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Progesterone receptor isoform expression in human meningiomas

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Abstract

The majority of meningiomas express the progesterone receptor (PR), and therefore meningiomas are considered to be progesterone-responsive. In addition, an association has been reported between PR and prognosis. At least two PR isoforms exist, PR-B (116–120 kDa) and PR-A (81 kDa), each of which are likely to have different biological functions. Knowledge of the differential expression of both isoforms is necessary to understand the effects of progesterone on meningioma growth. Therefore, in this study, PR-A and PR-B expression levels were determined in 61 human meningiomas by immunoblotting. Total PR expression levels were determined with a ligand binding assay (LBA) (total PR^{LBA}). Both PR isoforms and an additional PR 78 kDa protein (PR-78) were expressed in the meningiomas. Meningiomas expressing more PR-A than PR-B had significantly higher total PR^{LBA} levels ($P<0.001$). The PR-78 band intensity was negatively associated with that of PR-B ($r_s=-0.76$, $P<0.0001$). PR-78 may represent an endogenous degradation product, but a similar regulation pathway in the biogenesis of both PR-B and PR-78 is not excluded. Meningiomas contain both PR isoforms, but in highly variable ratios and this variability may have some biological significance. Most meningiomas express more PR-A than PR-B. Therefore in meningioma, assuming that PR-B is more transcriptionally active than PR-A, progesterone responsiveness could be based on transrepression rather than on transactivation of target genes, and progesterone blockade may only be effective in certain subsets of meningiomas.

Keywords: Progesterone receptor isoforms; Meningioma; PR-A; PR-B; PR-78; Immunoblot

1. Introduction

Progesterone may play a role in the development and proliferation of hormone dependent tumours, like breast cancer and meningiomas. Progesterone action is mediated via the progesterone receptor (PR), which can be found in 75% of the human meningiomas [1,2]. Meningiomas expressing high levels of the PR have a better prognosis and a higher survival rate [3–5]. PR might therefore serve as target for endocrine therapy [6]. To determine the biological significance of PR expression in meningiomas, the regulation of PR in meningiomas needs to be elucidated.

The progesterone receptor (PR) belongs to the family of ligand activated transcription factors. The PR is expressed as at least two isoforms, a full size protein (PR-B) and a 164 amino acid N-terminally truncated version of the B form, called PR-A [7,8]. The two isoforms are transcribed from two different translation initiation sites located on the same gene [9].

Besides PR-A and PR-B, an additional progesterone binding protein of 78 kDa (referred to as PR-78) was revealed by immunoblot analysis of breast cancer cytosols [10,11]. Yeates and colleagues reported that PR-78 is most likely not an artificial degradation, nor a phosphorylation product of PR-B or PR-A [11]. The origin and biological function of PR-78 remains to be elucidated.

While both PR-A and PR-B have similar DNA and ligand binding affinities [12], in most cell and promoter contexts both isoforms appear to exhibit different regulating properties. *In vitro* studies reveal that PR-B is a strong transactivator of progesterone-responsive genes, whereas PR-A is considered to be a dominant negative transrepressor of PR-B and other steroid hormone receptors [13–15]. The precise mechanism underlying the differential activities of PR-A and PR-B is not fully

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understood. PR-A, however, is missing the N terminally-located 'B Upstream Sequence' (BUS), resulting in a possible change in conformation and loss of phosphorylation sites [16]. The PR isoforms may have different affinities for co-factors and other proteins of the transcription machinery [17]. Because of the differential activities of PR-B and PR-A, the ratio of expression of both isoforms determines the final overall response of cells upon progesterone stimulation, and may be important for the regulation of growth and differentiation of endocrine-related tumours.

Meningiomas are tumours which are thought to be hormonally regulated [18]. They are generally benign intracranial tumours derived from arachnoidal cells in the meninges, the membranes covering brain and spinal cord. Meningiomas are the most common intracranial tumours with a 20% recurrence rate following current standard therapy, surgical resection with subsequent radiation therapy when necessary [19,20]. Growth of meningiomas is increased during periods with high progesterone levels in the circulation [21]. Meningiomas express high levels of total PR, as measured by a ligand binding assay (LBA) [1]. There is an association between total PR expression in the tumour and prognosis for the patient [3–5]. Several experiments, *in vitro* and *in vivo*, have been performed to inhibit cell growth using PR antagonists [6,18,22,23]. For development of endocrine therapy, however, the regulation and function of PR expression in meningiomas needs to be known.

In several previous publications, the presence of PRs in meningioma, based on a LBA, was reported [1,2]. The expression pattern of the PR isoforms in meningiomas, however, is not known. Both isoforms differentially mediate progesterone or PR antagonist signalling. Therefore, the differential expression of both PR isoforms in meningiomas is of great interest. This study was conducted to determine PR-A and PR-B expression levels with an immunoblot analysis. Results were compared with total PR expression, determined with a LBA (referred to as total PR^{LBA}), and tumour grade.

2. Patients and methods

2.1. Tissues

Sixty-one PR-positive human meningioma tissues were collected from 40 female and 21 male patients, the mean age at the time of surgery was 57 years (median: 60 years; range 45–81 years), operated at the University Medical Center Utrecht. The study was conducted in accordance with the guidelines of the local ethical committee. Tissues specimens were placed on ice immediately after removal from the patient. Representative specimens were frozen at −80°C until used for cytosol preparation.

2.2. Cytosol preparation

The tissue (250–500 mg) was chilled in liquid nitrogen, pulverised with a microdismembrator (Braun, Melsungen, FRG) and suspended in 2 ml of 10 mM phosphate buffer containing, 1.5 mM ethylene diamine tetra acentic acid (EDTA), 3 mM sodium azide, 10 mM 1-monothioglycerol and 10% (v/v) glycerol, at pH 7.5. The resulting homogenate was centrifuged at 0–4°C for 30 min at 100 000*g* to yield a clear cytosol. Total PR was measured by LBA immediately after cytosol preparation.

2.3. Receptor assay

Total tumour PR levels (total PR^{LBA}) were measured, as previously described, by the LBA and Scatchard plot analysis, according to the guidelines of the European Organization for Research and Treatment of Cancer (EORTC), Breast Cancer Cooperative Group [1,24]. The protein content of the cytosol was estimated with the method of Bradford, using reagents from Bio-Rad (Richmond, CA, USA) and serum albumin (Kabi, Diagnostica, Stockholm, Sweden) as a standard [25]. The lower cut-off level for PR positivity was set to be 10 fmol/mg protein. The between-assay variability of these assays is PR ($n=31$): 11.3% at 333 fmol/mg protein and for the protein assay ($n=31$): 5.8% at 3.4 mg/ml.

2.4. Protein electrophoresis and immunoblot analysis

Sixty-one cytosols with $PR^{LBA} > 10$ fmol/mg protein were used for the immunoblot analysis. PR isoforms were separated by electrophoresis through a 6.5% polyacrylamide (30% acrylamide/bis solution, 37.5:1) resolving gel and a 2.6% stacking gel both containing 0.10% sodium dodecyl sulphate (SDS), using a Mini Protean II apparatus (Bio-Rad, Richmond, CA, USA). The cytosol of each sample loaded on the gel contained 10 fmol PR, and was diluted to 80 μl with sample buffer. For cytosol samples with very low PR levels (<40 fmol/mg protein), the maximum amount of cytosol possible was used. Samples were heated for 8 min at 99°C and then loaded onto the gel. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, UK) at 125 V for 1.5 h at 4°C in 25 mM tris, 192 mM glycine, 20% (v/v) methanol as described by Towbin and colleagues [26]. The conditions used for transfer were chosen after pilot experiments which showed that shorter transfer times significantly altered the ratio of PR-A:PR-B in favour of PR-A (the lower molecular weight form). Increasing the transfer times beyond 1.5 h did not alter the observed ratio indicating that transfer was complete at this time.

After transfer, the membrane was blocked with 1.5 mM phosphate-buffered saline (PBS, pH 7.4) containing

5% low fat milk powder (Protifar; N.V. Nutricia, Zoetermeer, The Netherlands), and incubated with a specific antibody (AB) against human PR, monoclonal antibody Ab-4 (clone hPR a4, Neomarkers, Fremont, USA) at a 1:1000 dilution [27]. PR-A migrates as a single band, whereas PR-B migrates as a triplet, for analysis the sum of the triplet was used.

The specificity of hPR4a was verified using other antibodies against both isoforms (hPRa1, Neomarkers) and against only PR-B (clone hPR a6, Neomarkers). In order to establish the interassay reproducibility of each experiment, on each gel at least two identical amounts of cytosol of human myometrium were used as PR-positive controls. The molecular weight of each PR isoform was established with a molecular weight standard (BioRad, Richmond, CA, USA). After incubation with the PR antibodies, blots were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody at a final dilution of 1:2500 (Dako A/S, Denmark). Specific bands were visualised by using enhanced chemiluminescence detection substrates (0.01% (v/v) 250 mM luminol in dimethyl sulphoxide (DMSO), 0.004% (v/v) 90 mM p-loumaric acid in DMSO, 0.002% (v/v) H2O2 in Tris 0.1 M, pH 8.5) and blots were exposed to Kodak film (Biomax-ML, Kodak, Rochester, NY, USA). A control immunoblot, with and without a specific hPR4a PR antibody, of T47D (PR = 1800 fmol/mg protein), LNCaP (PR < 10 fmol/mg protein) and human myometrium cytosol revealed that the bands visualised were specific for PR.

Multiple exposures of each immunoblot were made, and results were used only from those where PR-A and PR-78 bands could be analysed separately, and that fell within the linear range of the film. Otherwise, a new assay was run using more diluted samples. Band intensities were measured densitometrically (Sharp JX330, Japan). The linear range of detection of PR on the immunoblot was established by a standard curve, made using increasing concentrations of control cytosol, and analysis by densitometry. A linear relationship between total PR concentration (based on LBA) and densitometric detection by immunoblot analysis could be established. The between-assay variability of PR in cytosols of human myometrium was 10.1% ($n = 31$).

2.5. Statistical evaluation

For each sample, the mean of the PR isoform expression was calculated from at least three independent immunoblot assays. Spearman rank sum tests (r_s) were used to compare total PR^{LBA}, PR-A, PR-B and 78-kDa band expression. Mann–Whitney non-parametric tests were performed to compare protein expression between the subgroups. P values of < 0.05 were considered statistically significant.

3. Results

3.1. Total progesterone receptor (PR) expression in human meningiomas based on ligand binding assay (LBA)

The PR concentration of 61 PR-positive meningiomas is depicted in Table 1. The average PR concentration of the cytosols used was 181 ± 24 fmol/mg protein (mean ± standard error of the mean (S.E.M.), $n = 61$). The total PR^{LBA} concentration fell in the range 10–755 fmol/mg protein with the median at 98 fmol/mg protein. PR-A expression was considered to be higher than PR-B expression when the total optical density of the band was higher (Table 1, PR-A > PR-B) and visa versa for PR-B > PR-A. No significant differences could be found between the histological meningioma subgroups and the total PR^{LBA} concentration.

3.2. PR isoforms

Fig. 1 shows a representative immunoblot of the two PR isoforms, PR-A (81 kDa) and PR-B (116–120 kDa) and an additional band PR-78. The PR-B isoform migration pattern depends on the phosphorylation status of the protein and can be seen as multiple bands.

Table 1
Progesterone receptor (PR) isoform expression in human meningiomas

Meningioma subtype	n	Total PR^{LBA} (fmol/mg p)[a]	PR-A > PR-B[b]	PR-B > PR-A[b]
Syncytial	16	183 ± 49	9	7
Transitional	4	82 ± 20	4	0
Atypical	3	66 ± 11	2	1
Malignant	1	23	1	0
Unknown	37	221 ± 36	24	13
Total	61	181 ± 24	40	21

Total PR^{LBA}, progesterone receptor determined with a ligand binding assay.
[a] Data are presented as the mean ± standard error of the mean (S.E.M.).
[b] PR-A or PR-B is considered to be higher than PR-B or PR-A expression when the optical density of the specific band was higher.

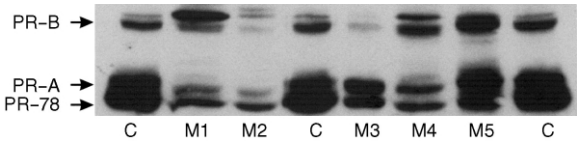

Fig. 1. Immunoblot analysis of the progesterone receptor (PR) isoforms in human meningioma cytosol. For each of the five samples depicted as M1 to M5, three control myometrium cytosols were used (C). Each lane contains equal amounts (10 fmol) of PR established with a ligand binding assay (LBA). PR-B (116–120 kDa) shows multiple bands depending on phosphorylation. Just below PR-A (81 kDa), a PR-78 bands migrates.

Sixty-six per cent of the tumours express more PR-A than PR-B (Table 1). No significant differences in the distribution of the two isoforms among the histological subtypes of meningiomas could be found. Fig. 1 also shows that there is a high variation (coefficient of variation of 30%) between the PR immunoblot band density (PR-A + PR-B + PR-78) and the calculated total PR^{LBA} levels of 10 fmol/mg protein. This was unexpected since the between-assay variability of the western immunoblot assay and of the LBA were 10.1% (at 79.5% PR-A, $n = 31$) and 11.3% (at 333 fmol/mg protein, $n = 31$), respectively.

3.3. The PR-78 isoform

An additional 78 kDa band migrating just below the PR-A band was detected in all 61 meningiomas. To analyse whether this 78 kDa band is associated with PR-A or PR-B, Spearman rank correlation analysis was performed for all the PR isoforms. Fig. 2 shows that PR-B and PR-78 are significantly negatively associated ($r_s = -0.76$, $P < 0.0001$). No association was found between PR-A and PR-78 ($r_s = -0.20$, $P > 0.5$). Besides PR-A and PR-B, because of its capability of ligand binding, PR-78 has also been included in the comparison with the total PR^{LBA}.

3.4. Association between the PR isoforms and total PR

Clinical studies that have compared PR concentrations in meningiomas with prognosis or recurrence rate, have found some relationship between these factors and total PR levels determined by a LBA (in this study referred to as total PR^{LBA}). The differential expression of PR isoforms may play an important role in these observations, therefore the association between total PR^{LBA} and the differential expression of the PR isoforms expression was determined.

PR-A and total PR^{LBA} were found to be significantly associated ($r_s = 0.45$; $P < 0.001$). Fig. 3 shows the relationship between the ratio PR-A:PR-B and the total PR^{LBA}. High expression levels of PR-A with respect to PR-B were found in all the tumours with a high total PR^{LBA} expression (> 200 fmol/mg protein). The group

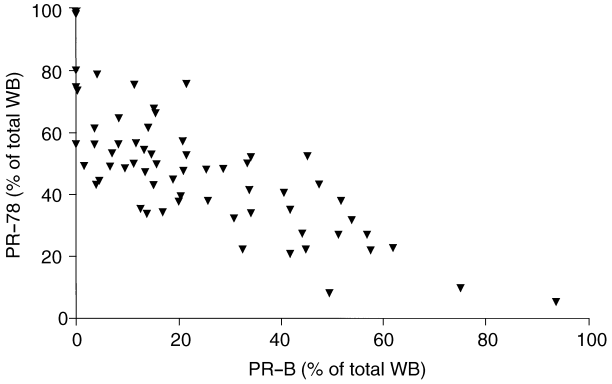

Fig. 2. The association between the band intensities of PR-B and PR-78. The densities of the PR-B band and PR-78 band are strongly negatively-associated. No association was found between the density of the PR-A and PR-78 bands. WB, Western immunoblot.

of meningiomas expressing less PR-A than PR-B, expressed significantly lower amounts of total PR^{LBA} than the group with more PR-A than PR-B (Fig. 4; $P < 0.001$). An association was found between PR-78 and total PR^{LBA} of $r_s = 0.3$ ($P = 0.03$). Meningiomas with a high total PR^{LBA} expression (> 200 fmol/mg protein) tended to express more PR-78 than PR-B.

4. Discussion

In this study, the distribution of PR isoforms was determined in human meningioma cytosols and compared with total PR levels determined with a LBA (total PR^{LBA}). The findings of this study showed that more than 65% of the tumours expressed more PR-A than PR-B. In addition, PR-A was positively associated with total PR^{LBA} levels. The differential expression of PR isoforms in meningiomas may have important implications for the clinical aspects of the disease.

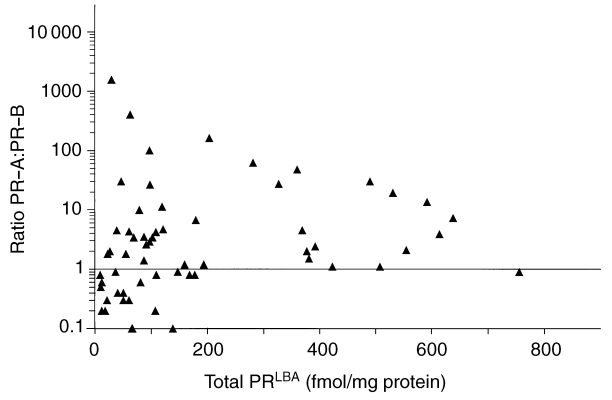

Fig. 3. The PR-A to PR-B ratio and the total PR^{LBA} expression in human meningiomas. The number of tumours that express more PR-A than PR-B is higher in meningiomas expressing more than 200 fmol/mg protein. In the group with a low total PR^{LBA}, both PR-A > PR-B and PR-B > PR-A expression ratios are observed.

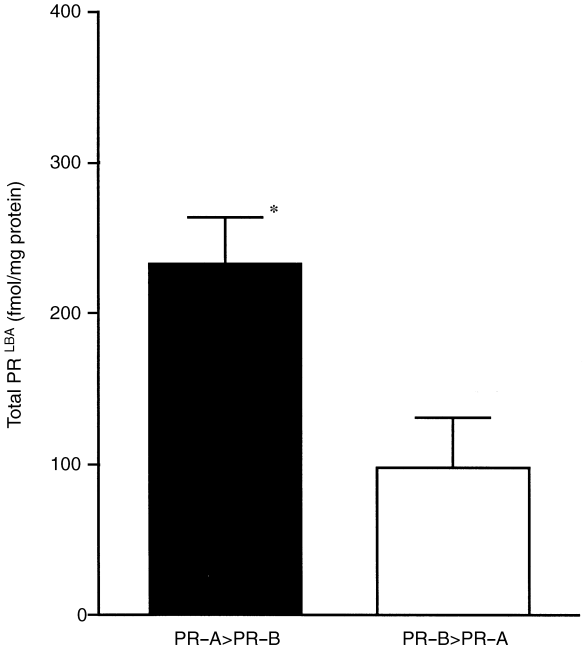

Fig. 4. Total PR^{LBA} expression in meningiomas with differing ratios of PR-A and PR-B. The group of meningiomas with a higher PR-A than PR-B band intensity (PR-A > PR-B, $n=39$) expressed significantly more total PR^{LBA} than the group with a higher PR-B than PR-A band intensity (PR-B > PR-A, $n=22$). *($P<0.001$).

Progesterone is thought to play a role in meningioma development and proliferation. Follow-up studies of patients with meningioma disease showed that the PR content of meningiomas may have clinical significance as a prognostic parameter for survival and recurrence. Hsu and colleagues and Brandis and coworkers, showed that the presence of PR is a favourable prognostic factor for meningiomas [3,4]. In agreement with Hsu, Fewings and colleagues, in a study of 62 meningiomas, found that PR-positive benign meningiomas were less likely to recur [5]. Since PR isoforms exhibit differential transcriptional activity, the overall response to progesterone or PR antagonists may depend on the PR isoform distribution.

In all our meningioma samples and the myometrium control, the presence of a PR-78 band migrating just below the PR-A isoform on the gel was detected. Graham and colleagues also reported the presence of this PR-78 band in a proportion of human breast cancer cytosols [10]. These authors found that the PR-78 band accounted for up to 20% of the total PR concentration. No association was found between PR-78 and PR-A or PR-B in these breast cancer cytosols. The origin of the PR-78 band has been reported not to be an artifact of the cytosol preparation, since deliberate degradation of the breast cancer cytosol samples did not lead to more PR-78 [10,11]. Yeates and colleagues performed additional experiments to characterise the origin of the PR-78 band. In breast cancers, they ruled out the possibility that PR-78 was a phosphorylated product of PR-A and PR-B. They also showed that PR transcripts, including splice variants, are unlikely to be implicated in the formation of the PR-78 isoform. PR-78 is, therefore, most likely not a direct product of translation of the *PR* gene. Photo-affinity labelling studies demonstrated specific binding of ligand to PR-78 [11].

In contrast to these findings, the results of our study showed a significant negative correlation between the expression of PR-B and the PR-78 isoform in meningiomas (Fig. 2). The absence of transcripts encoding for PR-78, and the negative association with PR-B, and not PR-A, are consistent with the idea that PR-B is endogenously degraded into PR-78 in meningiomas, but the data do not exclude that there may be a similar regulation pathway in the biogenesis of PR-B and PR-78. In addition, most of the antibodies used in literature were, apparently, not able to detect PR-78 in various other tissues. This may indicate that although PR-78 is capable of progesterone binding, PR-78 might miss some other epitopes besides the B upstream sequence. Nevertheless, the PR-78 protein is capable of ligand binding, therefore, it may have a biological function in mediating progesterone signalling. Experiments are underway to further address the origin of PR-78.

Our immunoblot data, in which equal amounts of PR were used, showed a high within-assay variability of the PR-A and PR-B band intensities (Fig. 1). Several possible explanations were ruled out by performing the appropriate control experiments; such as equalising the total protein levels. The amount of blood contamination also did not alter these differences. The presence of endogenous ligands in meningiomas might not be important since all tumours were from post-menopausal women, or from men. When the amount of PR-78 band is taken into account, this discrepancy decreases by 20%. Other PR proteins that bind ligand in the LBA, for instance PR-C, which could not be detected in our experimental subset, might explain a part of the remaining discrepancy.

The existence of PR-like proteins that are capable of binding progesterone might have important implications. If these isoforms, PR-78 and possible others, do not have a biological function, the total PR concentration used for clinical purposes is overestimated. However, if the PR-78 isoform interferes in the progesterone-signalling pathway, for instance by binding to cofactors, it might act as a transrepressor. In addition, PR-like proteins may interfere with the prognostic significance of steroid hormones in hormone-responsive cancers. In conclusion, PR-like proteins are present in tumour cytosols and their precise function and role in progesterone signalling needs to be elucidated.

PR is a phosphoprotein and contains at least seven phosphorylation sites. PR-B contains three more phosphorylation sites than PR-A located in the PR-B

upstream region [28–30]. Slower electrophoretic mobility on SDS-gels has been reported to be associated with increased phosphorylation [28,31]. In addition, in our immunoblot analysis of meningioma cytosol samples, variable differential expression of three closely migrating PR-B bands was observed. The biological relevance of this variability in the PR-B sub-band expression is not clear. Whether phosphorylation of total PR has a functional role remains poorly defined. Phosphorylation may not alter transcriptional activity of PR-B, but it could have other functions [29].

Growth of meningiomas seems to be influenced by progesterone levels and a lot of studies have achieved tumour growth remission by using progesterone agonists, progesterone depletion and, above all, progesterone receptor blockade by progesterone antagonists. Olson and colleagues reported inhibition of meningioma growth *in vitro* of three meningiomas after treatment with mifepristone (a PR antagonist), ranging from 18 to 36% after 28 days in culture [32]. We have shown that in meningiomas of 13 patients cultured for 8 days, the thymidine-labelling index significantly fell when increased concentrations of mifepristone were added [18]. Other authors, however, could not confirm these findings [33]. In clinical studies, responses were seen in patients with unresectable meningioma treated with mifepristone, 200 mg orally daily for 6–24 months. Grunberg and colleagues reported objective responses seen in 8 out of 20 patients accompanied by subjective improvement in 5 patients [34]. Lamberts and coworkers noted regression in 3 out of 10 patients, while headaches were improved in 5 patients [35]. Haak and colleagues also reported a case in which mifepristone was used successfully [36]. Whereas elevated levels of progestin in meningioma patients seems to stimulate tumour growth, a high dose of medroxyprogesterone acetate (MPA) resulted in a decrease in tumour size and PR expression in a study reported by Markwalder and colleauges [23,37]. However, Jääskeläinen could not confirm this in a study of 5 meningioma patients using the same dose of MPA [38]. Although most studies that used antiprogestins for treating meningioma have reported some responses, more than half of the treated patients did not show a response at all. Thus, data from both *in vivo* and *in vitro* reports are not yet conclusive.

In gynaecological cancers, progestins are used as therapeutic agents as reviewed by Gadducci and colleagues [39]. In metastatic breast cancer, progestins are used as third-line therapy. Progestins are also used in patients with advanced or recurrent endometrial cancer and regression and stabilisation of advanced recurrent low-grade endometrial stromal carcinomas. The theory behind the success is that progestins have anti-oestrogenic effects, like downregulation of the oestrogen receptor (ER) and stimulation of the conversion of oestradiol to the less oestrogenic oestrone. In addition, progestins seem to downregulate the anti-apoptotic factor bcl-2, and to suppress oestrogen-induced expression of vascular endothelial growth factor (VEGF)-subtypes.

It has been reported that for breast cancer PR-A expression is higher than that of PR-B [10]. In leiomyomas, both PR forms are also expressed and there was a predominance of PR-A over PR-B [40]. For endometrial cancers, 5 out of 11 cases seemed to have a predominant expression of PR-B mRNA. However, it is not yet known whether this is translated to the protein level [41]. The different response of meningiomas and gynaecological cancers to progestin administration could up to now not be clarified by PR isoform distribution. Firstly, not much is known about the ratio PR-A:B from clinical trials, and secondly in most of the gynaecological tumours PR-A expression also seems to be higher. The anti-oestrogenic effect of progestins, however, will not play a role in meningiomas since the majority of the tumours do not express appreciable levels of ER.

We suggest that progesterone blockade may only be effective in certain subsets of meningiomas. Meningiomas with a clear prevalence for PR-A or PR-B will respond in a different way to endocrine agents. If in meningioma PR-B is the more transcriptionally active of the two PR-forms, it could be anticipated that meningiomas with a prevalence for PR-A may be at least likely to respond to endocrine agents. The prognostic value of PR and the better outcome of meningiomas that express high levels of PR, may be due to transrepression activity of the prevalent PR-A isoform, and thus via a transrepression instead of a transactivation mechanism. Our initial idea: progesterone action via the PR activates progesterone-responsive genes in meningioma, needs to be reconsidered. Since there is no (pre-)clinical evidence that fully addresses the significance of PR isoform distribution and the response to endocrine therapy, further clinical investigations are needed.

In summary, meningiomas express both the PR-A and PR-B isoforms and an additional PR-78 product. The ratio of PR-A to the PR-B protein expression levels is highly variable and higher in the meningiomas with a high total PR^{LBA} expression. Perhaps this variability has some clinical and biological significance in meningioma disease.

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