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The Fetal Form of the Acetylcholine Receptor Distinguishes Rhabdomyosarcomas from Other Childhood Tumors

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The fetal nicotinic acetylcholine receptor (AChR) of muscle is an oligomeric membrane protein with subunit composition $\alpha_2\beta\delta\gamma$. After birth, the adult form, in which an ε-subunit replaces the γ-subunit, predominates, and expression of the fetal form is limited to thymic myoid cells, extraocular muscles, and denervated striated muscle. We looked for expression of AChR in rhabdomyosarcomas and other childhood tumors by reverse transcription polymerase chain reaction and immunohistochemistry. mRNA for the AChR γ-subunit was detected in all embryonal and alveolar rhabdomyosarcomas tested (n = 16) and in some tumors with a rhabdomyomatous component (n = 2) but not in other nonrhabdomyomatous tumors of childhood and adults (n = 45). The fetal form of the AChR was detected immunohistochemically in five of eight embryonal and four of eight alveolar rhabdomyosarcomas and in two Wilms' tumors with a rhabdomyomatous component but not in other tumors or in normal muscle. We conclude that reverse transcription polymerase chain reaction for AChR γ-subunit could be useful for the diagnosis of rhabdomyosarcoma of childhood and for the detection of micrometastases and minimal residual disease. In addition, the fetal AChR protein is the first extracellular tumor marker that can distinguish rhabdomyosarcomas from nonrhabdomyomatous tumors and from normal muscle. Our findings, therefore, imply that the fetal AChR may be a target for *in vivo* imaging and, as AChR internalization and degradation is increased by antibody-induced cross-linking, may also provide a sensitive and specific target for immunotherapeutic strategies. *(Am J Pathol 1998, 152:437–444)*

Pediatric soft-tissue sarcomas account for 10% of all childhood cancers,[1] and nearly 50% are rhabdomyosarcomas (RMSs).[1,2] Currently, these are classified according to the International Classification of Rhabdomyosarcomas.[3] Despite the availability of several immunohistological markers, such as myosin, desmin, myoglobin, actin, titin, dystrophin, MyoD1, and myogenin,[4–11] the differential diagnosis of RMS from other childhood neoplasms remains a challenge, particularly in tumors with a low degree of rhabdomyomatous differentiation or with regard to the detection of minimal residual tumors or micrometastases.

The combination of immunohistochemistry, cytogenetic analysis,[12] and molecular biology can improve the diagnosis in some cases, particularly in the many alveolar RMSs that have been shown to bear characteristic translocations, t(2;13) (q35;q14) and t(1;13) (p36;q14),[13–18] involving the PAX7 gene on chromosome 1, the PAX3 gene on chromosome 2, and the FKHR gene on chromosome 13.[19] In embryonal RMS, however, no consistent chromosomal abnormalities have been found.

Currently there is a mortality rate of 30% after modern chemotherapy,[26] and RMSs have not been amenable to immunotherapeutic trials. A marker that is both specific for RMS and expressed on the surface of the tumor cells would be useful in diagnosis, and such a surface marker could also provide a target for immunotherapy. Nuclear expression of MyoD1 and myogenin is found specifically in 90% of embryonal and alveolar RMSs,[5,11,20] but MyoD1 or myogenin proteins are not expressed on the surface of RMS cells.[20] Markers such as dystrophin,[21,22] which can be helpful in the diagnosis of RMS,[10] are intracellular and are shared between RMS and normal muscle that sometimes infiltrates other tumors.[23,24] Equally, dystrophin-associated protein complex (DAP), which has an extracellular domain,[25] is also shared between RMS and normal muscle.[21,25]

The nicotinic AChR of skeletal muscle consists of a pentameric ion channel, which is composed of four subunits, each with extensive extracellular domains[27] that are accessible to monoclonal antibodies and spontane-

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ous autoantibodies.[28–30] During development of the neuromuscular junction, a change from the fetal type ($\alpha_2\beta\gamma\delta$) to the adult type ($\alpha_2\beta\varepsilon\delta$) occurs when the γ-subunit is replaced by the ε-subunit.[31,32] After birth, the fetal type of the AChR is limited to myoid cells in the thymus[33–35] and some extraocular muscle fibers[36] but can be re-induced in normal skeletal muscle by denervation.

As RMSs consist of immature and noninnervated neoplastic myoblasts, our hypothesis was that the fetal type of the AChR, and specifically its γ-subunit, could be a tumor-specific marker distinguishing RMS from normal muscle and other childhood tumors. In this study we use reverse transcription polymerase chain reaction (RT-PCR) to show that hyperexpression of the AChR γ-subunit mRNA is found in all RMSs and is rare or absent in other nonrhabdomyomatous childhood tumors, normal muscle, normal tissues, and adult neoplasms. Furthermore, our immunohistochemical findings imply that the fetal AChR protein, due to its extracellular domain, is a promising target for *in vivo* immunoimaging and immunotherapy.

Materials and Methods

Materials

Sixteen RMSs and twenty-nine different other childhood tumors were studied using cryostat sections from snap-frozen tissue obtained on ice within 15 minutes to 4 hours of surgery. RMSs were classified according to the International Classification of Rhabdomyosarcomas.[3] Ten normal muscles and eight other normal tissues were derived from autopsies and biopsies. Autopsy material was obtained within 4 hours after death and checked by PCR analysis of glyceraldehyde phosphate dehydrogenase (GAPDH) message (22 cycles) for integrity of RNA. Biopsies were obtained within 15 minutes. The embryonal RMS cell line TE671[37] served as a positive control. Eighteen adult nonrhabdomyomatous tumors were obtained for frozen section diagnosis within 15 minutes after biopsy and used as negative controls. Clinical and pathological findings of the patients investigated are given in Tables 1 and 2.

RT-PCR

Total RNA was prepared from 100 mg of snap-frozen tissue cut into 10-μm sections on a cryostat or from 10^6 cells using the GTC method.[40] After cDNA synthesis with oligo-dT primers and MMLV reverse transcriptase (Gibco, Eggenstein, Germany), 1/20 of the reaction was amplified using *Taq* polymerase (Amersham, Braunschweig, Germany) and sequence-specific primers. The oligonucleotide primers of the acetylcholine receptor α-, β-, γ-, δ-, and ε-subunit (henceforth called α-AChR, β-AChR, γ-AChR, δ-AChR, and ε-AChR, respectively)[41–44] were as follows: forward (F) α-AChR, 5′ AAGCTACTGTGAGATCATCGTCAC 3′; reverse (R) α-AChR, 5′ TGACGAAGTGGTAGGTGATGTCCA 3′; Fβ-AChR, 5′ CCAGGAAGTGGCTGGGGTCGGGGA 3′; Rβ-AChR, 5′ TTCAAGGAAAGGGGTCTGGAGGGG 3′; Fγ-AChR, 5′ ATCTCAGTCACCTACTTCCCC 3′; Rγ-AChR, 5′ TACTTGCTGATGAGTGGCACC 3′; Fδ-AChR, 5′ GAGAAGGGCTACAACAAGGAGCTCC 3′; Rδ-AChR, 5′ TAGGTGACAGAGATGGGGCAGGAGG 3′; Fε-AChR, 5′ TATAGAAACCCTGCGAGTCCC 3′; Rε-AChR, 5′ GGGCACGATGATGTTAATGAC 3′. Amplifications were carried out at 68°C for α-AChR, β-AChR, and δ-AChR primers and at 65°C for γ-AChR and ε-AChR primers (35 cycles each). Primer pairs for GAPDH were used as control (60°C for 22 cycles).[45]

Semiquantitative RT-PCR

The RNA integrity was confirmed in all samples by the detection of a 920-bp GAPDH product in ethidium-bromide-stained gels. A semiquantitative PCR was established by adjusting all cDNAs to equal amounts of GAPDH transcripts. Ethidium bromide staining of the α-AChR, β-AChR, γ-AChR, δ-AChR, and ε-AChR amplification products revealed bands of the expected molecular size, and subsequent sequencing of the PCR products confirmed that the cDNA fragments were identical to published AChR subunit sequences.[41–44]

Cloning and Sequencing of the PCR Products

For sequencing of the PCR products, bands were cut from agarose gels and DNA was extracted with Jet-Sorb (Genomed, Bad Oeynhausen, Germany). Eluted DNA was cloned into the pGEM-T vector (Promega, Heidelberg, Germany), and the ligation mixture was transformed in JM 109 competent cells. DNA of recombinant colonies was isolated by mini-preparation[46] and sequenced by the cycle-sequencing method using dye terminators and the ABI 373A sequencer following the instructions of the manufacturer (Applied Biosystems, Weiterstadt, Germany).

Northern Blot Analysis

Five micrograms of total RNA was electrophoresed on a denaturing 1% formaldehyde-agarose gel and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The blots were hybridized with a ^{32}P-labeled probe specific for the γ-AChR 390- to 859-, ε-AChR 212- to 691-, and GAPDH 102- to 1056-nucleotide sequence and washed under standard conditions.[46] [α-^{32}P]dATP was purchased from DuPont (Bad Homburg, Germany). Radiolabeled probes were prepared using a random priming DNA labeling system (Stratagene, Heidelberg, Germany). The film was exposed for 48 hours at −70°C.

Southern Blot Analysis

Ten microliters of each PCR product was run on a 1.5% agarose gel containing ethidium bromide. For hybridization, probes specific for the ε- and γ-subunit of the AChR

Table 1. Pathological and Clinical Findings, RT-PCR, and Immunoreactivity for γ-AChR in Patients Whose Tumors Were Investigated

Case	Diagnosis	Sex	Age	Localization	γ-AChR	
					RT-PCR	MIB-8
7547/90	ER*	M	2 years	Hypogastrium	+	+
18325/95	ER*	F	7 years	Hypogastrium	+	−
25923/95	ER*	F	7 years	Tongue	+	+
11563/92	ER*	M	4 years	Mediastinum	+	+
17285/90	ER*	M	2 years	Small pelvis	+	−
11279/87	ER*	M	2months	NA	+	+
7863/95	ER*	M	55 years	Suprarenal body	+	−
3207/93	ER*	M	81 years	Thyroid gland	+	+
10162/97	AR*	F	13 years	Cervical region	+	+
15378/89	AR*	M	4 years	Testis	+	+
17940/94	AR*	M	4 years	Cervical region	+	−
14097/90	AR*	F	27 years	Angle of mandible	+	+
5750/93	AR*	M	15 years	Cervical region	+	−
11421/89	AR*	F	3 years	NA	+	+
32931/88	AR*	F	23 years	Right calf	+	−
18471/96	AR*	F	15 years	Mediastinum	+	−
22064/90	NB	F	3 years	Suprarenal body	−	−
16729/91	NB	F	3months	Suprarenal body/epigastrium	−	−
5964/92	NB	M	2 years	Epigastrium	−	−
17600/93	NB	M	5months	Suprarenal body	−	−
10796/97	NB	M	8 years	Kidney	−	−
11633/96-2	ES	F	41 years	Back muscle	+	+
11633/96-5	ES	F	41 years	Vertebral canal	−	−
18191/92	ES	F	1 year	NA	−	−
21591/93	ES	M	10 years	Right femur	−	−
24483/95	ES	M	18 years	Hip bone	−	−
8053/93	SS	F	11 years	NA	−	−
20897/96	OS	F	17 years	Femur	−	−
871/90	CS	M	11 years	Tibia	−	−
14744/89	WT	F	4 years	Left kidney	+	+
9496/94	WT	F	1 year	Left kidney	−	−
7454/94	WT	F	5 years	Right kidney	−	−
19002/93	WT	M	5 years	Right kidney	−	−
3839/96	WT	F	8 years	Left kidney	−	−
4841/90	WT	M	9 years	Left kidney	+	+
13126/93	GS	M	1 year	Testis	−	−
19217/93	TE	M	1 year	Testis	−	−
11037/96	SE	M	15 years	Testis	−	−
22046/96	MG	M	21 years	Testis	−	−
12531/93	ME	M	3 years	Paranasal sinus	−	−
27262/94	GN	F	5 years	Kidney	−	−
22946/95	SCHW	F	8 years	NA	−	−
7400/96	CAC	M	2 years	Suprarenal body	−	−
883/97	ACC	M	13 years	Orbit	−	−
3551/97	LBL	M	16 years	Lymph node	−	−
5171/96	LBL	M	4 years	Lymph node	−	−

ER, embryonal rhabdomyosarcoma; AR, alveolar rhabdomyosarcoma; NB, neuroblastoma; ES, Ewing's sarcoma; SS, synovial sarcoma; OS, osteosarcoma; CS, chondrosarcoma; WT, Wilms' tumor; GS, germinal stroma tumor; TE, teratoma; SE, seminoma; MG, malignant germinoma; ME, meningioma; GN, ganglioneuroma; SCH, schwannoma; CAC, carcinoma of adrenal cortex; ACC, adenoid cystic carcinoma; LBL, lymphoblastic lymphoma; NA, not available. *Classified according to the International Classification of Rhabdomyosarcoma (ICR).

and the GAPDH gene were labeled with [α-^{32}P]dATP from DuPont using terminal deoxynucleotidyl transferase (Gibco). PCR products were transferred onto a positively charged nylon membrane (Hybond N+, Amersham) by overnight alkaline-capillary blotting,[46] hybridized, and washed under standard conditions.[46] The film was exposed for 6 hours at −70°C.

Immunohistochemistry

The monoclonal mouse antibody MIB-8, raised against denervated human AChR,[29] binds specifically to the fetal form of the whole oligomer and to the γ-subunit on Western blots (L. Jacobson, D. Beeson, and A. Vincent, manuscript in preparation). The monoclonal rat antibody MAb 195 binds to the α-subunits of both adult and fetal human AChR.[38] The antibodies were used in a four-step immunoperoxidase labeling for single antigens in air-dried, acetone-fixed sections as described previously.[39] The working dilution for both monoclonal antibodies was 1:100. Furthermore, RMSs were routinely stained for desmin, actin, and titin.[7,8]

The statistical significance was determined by the Fisher exact test.

Table 2. Nonrhabdomyomatous Tumors and Nonneoplastic Tissues Investigated for the Expression of γ-AChR: Results of RT-PCR and Immunohistochemistry with MIB-8

Control tissues	Number of samples		Age (years)	γ-AChR	
	Female	Male		RT-PCR	MIB-8
Adult nonrhabdomyomatous tumors (n = 18)*	9	12	28–84	–	–
Normal muscle (n = 10)†	3	7	15–65	–	–
Normal tissue (n = 8)‡	5	3	21–84	–	–

* Three carcinomas of the breast, three carcinomas of the kidney, three leiomyosarcomas, three carcinomas of the stomach, three carcinomas of the lung, and three carcinomas of the ovary.

† Musculus (M.) biceps brachii, M. triceps brachii, M. extensor digitorum, M. sternocleidomastoideus, M. pectoralis major, M. latissiumus dorsi, M. rectus abdominis, M. quadriceps femoris, M. peronaeus longus, and diaphragm.

‡ Heart, lung, tonsil, liver, stomach, kidney, lymph node, and brain.

Results

γ-AChR mRNA Is Strongly Expressed in Rhabdomyosarcomas

Sixteen RMSs and ten normal muscles were examined by RT-PCR with AChR subunit-specific primers. Transcripts from α-AChR, β-AChR, δ-AChR, and ε-AChR subunit genes could be detected in all RMSs as well as in normal muscles in roughly similar quantities. By contrast, γ-AChR mRNA was overexpressed in all investigated RMSs compared with normal muscles (Figure 1). Expression of the γ-subunit was much greater than that of the ε-subunit. This finding was confirmed by Northern blot analysis with probes specific for the γ-AChR, ε-AChR, and GAPDH genes (Figure 2).

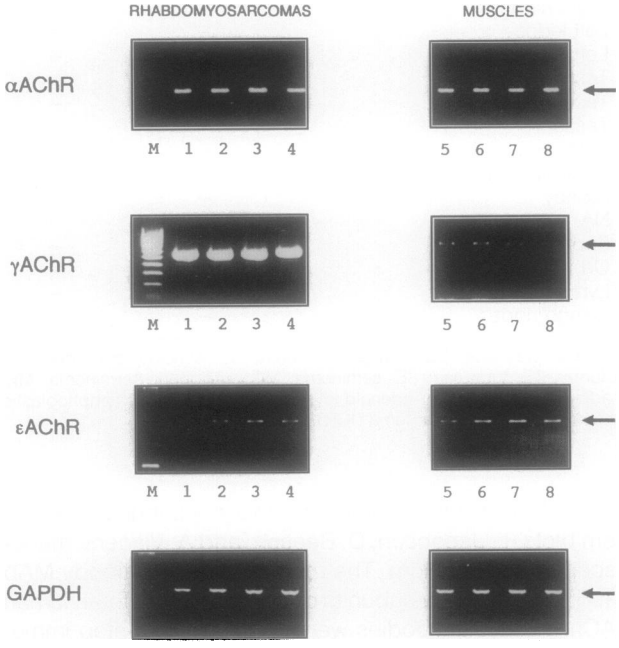

Figure 1. RT-PCR analysis of AChR subunit gene transcription in RMS and normal muscle (β-AChR and δ-AChR not shown). cDNAs were adjusted to identical contents according to GAPDH transcription (**arrow**). The α-AChR and ε-AChR are transcribed in similar quantities in RMS and normal muscle (**arrow**). By contrast, the γ-AChR was overexpressed in RMS compared with normal muscle (**arrow**). **Lane 1**, case 7547/90, ER; **lane 2**, case 11563/92, ER; **lane 3**, case 5750/93, AR; **lane 4**, case 11421/89, AR; **lane 5**, M. biceps brachii; **lane 6**, M. sternocleidomastoideus; **lane 7**, M. quadriceps femoris; **lane 8**, diaphragm.

Sensitivity of RT-PCR in Detecting AChR mRNA Expression

The usefulness of the RT-PCR technique for the detection of minimal residual disease, or for cytology, would depend on its sensitivity. To determine this, we performed serial dilutions of TE 671 RMS cells with Raji cells to obtain mixtures of 10^4, 10^3, 10^2, and 1 RMS cell(s) in 10^6 Raji cells for mRNA extraction. In addition, mRNAs extracted from normal muscle biopsies and from Raji cells were adjusted to identical mRNA contents according to GAPDH expression and mixed to make dilutions of 10^{-2} to 10^{-6} muscle mRNA in Raji cell mRNA. Duplicate samples were tested by RT-PCR and Southern hybridization (Figure 3). mRNA of α-AChR was detected in TE671 cells and in normal muscle at concentrations as low as 100 cells mixed with 10^6 Raji cells. The γ-AChR mRNA was detected in TE671 cells at the even lower concentration of 10 cells in 10^6 Raji cells. By contrast, in normal muscle

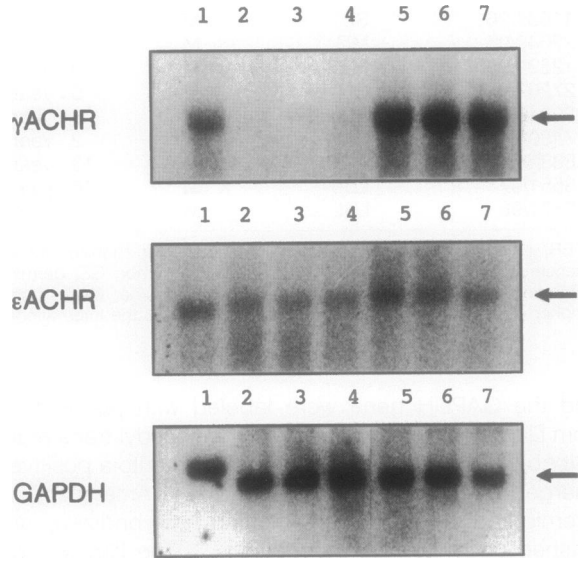

Figure 2. Northern blot analysis of TE 671 cell line, normal muscles, and RMS with probes specific for the γ-AChR, ε-AChR, and GAPDH. mRNAs were adjusted to equal amounts by GAPDH transcription (**arrow**). No significant difference was seen between RMS and normal muscle for transcription of the ε-AChR (**arrow**), whereas the γ-AChR was overexpressed in RMS compared with normal muscle (**arrow**). **Lane 1**, TE671 cell line; **lane 2**, M. biceps brachii; **lane 3**, M. rectus abdominis; **lane 4**, diaphragm; **lane 5**, ca 32931/88, AR; **lane 6**, case 17285/90, ER; **lane 7**, case 18325/95, ER.

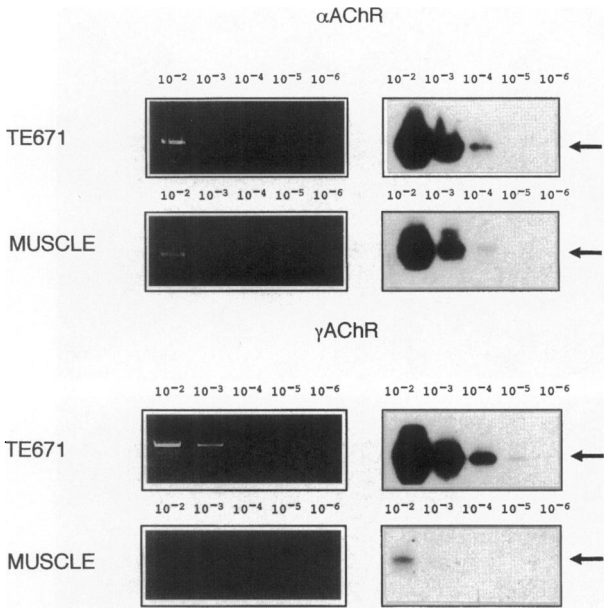

Figure 3. Sensitivity of RT-PCR for detection of α-AChR and γ-AChR mRNA from TE671 cells and from normal muscle mRNA mixed with mRNA from 10^6 Raji cells. PCR products were stained with ethidium bromide (**left**) and detected using Southern blot hybridization (**right**). In TE671 cells and in normal muscle, mRNA of the α-AChR was detected at concentrations as low as 100 cells in 10^6 Raji cells. The γ-AChR mRNA was detected in TE671 cells at the even lower concentration of 10 cells mixed with 10^6 Raji cells, whereas in normal muscle the γ-AChR was detectable at the concentration of 10,000 cells in 10^6 Raji cells. This indicates that γ-AChR is expressed approximately 1000-fold greater in rhabdomyosarcomas compared with normal muscle.

the γ-AChR mRNA was detected only at concentrations equivalent to or higher than 10,000 cells in 10^6 Raji cells. Therefore, there was an approximately 1000-fold difference in the γ-subunit expression between TE671 cells and normal muscle. Similar results were obtained when RMSs (case 17940/94, case 11421/89, and case 25923/95) were used instead of TE671 cells in analogous experiments.

γ-AChR mRNA Is Absent from Tumors without Rhabdomyomatous Differentiation

We next tested 29 childhood tumors, 18 adult nonrhabdomyomatous tumors, and 8 normal tissues for the expression of AChR transcripts. In all tumors and normal tissues, transcripts for the α-AChR, β-AChR, δ-AChR, and ε-AChR genes could be amplified, as shown for the α-AChR subunit in Figure 4, a and b. By contrast, the γ-AChR mRNA was detected only in two Wilms' tumors with a rhabdomyomatous component (cases 14744/89 and 4841/90) and in a muscle infiltrated by a Ewing's sarcoma (case 11633/96-2). Strikingly, another Ewing's sarcoma biopsy from the latter patient, derived from the vertebral canal and devoid of tumor-infiltrated muscle (case 11633/96-5), showed no amplification of the γ-AChR (Figure 4a). This suggests that γ-AChR mRNA might have been amplified from the infiltrated muscle rather than from the Ewing's sarcoma itself (see below).

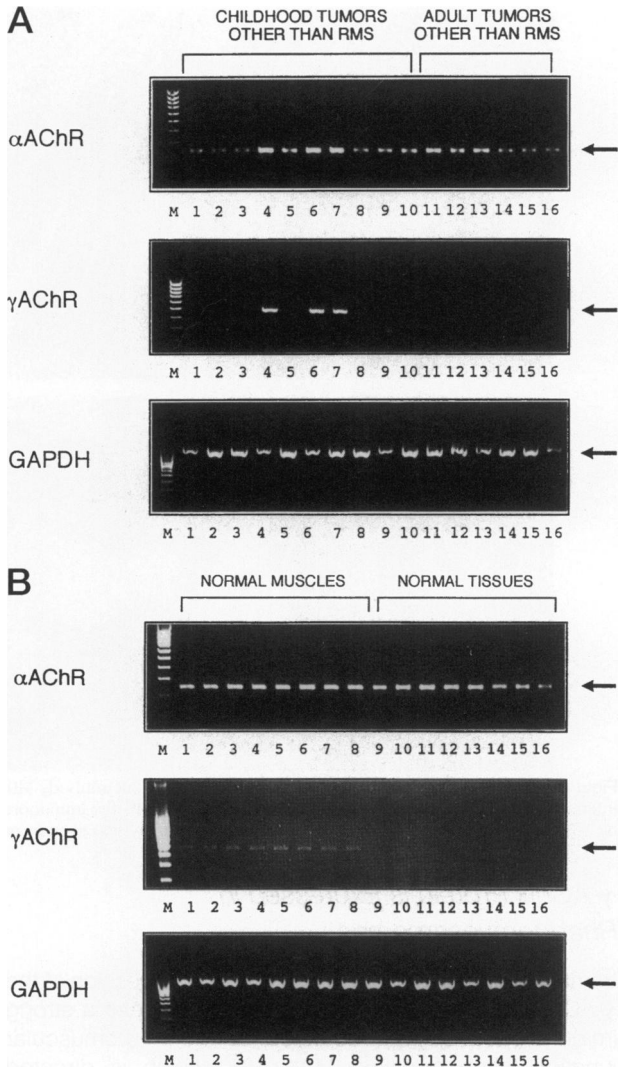

Figure 4. A: RT-PCR with primers specific for the α-AChR and γ-AChR of different childhood (**lanes 1** to **10**) and adult (**lanes 11** to **16**) tumors other than rhabdomyosarcomas. All investigated tumors showed amplification of the α-AChR (**arrow**). By contrast, the γ-AChR mRNA was detected only in two Wilms' tumors with a rhabdomyomatous component (**lane 6**, case 14744/89, and **lane 7**, case 4841/90) and in a muscle infiltrated by a Ewing's sarcoma (**lane 4**, case 11633/96-2) (**arrow**). In contrast, a biopsy from the same patient (**lane 5**, case 11633/96-5) derived from the vertebral canal without contaminating muscle, showed no amplification. **Lane 1**, NB, case 5964/92; **lane 2**, GN, case 27262/94; **lane 3**, SE, case 11037/96; **lane 4**, ES, case 11633/96-2; **lane 5**, ES, case 11633-5; **lane 6**, WT, case 14744/89; **lane 7**, WT, case 4841/90; **lane 8**, OS, case 20897/96; **lane 9**, CS, case 871/90; **lane 10**, LBL, case 3551/97; **lane 11**, carcinoma of the breast; **lane 12**, carcinoma of the kidney; **lane 13**, leiomyosarcoma; **lane 14**, carcinoma of the stomach; **lane 15**, carcinoma of the lung; **lane 16**, carcinoma of the ovary (for abbreviations see Table 1). **B:** RT-PCR with primers specific for the α-AChR and γ-AChR of normal muscles (**lanes 1** to **8**) and normal tissues (**lanes 9** to **16**). The α-AChR was amplified in all investigated muscles and nonmyogenous tissues (**arrow**), whereas the γ-AChR showed very few transcripts in muscles (**arrow**) and none in nonmyogenous tissues. **Lane 1**, M. biceps brachii; **lane 2**, M. triceps brachii; **lane 3**, M. sternocleidomastoideus; **lane 4**, M. latissimus dorsi; **lane 5**, M. quadriceps femoris; **lane 6**, M. rectus abdominis; **lane 7**, M. peronaeus longus; **lane 8**, diaphragm; **lane 9**, heart; **lane 10**, lung; **lane 11**, tonsil; **lane 12**, liver; **lane 13**, stomach; **lane 14**, kidney; **lane 15**, lymphnode; **lane 16**, brain.

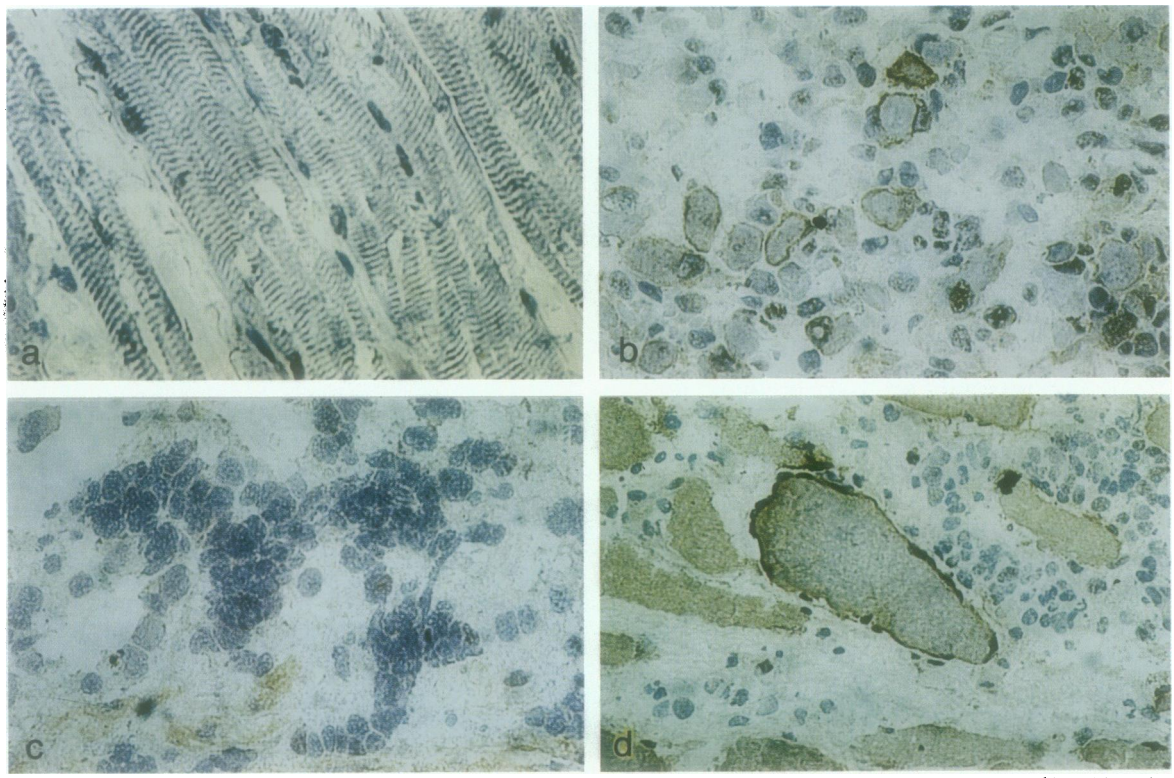

Figure 5. Immunohistochemical staining with the anti-γ-AChR antibody MIB-8 of normal muscle (**a**), AR case 11421/89 (**b**), NB case 5964/92 (**c**), and a muscle infiltrated by a Ewing's sarcoma case 11633-2/95 (**d**). In RMS, the immunoreactivity was clearly restricted to the cell surface (**b**) as well as in a muscle infiltrated by a Ewing's sarcoma (**d**). By contrast, no immunoreactivity was seen in normal muscle (**a**) or neuroblastoma (**c**). Immunoperoxidase; magnification, ×400.

γ-AChR Protein Is Expressed in Rhabdomyosarcomas

Immunostaining with MIB-8 revealed no expression of the γ-AChR on normal muscle (Figure 5), whereas a strong immunoreactivity was detected at the neuromuscular junction when applying MAb 195, which is directed against the α-AChR (not shown). By contrast, 9 of 16 RMSs showed positive staining with MIB-8 (see Table 1). Immunoreactivity tended to be more intense in the more differentiated appearing myoblasts and was particularly strong in strap-shaped rhabdomyoblasts. Immunoreactivity was mainly restricted to the cell surface as expected for binding to a membrane protein. The positive PCR results for two Wilms' tumors with a rhabdomyomatous component (case 14744/89 and case 4841/90, not shown) and a muscle infiltrated by a Ewing's sarcoma (case 11633/96-2, Figure 5) were also confirmed by immunohistochemistry.

Discussion

We used both RT-PCR and immunohistochemistry to look at expression of the γ-subunit that specifies the fetal form of the AChR and found that the γ-subunit was strongly transcribed in 16 of 16 RMSs and also in tumors with a rhabdomyomatous component. By contrast, very few transcripts of the γ-AChR were detected in normal muscle, and no mRNA for the γ-AChR could be found in different normal tissues, various nonrhabdomyomatous childhood tumors, and adult neoplasms. Therefore, the transcription of the γ-subunit gene of the AChR is significantly associated with a rhabdomyomatous differentiation ($P < 0.001$, Fisher exact test). mRNA for the other AChR subunit genes were detectable by PCR in normal muscle but were also found in nonrhabdomyomatous tumors (Figure 4a) and in many normal tissues, as reported previously by Hara et al.[33] The ratio of α-AChR/γ-AChR, determined by semiquantitative PCR, was 100:1 in normal muscle but 1:10 in the rhabdomyosarcoma cell line TE671 and in all RMSs tested, indicating that the γ-AChR is overexpressed approximately 1000-fold in RMSs compared with normal muscle. Thus, we propose that γ-AChR mRNA is a useful tumor-specific marker for RMS and for other tumors with a rhabdomyomatous differentiation.

In most alveolar RMSs, a characteristic translocation t(2;13) (q35;q14) can be detected by PCR in approximately 60 to 70% of cases.[14–18] For embryonal RMS with its subtypes, such an unequivocal marker has not been available. Although myogenin and MyoD1 are expressed at the protein levels in RMS[5,20] and the myogenin and MyoD1 genes, like the γ-AChR gene, are transcribed after birth at very low levels in normal skeletal muscle,[32,48–53] their transcripts could also be detected in some nonrhabdomyomatous small round-cell tumors.[47] Thus, the PCR-based detection of γ-AChR message should be useful in the accurate diagnosis of difficult primary tumors, and due to the high sensitivity of the method (Figure 3), it might also help with the detection of

micrometastases and minimal residual disease. Moreover, in positive cases, the semiquantitative determination of α-AChR/γ-AChR mRNA ratios should help to distinguish between RMS and contaminating normal muscle (Figure 3). In this situation, however, it would be important to bear in mind that infiltration of normal muscle by a nonrhabdomyomatous tumor may cause denervation, leading to overexpression of the γ-AChR, as suggested in case 11633/96-2 (Figure 4a).

The γ-subunit of the fetal AChR was shown to be expressed at the protein level in 9 of 16 RMSs and in two Wilms' tumors. γ-AChR immunohistochemistry in RMS is therefore a less sensitive method than the PCR-based approach and also less sensitive than immunohistochemistry for the contractile proteins desmin, dystrophin, MyoD1, or myogenin.[4–11,20] However, in contrast to the results of MyoD1 and myogenin expression,[20] γ-AChR immunoreactivity appeared stronger in the more differentiated rhabdomyoblasts and strap-shaped rhabdomyoblasts. Therefore, immunolabeling with fetal AChR-specific antibodies could be used, for instance, to evaluate the degree of RMS maturation before and after chemotherapy treatment.[54]

Immunostaining indicated that, as expected, the fetal AChR was predominantly at the RMS cell surface, but it was also, as expected, absent from nonrhabdomyomatous tumors and normal muscle (Figure 5). Therefore, the fetal AChR protein is the only rhabdomyosarcoma marker described so far that is both tumor specific (like the nuclear antigens MyoD1 and myogenin)[5,20] and expressed on the outer cell membrane.[27] As a consequence, radiolabeled anti-γ-AChR antibodies, such as MIB-8, might be useful tools for imaging RMS *in vivo*. The potential safety of this procedure is demonstrated by a recent study in which spontaneous maternal antibodies specific for the fetal AChR were implicated in causing paralysis and contractures in the developing fetus, without symptoms in the mother.[55] Moreover, as internalization of surface-expressed AChRs for degradation is enhanced by antibody cross-linking,[56] fetal-type AChRs may also be tumor-specific targets for immunotherapeutic strategies.

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