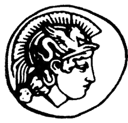

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The truncated estrogen receptor alpha variant lacking exon 5 is not involved in progesterone receptor expression in meningiomas

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Abstract

Human meningioma tissues are mostly estrogen receptor (ER) negative and progesterone receptor (PR) positive in ligand binding and enzyme immuno assays. To explain this apparently ER independent PR expression, we investigated the existence of a 'hidden' ER variant, which would be capable of activating transcription of the PR gene. Total RNA of seven meningiomas, two breast cancer tissues and of MCF7 cells was analyzed by RT-PCR using primers situated in exon 4 and exon 6. Differential hybridization of the PCR transcripts with probes in exon 4 and 5 respectively, revealed a wild type ER (wtER) fragment and an exon 5 deleted ER variant (ERΔ5). PCR products of two meningiomas were cloned for sequence analysis. The result confirmed the existence of a wtER and ERΔ5.

RT-PCR followed by Southern analysis was performed on mRNA of 23 meningiomas to determine the amount of ERΔ5 relative to wtER, which was compared to the PR content of the tissues. In contrast to our initial hypothesis and literature data on breast cancer, there was no relationship between the ERΔ5/wtER ratio and PR protein concentration. It is therefore concluded that ERΔ5 mRNA does not play the dominant role in PR synthesis in meningioma tissue. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Alternatively spliced estrogen receptor; Meningioma; Progesterone receptor expression

1. Introduction

Meningiomas are tumors that arise from the arachnoid cells of the leptomeninges.

Like in breast cancer, female sex hormones are thought to be involved in the etiology of meningiomas. Meningiomas differ considerably from breast cancer tissue however, since the vast majority of these tumors expresses PR in the virtual absence of ER [1,2]. We have previously demonstrated the presence of an ER-like protein in ER+/PR+, ER−PR+ and ER−/PR-meningiomas [3].

ER itself, a member of the steroid/thyroid/vitamin D superfamily of nuclear receptors, acts as a ligand activated transcription factor that binds to an estrogen responsive element (ERE) to mediate transcription of specific genes such as PR. This mechanism is accepted nowadays for the ER in breast and uterine tissues, it could not explain however the ER−/PR+ phenotype found in approximately 5% of the breast tumors. Using PCR analysis wtER and exon deleted ER transcripts were found in these, at protein level, ER negative tissues. By analogy with breast cancer tissue it was hypothesized that such ER variants might contribute to the apparent autonomous PR transcription in the ER−/PR+ meningioma tissues [4]. We initiated a

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search for such ER variants in human meningiomas. In addition to a wild type ER we detected an ERΔ4 and an ERΔ7 variant [5]. We found the ERΔ4 protein to have no transcriptional activity [6] and the ERΔ7 protein reportedly is dominant negative [7]. It was considered very unlikely therefore that these variants would account for the estrogen independent PR synthesis [6]. In breast tumor tissue an ERΔ5 variant has been described that constitutively stimulates estrogen-responsive genes in a yeast reporter system [8] and in stably transfected MCF7 cells [9]. In the present study, we investigated the prevalence of such an ERΔ5 variant in meningioma tissue in search for an explanation for the ER−/PR+ phenotype of most meningiomas.

2. Materials and methods

2.1. Tissues and cells

The breast cancer cell line MCF7 was routinely grown in RPMI-1640 with 10% fetal calf serum (Gibco BRL, Paisley, UK) with phenol red. Human meningioma and breast cancer tissues were placed on ice immediately after removal from the patients. Specimens were frozen at −80°C until they were used for RNA extraction. Steroid receptors were measured in cytosols by ligand binding assay or enzyme immuno assay as described earlier [10].

2.2. RNA isolation

Total RNA was prepared from 250 mg of tissue or 1×10^6 MCF7 cells [11]. The concentration of RNA was determined spectrophotometrically and its integrity assessed by gel electrophoresis.

2.3. CDNA synthesis

One microgram of total RNA was denatured for 10 min at 70°C. cDNA synthesis was carried out in 20 μl 10 mM Tris-HCl (pH 8.3) containing 50 mM KC1, 5 mM $MgCl_2$, 0.5 mM dNTP, 100 ng random hexamer primer, 20 U RNAse inhibitor (Boehringer, Mannheim, Germany) and 100 U of M-MLV RT superscript (BRL, Bethesda, MD, USA). After denaturation of the RNA the reverse transcription was performed in one cycle: 45 min of incubation at 42°C, 5 min enzyme inactivation at 95°C and 5 min at 4°C.

2.4. PCR amplification

2.4.1. To determine the existence of ERΔ5

In the PCR reaction 10 μl of cDNA product was used in a nested primer strategy. PCR amplification was carried out in 50 μl 10 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 1.5 mM $MgCl_2$, 250 ng primers, 0.5 mM dNTP and 0.5 U Ampli Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) . The nucleotide sequences of primers and probes used are presented in Table 1. Primers 1 and 2, situated in exon 2 and 6 respectively, were used during the 35 cycles of amplification. For the second round of amplification (35 cycles) we used 10 μl of template PCR product and the set of primers, 3 and 2 situated in exon 4 and 6. Each cycle consisted of: 1 min of denaturation at 94°C, 2 min of annealing at 58°C and 3 min of extension at 72°C.

2.4.2. Determination of the percentage ERΔ5/wtER

PCR amplification was carried out with 5 μl of cDNA product, primer set 3 and 2 and 1 U of Ampli Taq Gold®: 10 min at 95°C followed by 30 cycles of: 30 s at 95°C, 30 s at 58°C, 1 min at 72°C. Negative controls of every sample were added during cDNA synthesis leaving out RT superscript beside a control containing distilled water instead of RNA. These negative controls were amplified during PCR and put on gel for Southern blot analysis. PCR synthesis was performed in the exponentional phase. Through the parallel amplification of wtER and ERΔ5, the percentage ERΔ5/wtER gives an indication of the expression of ERΔ5.

2.5. Southern hybridization

Twenty microlitres of PCR product (primers 2 and 3) was applied to a 2% agarose gel and electrophoresed for 2 h at 100 V. After denaturation in 0.5 M NaOH for 30 min, the gel was neutralized in 0.5 M Tris-HCl (pH 7.2) containing 1.5 M NaCl and 0.001 M EDTA for 30 min. PCR products were transferred under vacuum to a Hybond®-N membrane (Amersham, Little Chalfont, UK). The membrane was prehybridized for 1 h at 55°C in 5 × SSPE, 5 × Dennhardt's

Table 1
Nucleotide sequence of primers and probes used

PCR primers:
Primer 1 cDNA 172–178 5′-GGAAGTATGGCTATGGAATCT-3′
Primer 2 cDNA 423–430 5′-CCAGCAGCATGTCGAAGATC-3′
Primer 3 cDNA 284–290 5′-GGAGACATGAGAGCTGCCAAC-3′
Primer for sequence analysis:
Primer 6 cDNA 380–388
5′-CATCAGGATCTCTAGCCAGGCACATTC-3′
Probes for Southern hybridization:
Probe 4 cDNA 347–354
5′-ACCAACCTGGCAGACAGGGAGCTG-3′
Probe 5 cDNA 380–388
5′-CATCAGGATCTCTAGCCAGGCACATTC-3′

solution, 0.5% SDS and 20 μg/ml of sheared herring sperm DNA. Hybridization was performed overnight at 50°C with the ^{32}P end-labeled internal probe 4 (situated in exon 4) or probe 5 (situated in exon 5). After hybridization the membrane was washed in SSPE 2 ×, SDS 0.1%. Autographs prepared with various exposure times to rule out saturation levels were scanned with a Sharp JX-330 (Pharmacia Biotech). Contours (OD × mm^2) of wtER and ERΔ5 bands were compared to a control sample in the various blots.

2.6. Cloning and sequence analysis

PCR products amplified with the primers 2 and 3 from cDNA of two meningiomas, revealed two bands of 438 bp and 300 bp on an agarose gel. Both bands were excised from the gel and purified (Gel extraction kit, QIAGEN, Chatsworth, USA). The fragments were cloned in pMos*Blue* T-vector (Amersham). 2.5 μg of pMOS*Blue*-PCR product was denatured and ligated with 5–10 pmol of the external primers M13 and T7 or the internal primer 6, situated in exon 6. Labeling reactions were performed using the Sequenase® PCR Product Sequencing Kit (Amersham) and [α-^{35}S]dATP. The labeled fragments were loaded on a 8% acrylamide gel containing 8 M urea, electrophoresed and visualized by autoradiography.

3. Results

From cDNAs of seven meningiomas, two breast cancer tissues and MCF7 cells, we amplified exon 4–6 by PCR using primers 2 and 3. The PCR product was electrophoresed on agarose gel, transferred to a Hybond N membrane and hybridized with probe 4 situated in exon 4 or with probe 5 situated in exon 5. After hybridization with probe 4, the expected wild type fragment of 438 bp and another fragment of 300 bp, the possible exon 5 deleted variant were clearly seen in all samples (Fig. 1 top panel). Hybridization with probe 5 only revealed the wild type product of 438 bp (Fig. 1 lower panel) . For sequence analysis we cloned the 438 bp and the 300 bp PCR transcripts of two meningiomas in the pMos*Blue* T-vector. The sequence of the 438 bp wtER transcript generated by amplification of exon 4–6 was identical to the sequence published of the MCF7 ER [12] with the exception of a single base change at Val 400 [GTG] to Gly 400 [GGG] in the meningioma tissue. As expected in the 300 bp ER transcript exon 5 was found to be deleted as evidenced by the 4–6 junction sequence (Fig. 2).

Contour measurements of ERΔ5 and wtER bands in 23 meningiomas and calculating percentages of ERΔ5/wtER revealed mean values in 4 ER−/PR− tissues of 22.9% (range: 11.5–44.6%), in 18 ER−/PR+ meningiomas 37.1% (range: 19.1–55.9%, PR: 11–563 fmol/mg P) and in one ER+/PR+ meningioma: 30.8% (ER/PR: 26/495 fmol/mg P) . Negative protein levels are considered to be < 10 fmol/mg P in ligand binding

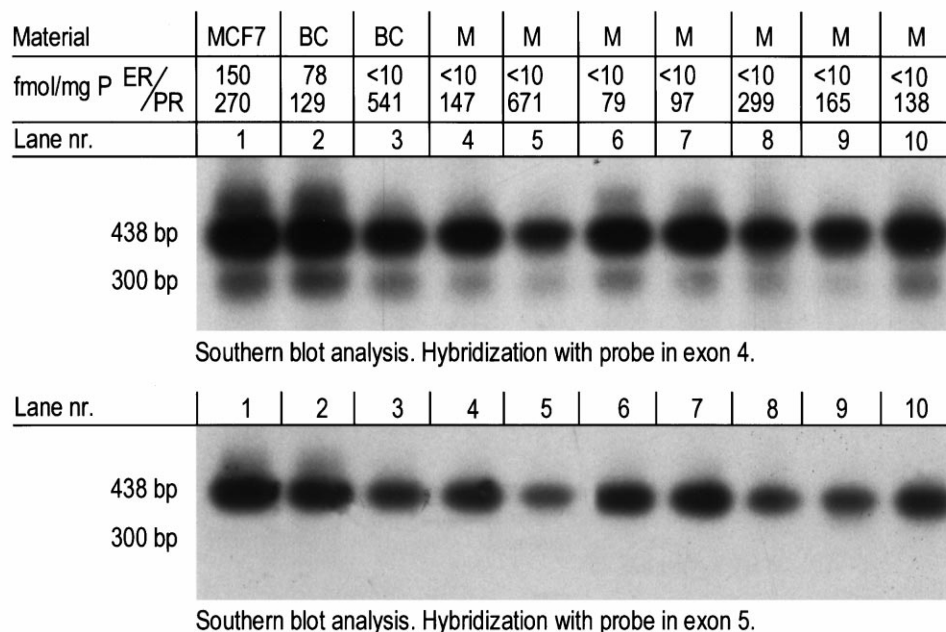

Fig. 1. Southern blot hybridization of RT-PCR products revealing exon 5 deletions in mRNA from MCF7 (lane 1), breast cancer tissues (lanes 2 and 3) and meningioma tissues (lanes 4–10). Hybridization was performed with probes specific for ER exon 4 (top) or exon 5 (bottom).

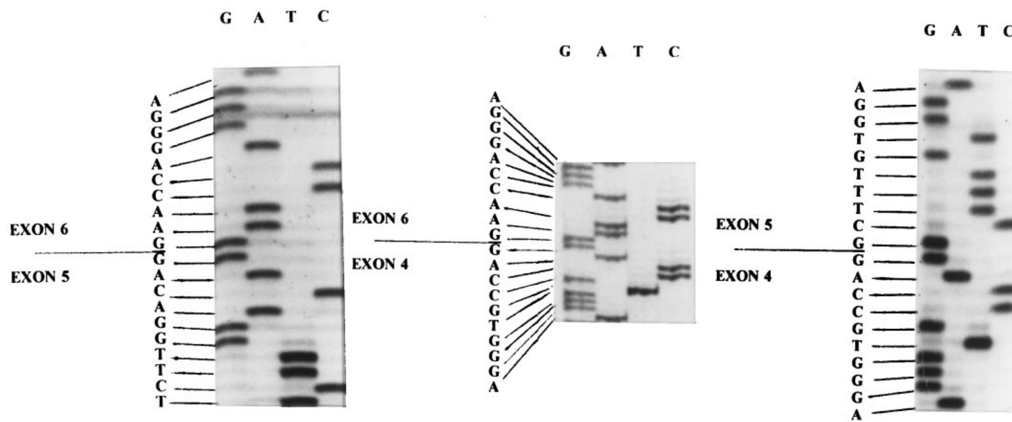

Fig. 2. Results of dideoxy sequence analysis of ER exon 5 deletion variant in meningioma tissue following cloning in pMos*Blue* T-vector. Exon 6/5 and exon 5/4 boundary from the wtER. Exon 6/4 boundary from the ERΔ5 variant.

assays (Fig. 3). No relationship was found between the PR concentration and the percentage ERΔ5/wtER (Fig. 4).

4. Discussion

In spite of the fact that female sex steroids are considered to play a role in the etiology of breast cancer as well as meningioma, in terms of receptor phenotype meningioma tissues are comparable only to a small group of breast cancer tissues (2–11%) which are ER negative/PR positive. By RT-PCR, different groups of investigators detected mRNA coding for ER variants and wtER in these ER negative tissues or cell lines. It was suggested that a mutant form of the estrogen receptor, although not capable of binding its ligand and not detectable by ER ligand binding assay, could still be responsible for the expression of the PR. Sofar ER variants lacking exon 2, 3, 4, 5 or 7 have been found in many different tissues like breast tumor tissues and cell lines and in human pituitary adenomas [13–16], ERΔ4 and ERΔ7 in meningioma tissue [5,6] and pre- and postmenopausal endometrium and endometrial carcinoma [17]. Even ER-like transcripts containing two or three entire exon deletions have been detected [16,18]. ER variants Δ2, Δ3, Δ4 and Δ7 were not capable to activate transcription in a dominant way.

ER mRNA with an exon 5 deletion was first detected by Fuqua et al. [4,8] in ER− and ER+ breast cancer tissues. Other investigators also isolated this mutant from breast cancer tissues and cell lines of ER−/ER+ phenotype and found the ERΔ5 fragment sometimes even predominantly coexpressed with the wtER [19–21]. The involvement of ERΔ5 in PR synthesis in breast cancer tissue is further supported by the observation of Zhang et al. [19] that ER−/PR− breast cancers do not express ERΔ5 or wtER at all.

The exon 5 deletion leads to a truncated partially out-of-frame protein of approximately 40,000 D due to the introduction of a stop codon in exon 6. The predicted protein lacks the majority of the hormone binding domain (HBD) but still possesses constitutive transcriptional activity in a yeast expression system of normally estrogen dependent genes [8] and also in

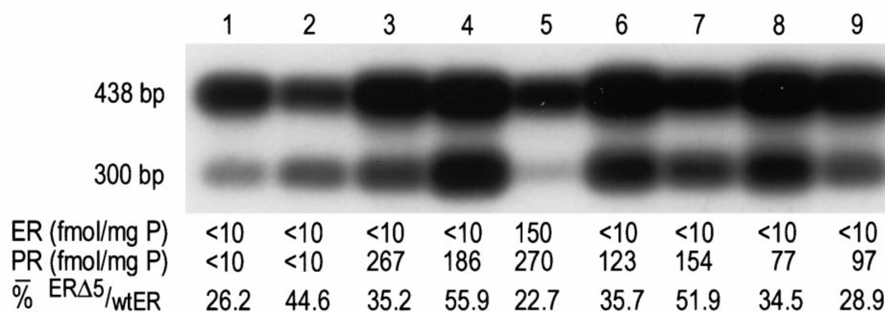

Fig. 3. Southern blot hybridization of PCR products (primer set 2 and 3). The upper band is wtER (438 bp) and the lower band is ERΔ5 (300 bp). Percentages ERΔ5/wtER given are the mean of contour measurements of three audiographs of different intensity. Lanes 1–4 and 6–9 are products of meningioma tissues, lane 5 is from MCF7 cells.

MCF7 cells this ERΔ5 variant has been reported to be able to express PR in a constitutive way [9].

The results of the functional analysis of the ERΔ5 variant raised the question whether such an ERΔ5 variant was also present and transcriptionally active in meningiomas and thus could explain their ER−/PR+ phenotype.

Northern blot analysis of ER RNA did not show a wt or a variant transcript probably due to the low expression of ER (Data not shown).

Using the more sensitive RT-PCR technique followed by the Southern blot analysis we could detect wtER and ERΔ5 fragments in all 23 meningiomas (one ER+/PR+, 18 ER−/PR+ and 4 ER−/PR−), two breast cancer tissues (ER−/PR+ and ER+/PR+) and MCF7 cells. Sequence analysis performed on two of these meningiomas confirmed the identity of both fragments (Fig. 2). Most likely the ERΔ5 is the result of alternative splicing. Our finding of an wtER mRNA detected by RT-PCR in 100% of the meningiomas was in agreement with the reports of Speirs et al. ($N = 20$) [22] and Magrassi et al. ($N = 12$) [23] and seems to be tissue specific. We expected to find none or a lower expression of ERΔ5 in ER−/PR-meningioma tissues compared to PR+ tissues. Surprisingly, in contrast to Zhang et al., in all 23 meningiomas, even in the ER−/PR− tissues, we found wtER and ERΔ5, while wtER was predominantly present.

So far no classical ERE has been identified in the promoter region of the PR gene, but the presence in meningioma of an ER-like protein capable of binding to an synthetic estrogen responsive element (ERE) has been shown [3]. In ER−/PR- meningiomas such an ER-like protein was also found but peculiarly, this does not lead in vivo to induction of PR synthesis to a detectable level using a ligand binding assay. It remains to be established whether this ER-like protein [3] is identical or related to the ERΔ5 variant described in the present paper and whether these proteins as expressed at very low levels have a biological relevance. Using immunoprecipitation followed by Western blot analysis Castles et al. [19] found a truncated protein of M_r 42,000 coding for an exon 5 deleted ER in ER negative BT-20 cells. This ERΔ5 variant possesses constitutive transcriptional activity to an estrogen regulated reporter gene construct in a yeast expression system (RNase protection assay). In contrast to previous reports of Fuqua et al. [9] Rea and Parker concluded that ERΔ5 was unable to constitutively activate expression of pS2 and PR when it was stably transfected in MCF7 cells [24]. Ohlsson et al. [25] showed that ERΔ5 when cotransfected with wtER in the ER negative cell line HMT-3522Sl had a dominant negative activity. Northern blot analysis revealed a high ERΔ5 mRNA expression while the ERΔ5 protein was not always detectable suggesting that this protein had a decreased stability. Although we found an ER-like protein in PR negative meningioma tissue and by RT-PCR an ERΔ5 mRNA, it remains to be elucidated if these transcripts are or will be translated to a

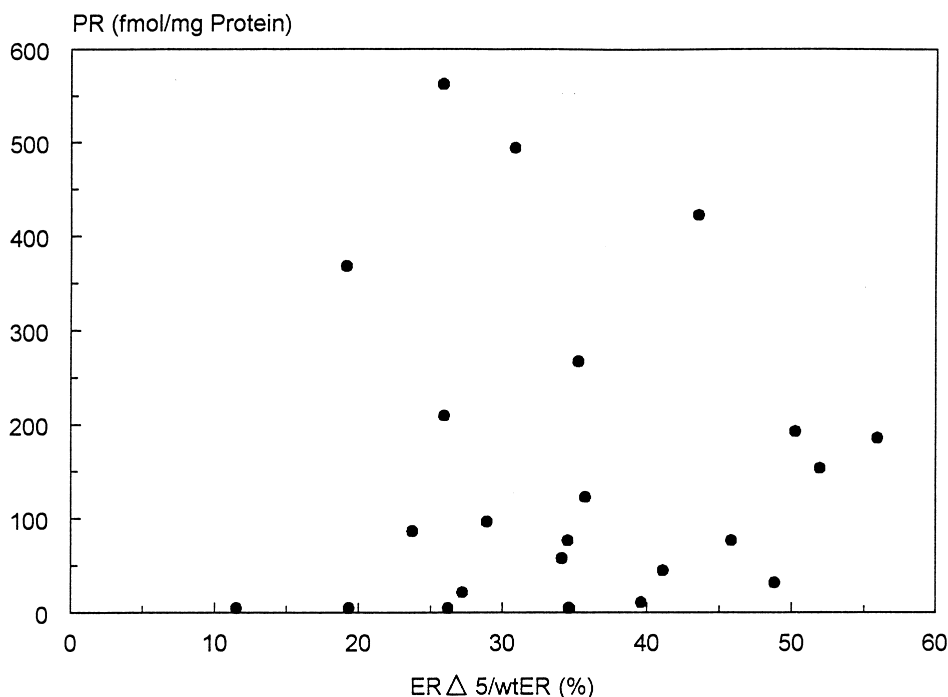

Fig. 4. Percentage ERΔ5/wtER vs PR (fmol/mg P) in 23 meningiomas.

biologically active protein that could be one of the possible components that is involved in PR synthesis. The major reason to reconsider our initial hypothesis that ERΔ5 per se is related to autonomous PR expression comes from the results of the ER−/PR− tissues in which a similar ERΔ5 mRNA signal was observed as in ER−/PR+ tissues.

In conclusion the presence of ERΔ5 appears to be much less important in the induction of the PR in meningiomas than we initially thought. This means that other, as yet unidentified, mechanisms lead to the expression of PR in this highly interesting tissue.

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