\alpha$ -subunit cDNAs of neuronal AChRs, because the homology of the amino acid sequences between the muscle and neuronal receptors was rather low in the amplified region [12]. These results indicate that the muscle  $\alpha$ -subunit mRNA is expressed in the neoplastic epithelial cells derived from MG-associated thymomas.

The specific expression of the  $\alpha$ -subunit mRNA in neoplastic epithelial cells was confirmed by the following procedures. The passages of the neoplastic epithelial cells in culture excluded the contamination by myoid cells, because the myoid cells could not be cloned under our culture condition. All cultured cells were immunohistologically stained with anti-keratin antibodies, indicating that they were of epithelial cell-origin. PCR produced an  $\alpha$ -subunit fragment of 245 bp, ruling out the contamination of the genomic DNA, because the amplification of  $\alpha$ -subunit genomic DNA resulted in the product of 650 bp including exon 5, intron and exon 6 (fig.4).  $\alpha$ -subunit fragments were amplified using total RNAs obtained from hyperplastic thymuses (fig.2). Since epithelial cells are not the main constituent in these thymuses, we can not confirm whether the epithelial cells synthesize  $\alpha$ -subunit mRNA. It may be possible that the  $\alpha$ -subunit mRNAs were derived from myoid cells because the cells are suggested by immunological studies to possess the receptors.

In contrast to our observations of  $\alpha$ -subunit mRNA expressions in neoplastic epithelial cells, the mature AChRs are not evidenced on the cells. Marx et al. showed that the monoclonal antibody against  $\alpha$ -subunit of muscle AChR recognizes a protein quite distinct from any known AChR chain in epithelial cells [13]. The explanations for the discrepancy of the expressions at RNA and protein levels include; AChR subunit genes are

rearranged, and RNA transcripts from the genes are out of phase inside the neoplastic epithelial cells [14]. Therefore AChRs are not expressed on the neoplastic cells. There is, however, no evidence supporting this explanation. Neither unusual rearrangements of the AChR gene nor major deletions are detected inside the AChR gene [15] .

Alternatively, the posttranslational modifications of the epithelial AChR are different from that of muscle AChR, and the immunogenicities of the two receptors are different. Based on this hypothesis and our data, we speculate that the neoplastic changes of a large number of epithelial cells in thymomas start to express AChR, and the neoplastic cells provide loci responsible for the initiation of the antibody production to muscle AChR in thymoma associated MG patients.

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FIGURE LEGENDS

Fig.1. Northern blot analysis of total RNAs from muscle and thymic tissues.

Fig.2. Polyacrylamide gel electrophoresis of PCR products of RNAs derived from thymic tissues and neoplastic epithelial cells (upper) and Southern blot analysis (bottom).

Fig.3. Dot blot analysis of PCR-amplified RNA .

Fig.4. PCR amplifications of genome and  $\alpha$ -subunit cDNA, and reverse transcription and PCR of serially diluted RNAs from muscle tissue. Doses of template RNAs are shown at the top of the lanes.

Fig.5. Nucleotide sequence coding for the putative acetylcholine binding site.

Fig. 1

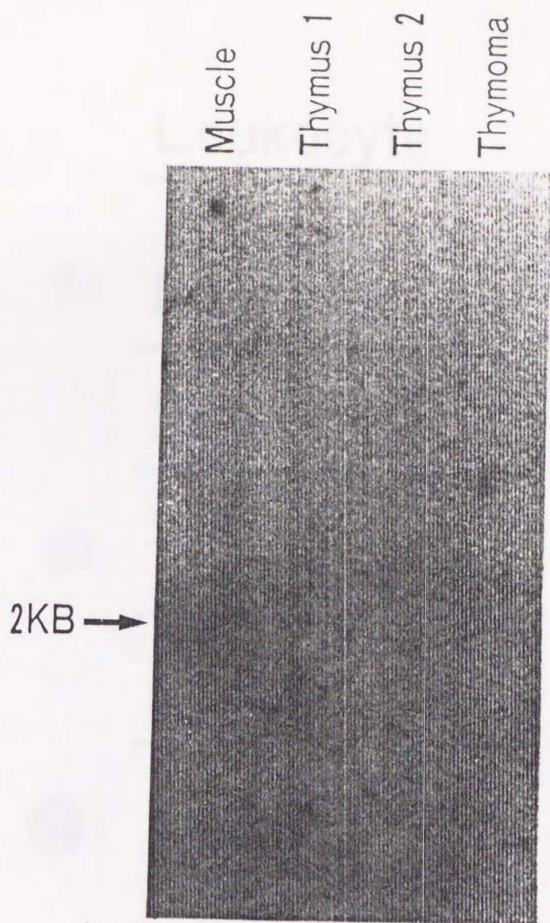


Fig. 2

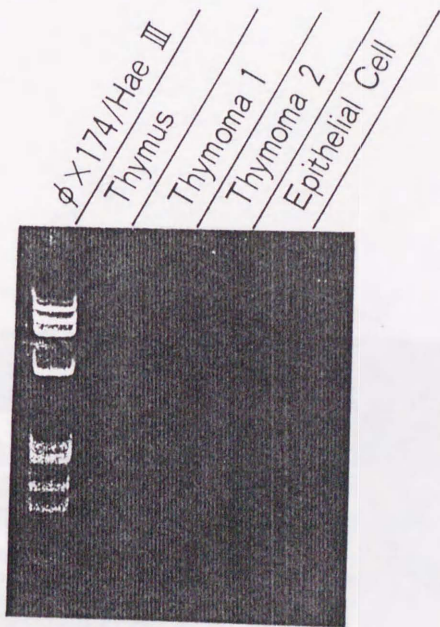


Fig. 3

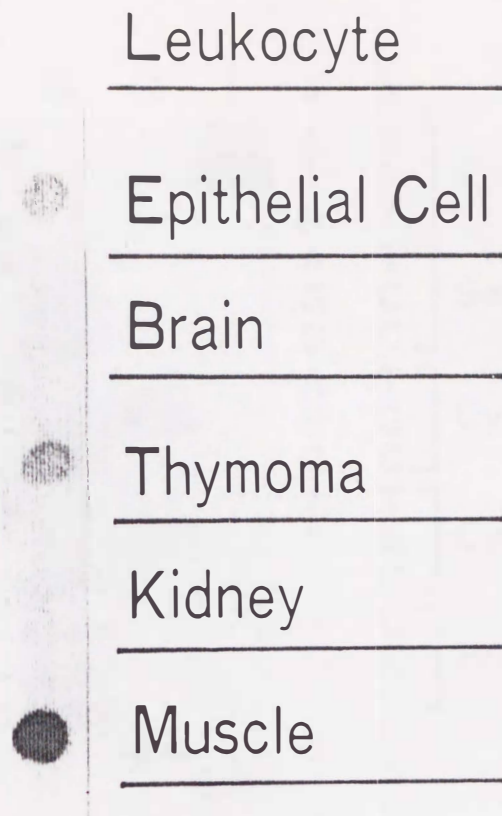


Fig. 4

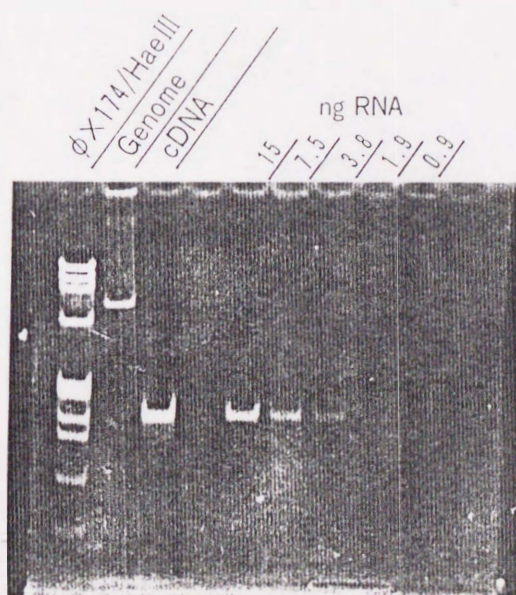


Fig. 5



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