

# Progesterone Receptor Expression in Meningiomas

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## ABSTRACT

The possibility that the female sex steroid progesterone plays a role in meningioma proliferation has been suggested by a number of investigators; and it had been shown that many meningiomas have high affinity progesterone binding sites. The aim of this study was to examine the expression of progesterone receptor mRNA and correlate it with the nuclear localization of progesterone receptor by immunocytochemistry in a large number of meningiomas. Thirty-three meningiomas were examined for the presence of measurable amounts of progesterone receptor mRNA by Northern blot analysis and 11 of these were analyzed for receptor protein by immunohistochemistry. These were compared with normal arachnoid and an arachnoid cell line.

Sixty-four % of the meningiomas expressed progesterone receptor mRNA. This occurred in a similar pattern to what has previously been shown for T-47 D cells, a breast carcinoma cell line with 11.4-, 6.1-, 5.2-, 4.5-, 3.2-, and 2.5-kilobase mRNA species. This suggests that progesterone receptor expression is not tumor specific. There was a marked predominance of women among those patients whose tumors expressed progesterone receptor; 81% were female and 19% were male. The immunohistochemistry data correlate well with the Northern blot analysis. The staining was clearly nuclear, suggesting that the receptor is in a location to be activated.

These data suggest that progesterone receptor mRNA and protein is expressed in meningiomas and support the concept that progesterone may play an important role in meningioma growth.

## INTRODUCTION

Meningiomas are common intracranial neoplasms with biological characteristics that distinguish them from most other intracranial new growths. They enlarge slowly and rarely infiltrate the brain; however, they display a wide range of behaviors (1, 2). The tumors in most cases are thought to arise from the meningothelial cells that make up the arachnoid villi of the meninges, with the membranes covering the brain and spinal cord. They comprise 18% of all primary intracranial tumors and 25% of all intraspinal tumors (1, 2). Most meningiomas are benign tumors, but some meningiomas have malignant characteristics with cortical invasion and a high recurrence rate. Meningiomas are most likely to occur in the 50- to 60-year-old age group and their incidence is twice as high in females than it is males. In females their growth appears to be accelerated during the luteal phase of the menstrual cycle and during pregnancy. Several investigators have reported an increase in size or symptoms of meningiomas in the second and third trimesters which is resolved at the termination of the pregnancy (3).

There is also an association between meningiomas and breast cancer. Two separate studies have demonstrated a higher occurrence of meningiomas in patients with breast cancer than expected. A study from data collected from the Connecticut Tumor Registry (4) demonstrated a positive correlation between breast cancer and meningiomas (8 observed versus 3.4 expected cases). Data collected from the Los Angeles County Tumor Registry also showed a positive correlation between the two diseases (5).

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There have been many reports which have examined the presence of estrogen and progesterone receptors in meningiomas (6-20). Some groups find measurable amount of estrogen receptors in the cytosol, while others do not. On the other hand, most binding studies which have been conducted have demonstrated the presence of cytosolic progesterone receptors. The question which still remains in the literature is whether the progesterone receptors that are present in meningiomas are functional. Schwartz *et al.* (18) suggested that progesterone and estrogen receptors in meningiomas may not meet the specific criteria for true steroid receptor proteins. Schrell *et al.* (17) found that 49 of the 50 tumors had high affinity binding sites for progesterone by the DCC<sup>2</sup> assay. In the same tumor cytosols, solid phase enzyme immunoassay also demonstrated the presence of progesterone receptors. However, only five of the tumors had positive staining for progesterone receptors by immunohistochemistry suggesting that progesterone receptor is absent from the nucleus, which is where the active form of the receptor should be.

The aim of this study was to examine the expression of progesterone receptor mRNA and correlate it with the nuclear localization of progesterone receptor by immunohistochemistry in a large number of meningiomas.

## MATERIALS AND METHODS

**Tissue Samples/Cell Culture.** For Northern blot analysis tissues were collected at the time of craniotomy for meningioma resection and were immediately snap-frozen in liquid nitrogen and subsequently stored in liquid nitrogen. Nonmalignant tissue was obtained from patients undergoing temporal lobectomy for uncontrollable seizures, as a comparison. Each sample was taken from a specimen which was part of the specimen used by the neuropathologist for diagnosis. These tumors were reviewed by Dr. Matthew Frosch and classified as syncytial, transitional, or fibroblastic by standard criteria (1). T-47D breast carcinoma cells were obtained from the American Type Culture Collection and maintained in RPMI 1640, 10% fetal bovine serum, and 0.2 IU insulin/ml.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated by the method of Chirgwin (21). Tissue samples were placed in 4 M GTC and then homogenized with the use of a Polytron until they were totally disrupted. For cells, medium was aspirated and cells were washed twice in ice-cold PBS (0.06 M NaH<sub>2</sub>PO<sub>4</sub> and 0.15 M NaCl, pH 7.3) and 3.3 ml of GTC was added to each flask. The cells were scraped into the GTC and DNA was sheared. After centrifugation for 10 min at 3,000 rpm, (Beckman RT 6000) the supernatant was layered over 5.7 M cesium chloride and centrifuged in a Beckman ultracentrifuge in a SW 50.1 rotor at a speed of 38,000 rpm at 22°C for 16 h. The RNA pellet was dissolved in 0.3 M sterile sodium acetate and the RNA was ethanol-precipitated. Twenty µg (A<sub>260</sub>) of total RNA for each sample were subjected to electrophoresis and diffusion-blotted onto Duralon nylon membrane (Stratagene, La Jolla, CA; Ref. 22). The RNA was cross-linked to the Duralon using UV light (Stratalinker; Stratagene). Blots were prehybridized for 2 h at 42° (50% formamide, 5 × SSC, 10 × Denhardt's solution, 50 mM NaPO<sub>4</sub>, 1% SDS, and 10 µg/ml Sigma free acid) and hybridized overnight at 42°C (50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM NaPO<sub>4</sub>, 0.5% SDS, 5% dextran sulfate, and 20 µg/ml Sigma free acid) with 10<sup>6</sup> cpm/ml of <sup>32</sup>P-labeled complementary DNA probe. Northern blots were sequentially hybridized with progesterone receptor mRNA and then with β-actin. Blots

<sup>2</sup> The abbreviations used are: DCC, dextran-coated charcoal; GTC, guanidinium thiocyanate; PBS, phosphate-buffered saline; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; RT, room temperature; BSA, bovine serum albumin.

were washed (1 × SSC/1% SDS at RT for 15 min, 0.5% SSC/0.5% SDS at RT for 15 min, and twice in 0.1 × SSC/0.1% SDS at RT and 0.1% SSC/0.1% SDS at 50°C for 30 min) and then subjected to autoradiography.

**DNA Fragments.** The probes used for hybridization were a 3000-base pair *SphI-EcoRI* human progesterone receptor insert kindly provided by Dr. Geoffrey Greene and a 1800-base pair human  $\beta$ -actin fragment provided by Larry Kedes. The complementary DNA fragments were labeled with <sup>32</sup>P using random primer translation to achieve a specific activity of 0.5–1.0 × 10<sup>9</sup> cpm/ $\mu$ g DNA (23).

**Immunocytochemistry for Progesterone Receptor in Cell Lines.** T47-D cells were grown as monolayers in eight well Permax chamber slides (Nunc, Inc.) and immunocytochemistry was performed as previously described (24). Briefly, cells were fixed by incubation for 15 min with 3.7% formaldehyde-PBS followed by cold acetone for 15 s. The fixed cells were then incubated with 1% BSA-PBS for 1 h at RT. Cells were next permeabilized with 0.1% Triton X-100, washed, and incubated overnight with a mouse IgG progesterone receptor antibody (BioGenex Laboratory) diluted 1:10 in 1% BSA-PBS. Control incubations included both 1% BSA-PBS and a mouse monoclonal IgG to *M*<sub>r</sub> 68,000 neurofilament protein, an unrelated antigen. The next day cells were incubated for 4 h at 4°C with biotinylated sheep anti-mouse IgG (Amersham) diluted to 33  $\mu$ g/ml in 1% BSA-PBS, followed by incubation for 1 h at 4°C with streptavidin fluorescein (Amersham) diluted 1:50 in 1% BSA-PBS. All incubations were followed by three washes of 30 s each in PBS.

**Immunohistochemistry for Progesterone Receptor in Tumors.** Fresh tissue was embedded in OCT embedding medium and was stored at –20°C. Cryostat sections (4  $\mu$ m) were mounted on poly-L-lysine-coated glass slides. Tissue was fixed for 15–20 min in 3.7% formaldehyde-PBS, followed by acetone for 15 s. The fixed tissue was next incubated with 1% BSA-PBS for 1 h at RT followed by a 1-h incubation with the progesterone receptor antibody. After 1 h slides were washed and subsequently incubated with biotinylated sheep anti-mouse for 1 h at RT. Lastly, slides were incubated with streptavidin fluorescein for 1 h at RT. Dilutions, washing, and controls are similar to those described for cells.

**RESULTS**

**Northern Blot Analysis.** Thirty-three meningiomas were examined for the presence of progesterone receptor mRNA by Northern blot analysis. RNA was also isolated from T-47D cells which were used as a positive control on each Northern blot. In meningiomas we observed at least six mRNA species of approximately 11.4, 6.1, 5.2, 4.5, 3.2, and 2.5 kilobases under high stringent washing conditions (Fig. 1). This is a similar pattern to what has previously been reported for T-47D cells and human endometrium (25) suggesting that the different RNA are not tumor specific. We examined the expression of

the progesterone receptor mRNA in 22 females and 11 males ranging in age from 26 to 86 years (Table 1). The level of expression of the progesterone receptor mRNA varied dramatically. Sixty-four % of the tumors examined showed some level of expression; of these 81% were female and 19% were male. By contrast, of the 36% that were negative, only 42% were female and 58% were male. There is a significant sex difference between men and women in the tumors which are positive and negative for progesterone receptor by  $\chi^2$  (*P* < 0.05). Women have significantly more tumors with progesterone receptor than do men. Normal arachnoid and a human leptomeningial cell line (26) were negative for progesterone receptor mRNA by Northern blot analysis (data not shown). There was no relationship between receptor expression and histological types.

**Immunocytochemistry.** The conditions for using the mouse IgG progesterone antibody from ascities (BioGenex Laboratories, San Ramon, CA) were first worked out in T-47D monolayer cultures. Under these conditions described in “Materials and Methods” intense staining of the nucleus was observed (results not shown). The results for immunostaining in 11 cases of meningiomas are summarized in Table 1. Six meningiomas which were positive for progesterone receptor by mRNA analysis were also positive for progesterone receptor by immunocytochemistry. They all showed very intense nuclear staining with little to no staining observed in the cytoplasm. Fig. 2A illustrates the immunostaining of one positive staining meningioma (case 1). Fig. 2B is the same tumor stained with *M*<sub>r</sub> 68,000 neurofilament protein, an unrelated antigen. Five meningiomas which were negative for progesterone receptor by Northern blot analysis were examined for the presence of progesterone receptor by immunocytochemistry. Four of the five specimens examined showed no evidence of positive immunofluorescence staining. One specimen (case 24) which was negative by Northern blot analysis showed a few scattered cells which were positive for progesterone receptor.

**DISCUSSION**

The role of steroid hormones in the progression of meningiomas is still an area of controversy in the literature. Many studies have examined the presence of both estrogen and progesterone receptors by competitive receptor binding assay. If the data from all the studies are combined, 28% of meningiomas have estrogen receptor binding and 72% have progesterone receptor binding (27). Using solid phase enzyme immunoassay, *in situ* hybridization, and immunohistochemistry,

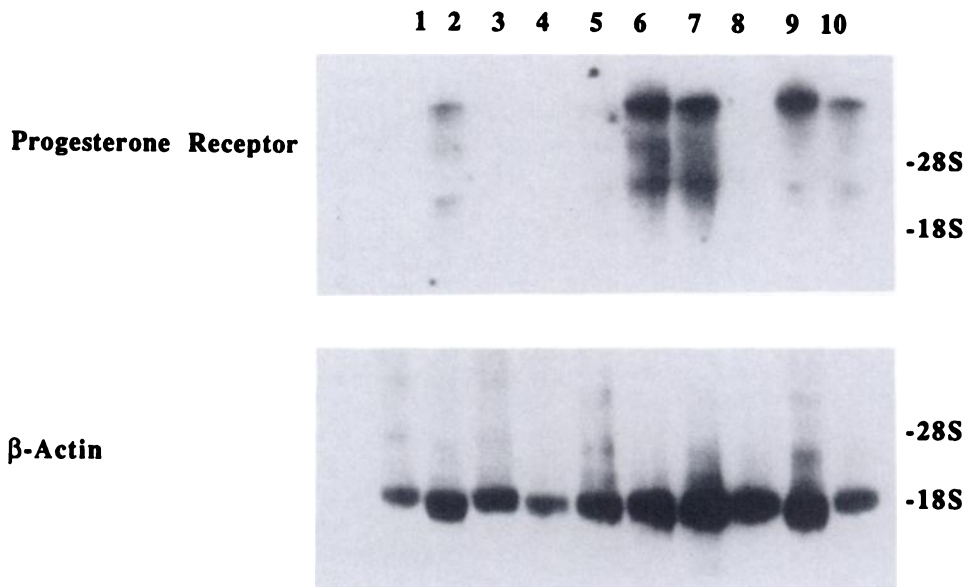


Fig. 1. Top, representative Northern blot showing the level of expression of progesterone receptor in 10 meningiomas. Bottom, the same Northern blot reprobbed with  $\beta$ -actin, showing that there is RNA loaded equivalently in each lane. Note the multiple species of message is similar to that shown in T-47 D cells (Ref. 25; data not shown).

Table 1 Progesterone receptor profiles

Case	Age	Sex	mRNA levels	Immunocytochemistry	Histological type
1	64	F	++++	++++	Syncytial with few psammoma bodies
2	54	M	++++		Transitional/syncytial
3	27	F	+++	++++	Syncytial
4	43	F	+++		Transitional
5	48	F	+++	++++	Syncytial with rare fibroblastic areas
6	67	F	+++	++++	Syncytial with rare fibroblastic areas
7	69	F	+++	++++	Transitional
8	63	M	+++	++	Syncytial with rare fibroblastic areas
9	27	F	++		Transitional
10	39	F	++		Transitional
11	39	F	++		Syncytial/transitional
12	63	F	++		Transitional
13	65	F	++		Transitional with psammoma bodies
14	61	M	++		Transitional
15	26	F	+		Transitional
16	35	F	+		Syncytial
17	54	F	+		Fibroblastic with few psammoma bodies
18	55	F	+		Fibroblastic/transitional
19	56	F	+		Transitional with few psammoma bodies
20	86	F	+		Syncytial with rare fibroblastic areas
21	70	M	+		Syncytial/transitional with bone invasion and mitosis
22	56	F	-	-	Fibroblastic with minor transitional
23	65	F	-		Transitional with few psammoma bodies
24	67	F	-	+/-	Transitional
25	78	F	-		Syncytial
26	78	F	-	-	Transitional
27	28	M	-	-	Malignant/syncytial
28	45	M	-		Hemanangiopericytoma
29	54	M	-		Syncytial; areas of sclerosis
30	63	M	-	-	Syncytial/transitional
31	64	M	-		Syncytial with areas of transitional
32	65	M	-		Fibroblastic/transitional
33	71	M	-		

which are much more sensitive methods than the standard DCC method, Schrell *et al.* (17) failed to demonstrate the presence of estrogen receptors in meningiomas.

By contrast, most binding studies have been able to detect progesterone receptors. The area that remains controversial is whether the progesterone receptor present in meningiomas is functional. Schwartz *et al.* (18) have suggested that the estrogen and progesterone receptors which are present in meningiomas do not meet the specific criteria of true steroid receptors, in that competition studies demonstrated a lack of steroid specificity. Other investigators have noted a strong correlation between biochemical and immunohistochemical determinations of progesterone receptor content using both DCC and nuclear binding and immunoperoxidase methods (8, 10).

Investigators have tried to answer the question of functionality of the receptor by examining the effect of progesterone and the progesterone antagonist RU486 on meningioma growth in culture. Most of the studies demonstrated some effect of progesterone and RU486 on meningioma proliferation, suggesting that the receptors that are present can be activated by ligand (28–33).

We have demonstrated the presence of progesterone receptors in meningiomas by two independent methods, Northern blot analysis for messenger RNA and immunohistochemistry for the protein product. To our knowledge, this is the first article which has demonstrated the expression of progesterone receptor mRNA in tissue specimens of human meningiomas. The size of the mRNA seen in meningiomas is very similar to that observed in the breast carcinoma cell line, T-47D (25). The largest message which is 11.4 kilobases is most abundant in both meningiomas and breast tumor cell lines. It is thought that all the mRNA species code for progesterone receptor or closely related proteins. The origin of the different size mRNAs is not known; possible explanations include multiple polyadenylation sites, differences in the 5' untranslated region, or alternative exon splicing.

In our study, 64% of the tumors examined showed some level of progesterone receptor expression. It is well documented that the incidence of meningiomas is 2 times higher in women than in men for intracranial tumors and 9 times higher in spinal meningiomas (1). Our study also demonstrates a sex difference in the expression of progesterone receptor mRNA levels, in that a higher percentage of women with meningiomas have progesterone receptor mRNA expression than do men. These results are in agreement with those of Cahill *et al.* (9). They found that 8 of 16 tumors in women had significant progesterone receptor levels; in contrast, only one of the seven tumors in men had significant progesterone receptor levels. Other studies (7, 8, 11, 14) have not found a positive correlation between high progesterone receptor levels and the sex of the patient. These discrepancies could be accounted for by the different techniques which are used in each of the studies. Some of the studies used the DCC assay, while others used sucrose gradient centrifugation to determine progesterone receptor content. In the binding studies receptor quantities are expressed in different units and the authors do not define the quantity of receptor which they considered "significant." In our study the presence of progesterone receptors was determined by Northern blot analysis which is a very different technique from most of the studies which have determined progesterone receptor levels by ligand binding. These discrepancies could account for the differences in the data.

In the second part of our study we wanted to determine the location of the progesterone receptor. It is generally believed now that both unoccupied and occupied steroid receptors reside in the nucleus; therefore for the receptor to be functional it must be present in the nucleus. Immunohistochemistry allows us to examine both the cytoplasm and nucleus for the presence of progesterone receptors. In our studies, the progesterone receptor was confined to the nucleus. Our results are in agreement of those of other laboratories (34–36) which show the presence of nuclear progesterone receptors in meningiomas

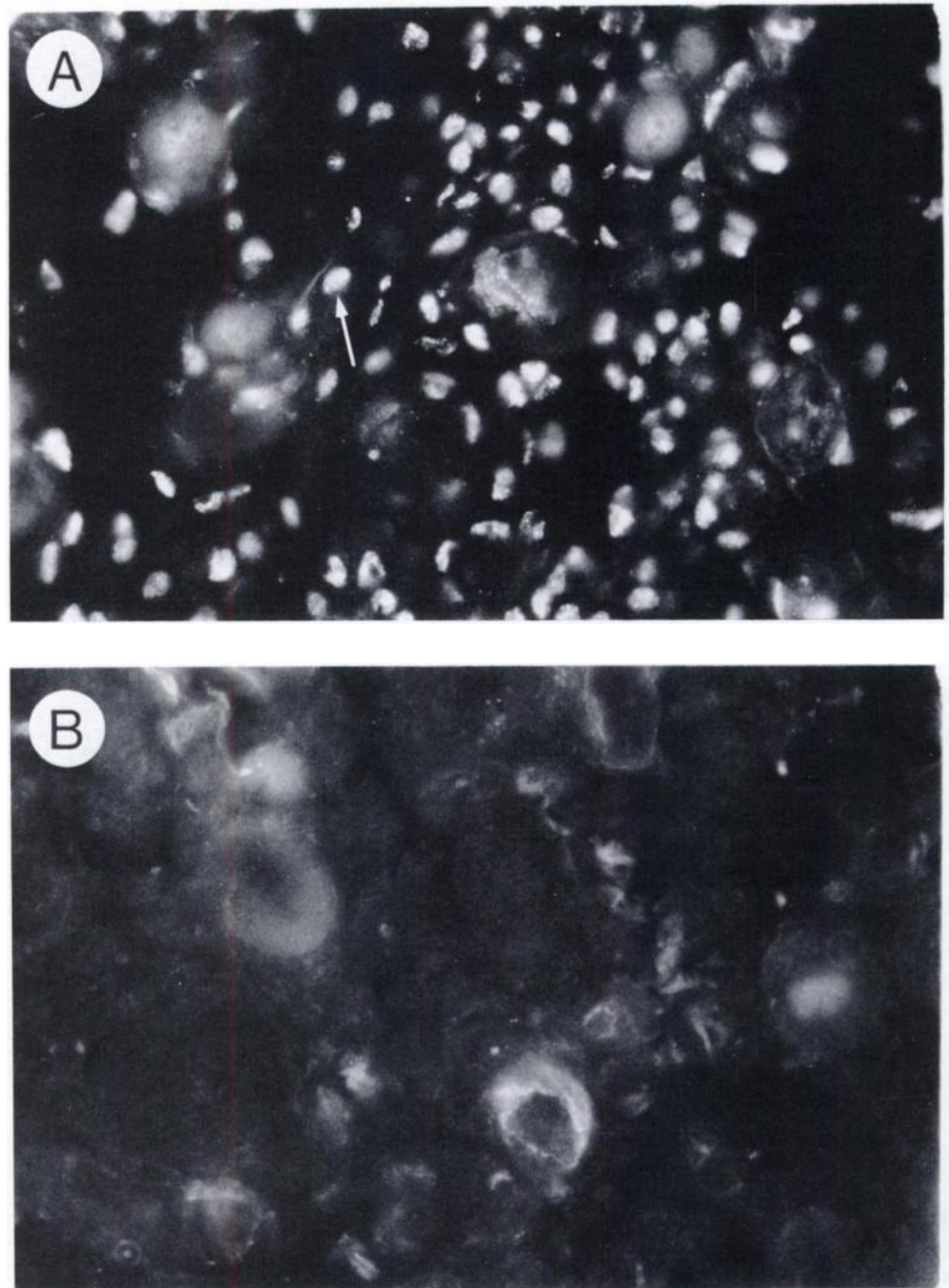


Fig. 2. Case 1 stained with progesterone receptor antibody (A); arrow, positive staining in the nucleus. The same tumor stained with M, 68,000 neurofilament protein (B), an unrelated antigen which serves as a control.

by the use of a highly specific monoclonal antibodies. In contrast, Schrell *et al.* (17) observed only slight cytoplasmic immunoreactivity and very little in the nucleus suggesting that the progesterone receptor may be inactive. The discrepancies in these studies may be due to differences in the primary antibody. In our studies a mouse monoclonal antibody (BioGenex Laboratories) raised against a synthetic peptide of progesterone receptor was used. In contrast, Schrell *et al.* (17) used a mouse monoclonal antibody raised against purified progesterone receptor from the cytosolic fraction of rabbit uteri.

In one specimen there was a slight discrepancy between the results we obtained by Northern blot analysis and immunohistochemistry. For sample number 23, no signal was detected by Northern blot analysis, but a few scattered cells stained positive for progesterone receptor by immunostaining. This difference can be accounted for by the fact that if only a few cells are synthesizing the mRNA of interest it would be very hard to detect any signal by Northern blot; more sensitive techniques are needed such as polyadenylate RNA isolation or RNase

protection. By contrast, with immunohistochemistry each individual cell is examined for positive staining.

In our studies the majority of the tumors were syncytial or transitional (Table 1), and there was no clear relationship between the presence of progesterone receptor and histological type of the tumor. Some studies have suggested that more aggressive meningiomas have higher levels of receptor (9); other studies (7) like ours have shown no relationship between histological type and the presence of progesterone receptors.

These studies show that progesterone receptors are present with some distinct differences with regards to the sex of the patient. The receptors are likely to be functional since they are found in the nucleus although a formal *in vitro* or *in vivo* assay of progesterone receptors functionality remains to be done. These data may have considerable importance, since progesterone receptors blockade may have a role to play in the inhibition of meningioma growth. When 14 patients with unresectable meningiomas were treated with mifepristone (RU486),

five showed signs of improvement (37). The clinical use of progesterone antagonists may have an important role in the management of recurrent or inoperable meningiomas.

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