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MICROVESSEL DENSITY AND CPG ISLAND METHYLATION OF THBS2 GENE IN MALIGNANT OVARIAN TUMORS

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We aimed to investigate the role of thrombospondin-2 (THBS2) related angiogenic activity in malignant ovarian tumors and to determine if aberrant methylation associated inactivation is involved in down-regulating THBS2 expression in ovarian cancer. The methylation status of the THBS2 promoter region and microvessel density (MVD) was studied in 70 malignant ovarian tumors and in 15 control ovarian samples. A methylation specific PCR (MSP) method was used to distinguish methylated from unmethylated DNA in the promoter regions of the THBS2 gene. MVD was assessed with anti-CD34 antibodies and the results were compared between tumors with average (AVD) and high (HVD) microvessel density. Alterations in the expression of thrombospondin-2 were more often seen in early (FIGO stage I and II) than in late stage tumors (66% vs. 30%, p=0.01). Age, menopausal status, the histological type and tumor grade did not correlate with thrombospondin-2 expression, however, silencing of THBS2 gene was more often seen in higher rather than in lower grade (50% vs. 28%) cancers and in nonserous rather than in serous (43% vs. 32%) tumors. In 81% of THBS2 mRNA-negative tumors, a hypermethylated promoter region of THBS2 was found (p=0.00003). An unmethylated product of the MSP reaction was more often detected in high grade tumors (93% vs. 76%, p=0.04). The incidence of THBS2 hypermethylation was not related to the tumor histological type, but unmethylated THBS2 was more often found in serous rather than in nonserous tumor (96% vs. 74%, p=0.01). The median MVD in malignant the tumor samples was 21.7 (range: 7.6-55.2). In the group with HVD, 54% were THBS2 mRNA negative, conversely, in the group with AVD tumors only 26% of the cases had undetectable THSB2 mRNA. A significant correlation between microvessel density and the expression of thrombospondin-2 (p=0.009) was found. In the samples with HVD, 51% had hypermethylated THBS2, however methylation pattern had no significant influence on microvessel density. In conclusion, hypermethylation might be responsible for altered expression of thrombospondin-2 in ovarian cancer. The THSB2 methylation pattern had no significant influence on microvessel density.
INTRODUCTION

Neovascularization is a common feature of many human cancers including malignant ovarian tumors. The phenomenon of new blood vessel formation plays an important role in the metastatic process because tumor cells easily enter the circulation via microvessels (1). Angiogenesis is regulated by a wide variety of angiogenesis stimulators (2, 3). The thrombospondins (TSPs) are known to be angiogenesis inhibitors and/or promoters, and, therefore, are important factors in tumor proliferation (2,3). There are a family of extracellular matrix glycoproteins consisting of five members in vertebrates, TSP-1, -2, -3, -4, and -5 (4-7). TSP-1 and TSP-2 are structurally more similar to each other than to TSPs 3–5 and are, therefore, considered to be constitutes of a subfamily (8). They are homotrimeric proteins composed of three identical 150-kDa monomers connected by disulfide bridges (9, 10). This similar structure is reflected in the finding that both TSP-1 and TSP-2 interact with a number of the same cell surface receptors, including heparan sulfate proteoglycans, low density lipoprotein-related receptor protein and integrin (11).

Thrombospondins are produced by stromal fibroblasts, endothelial cells and immune cells. TSP-1 inhibits angiogenesis through direct effects on endothelial cell migration and survival, and through indirect effects on growth factor mobilization (12). Thrombospondin-2 has also been shown to inhibit the angiogenic activity, mitogenesis and formation of focal adhesions in endothelial cells, as well as the spreading of adrenocortical cells in vitro, however, functional properties of TSP-2 in vivo remain elusive (13-16). TSP-1 and TSP-2 that are present in tumor microenvironments also act to suppress tumor cell growth through the activation of transforming growth factor-β in those tumor cells that are responsive to TGF-β (17). In general, the expression of gene coding thrombospondins -THBS is decreased in tumor cells that contain mutations that affect various oncogenes and tumor suppressor genes. In several tumors types like melanoma, bladder carcinoma and colon carcinoma samples, decreased expression of THBS1 correlated with mutations in p53 gene (18,19). In endometrial carcinoma, downregulated expression of THBS2 was found to be correlated with the lymphatic vascular space and cervical involvement, and was inversely related to disease-free survival (20). Decreased thrombospondin-1 and-2 expression also correlates with decreased tumor angiogenesis (19). Intratumoral microvessel density (MVD) determined by staining endothelial antigen like factor-VIII, CD31 or CD34 on histological sections might be used as a quantitative measure of angiogenesis (21). The degree of tumor angiogenesis, as assessed by MVD, has...
emerged as a powerful candidate for determining the prognosis of patients, and as a predictive tool in many solid tumors.

The mechanisms controlling the expression of thrombopondins are not completely understood. Li et al. proposed that alterations in DNA methylation could play a role in regulation of THBS gene expression (22). DNA is hypermethylated in the so called “CpG islands”, which are short stretches of guanine cytosine (GC)-rich sequences frequently located in the promoter and the first exon of genes (23). This epigenetic event is somatically heritable and increased density of the methylated CpG sites within a promoter region can accumulate over time during tumorigenesis. The DNA hypermethylation may provide an alternative pathway to gene deletion or mutation for the loss of tumor suppressor gene function (24). Methylated DNA also represents a promising biomarker for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignancies, and the spectrum of genes involved suggests that specific tumors may have their own distinct pattern of methylation (25-27).

In this study we investigated the possible role of trombospondin-2 related angiogenic activity of malignant ovarian tumors measured as MVD. To determine whether aberrant methylation-associated inactivation is involved in down-regulating thrombospondin-2 expression in ovarian cancer, we analysed the methylation status of THBS2.

MATERIALS AND METHODS

The studied group comprised of 70 women with malignant ovarian tumors who underwent surgery at the Ith Department of Gynecology of the Medical University in Lublin between the years 2004 and 2006. Tumor tissue was collected from consenting patients undergoing surgery. Ten samples were cut from benign ovarian tumors and five samples taken from normal ovaries were used as controls. Representative tumor samples were immediately snap-frozen in liquid nitrogen until further processing. All specimens were subjected to histological examination in order to confirm the diagnosis of ovarian cancer. The histological type and grade of all tumors were classified according to the criteria of the World Health Organization (WHO). The clinical stage of each malignant tumor was established according to International Federation of Gynecology and Obstetrics criteria. The proportion of malignant cells in all tumor tissues used in this study was more than 50%.

Detection of THBS2 hypermethylation

Genomic DNA was extracted using the DNAeasy Qiamp Mini Kit (Qiagen, USA) according to manufacturer’s protocol. For DNA modification we used the “CpG Modification Kit”(Qiagen, USA) according to the manufacturer’s instructions. Two µg’s of genomic DNA from each sample were modified by sodium bisulfite treatment, which converts all of the unmethylated cytosines to uracils, whereas the methylated cytosines remained unchanged. Modified DNA was amplified according to the method described by Herman et al. with two sets of specific primers designed to distinguish methylated DNA from unmethylated DNA in the promoter regions of the
THBS2 gene (28). The primer’s sequences designed by Whitcomb et al. and PCR conditions are available upon request (27).

**Analysis of THBS2 expression by RT-PCR method**

Tissue samples were homogenized in 1 ml of Trizol Reagent (Life Technologies, USA; 1 ml/35–45 mg of tissue). The homogenized samples were incubated for 5 min at room temperature. Following addition of 0.2 ml of chloroform, the samples were vigorously shaken for 15 s and incubated at room temperature for another 3 min. The samples were then centrifuged at 13.500 rpm for 15 min at 4°C. After the centrifugation, the aqueous phase containing total RNA was transferred to a fresh tube, and the same volume of isopropyl alcohol was added to the tube. The samples were incubated for 10 min at room temperature, and the RNA was precipitated by centrifugation. The RNA pellets were washed with 1 ml of 75% ethanol and dissolved in 40 µl of RNase-free water.

RT-PCR analysis was performed using a standard one-step RT-PCR kit (Qiagen), according to the manufacturer’s instructions, with primers specific for human THBS2 mRNA and beta-actin mRNA. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers for beta-actin. The primer’s sequences and PCR conditions are available upon request.

**Microvessel density assessment**

MVD analysis was performed on 70 paraffin wax-embedded representative tumor tissue sections fixed in 10% neutral buffered formalin. In all samples, monoclonal mouse anti-human CD34 antibody (clone QBEND/10; DAKO, Denmark) was used for MVD assessment. Following antigen retrieval with the citrate buffer (0.01 M, pH 6.0), two cycles of heating in a microwave oven (each of 5 min, at 750 W) were performed. Tissue sections were incubated at 37°C with the primary antibody for 1 h (dilution: 1:25). The slides were then incubated with the anti-mouse secondary antibody conjugated with streptavidin-biotin-peroxidase complex (LSAB2/HRP kit; DAKO, Denmark), and color reaction was developed using DAB (3'-3-diaminobenzidine tetrahydrochloride (Sigma, USA) chromogen substrate for 5 min at 37°C. The sections were counterstained with Mayer’s hematoxylin. In each case, negative control was applied by replacing the original antibody with PBS or nonimmune serum. Sections of normal ovary were used as a positive control. Microvessel density counting was performed by an experienced pathologist who had no knowledge of the patient’s clinical data. All histological studies were performed with Olympus CX41 (Japan) microscope equipped with digital camera image acquisition system (Olympus DP12). Initially, the most vascularised tumor areas were selected under low power magnification (x40 and x100) to identify the areas containing the greatest number of stained vessels (so called “hot spots”). MVD was assessed under 200x magnification according to a method described by Weidner et al. (29) with the use of “count point” option of built-in software in five different vascular “hot spots”. Each brown stained cell or cell cluster that was clearly separated from adjacent microvessels, tumor cells and other connective tissue elements was considered as a single countable microvessel. Vessels characterized by thick muscular walls or with lumen greater than 20 µm in diameter were excluded from the count. The results were expressed as an MVD count per high power field (HPF) of microscope. To facilitate statistical analysis all cases were next subdivided into two groups with an average microvessel density (AVD), where MVD≤21.7 per HPF and with high microvessel density (HVD), where MVD>21.7 per HPF. Fig. 1 presents examples of two typical types of ovarian cancer microvessels stained with anti-CD34 antibody. The results were compared with the use of χ² or Spearman rank correlation tests where appropriate (Statistica 6.0, Statsoft, USA) and P<0.05 was considered statistically significant throughout the study.
The RT-PCR products of THBS2 mRNA were found in all control samples and in 43 (62%) of ovarian cancer samples. In the remaining 27 (38%) samples no detectable expression of THBS2 mRNA was found (Fig. 2AB, Table 1). Alterations in the expression of thrombospondin-2 gene were more often seen in early (FIGO stage I and II) rather than in late stage tumors (66% vs. 30%, p=0.01). Abnormal expression of THBS2 was not related to age and the menopausal status of the patients. The histological type and grading of tumors were not correlated with thrombospondin-2 expression, however, silencing of THBS2 gene was more often seen in high rather than low grade (50% vs. 28%) cancers. To identify a possible link between the gene’s promoter DNA hypermethylation and expression of THBS2, we examined methylation status of the DNA obtained from tumor samples. With the use of the MSP method we found that in 35 of 70 (50%) cases of ovarian cancer, hypermethylated product of MSP amplification was present. Moreover, in 59 (84%) unmethylated product of
MSP was also detected. In 25 samples both products of amplification with methylated or unmethylated primer sets were found. In these cases 15 (60%) of the tumor samples were THBS2 mRNA negative. In 81% (22 of 27 cases) of tumors with altered expression of thrombospondin-2 the hypermethylated products of MSP were found and it was noted to be statistically significant ($\chi^2 = 17.42 \ p=0.00003$). Aberrant methylation of THBS2 was more often seen in tumors of premenopausal rather than postmenopausal women (61% vs. 41%), however, the difference did not reach statistical significance (p=0.09). The unmethylated product of the MSP reaction was more often detected in well differentiated tumors rather than in low grade tumors and it was noted to be

Fig. 2.
Immunohistochemical staining for CD34 (brown spots) used for microvessel density assessment in two types of ovarian cancer: (A) high grade ovarian serous carcinoma with average microvessel density (AVD) and (B) mucinous ovarian cancer with high microvessel density (HVD). Original magnification x200.
statistical significant (93% vs. 76%, p=0.04). Hypermethylation was not related to the clinical tumor stage. The incidence of THBS2 hypermethylation was not related to the histological type of the tumor, but unmethylated THBS2 was more often found in serous than in nonserous tumor (96% vs. 74% p=0.01).

The mean number of microvessels assessed in malignant tumors with CD-34 antibody was 21.7 (range: 7.6-55.2). There were 33 cases with high microvessel density (where MVD>21.7) and in this group 54 % (18) showed downregulation of thrombospondin-2 expression. Conversely, in tumor samples with average microvessel density only 9 (24%) cases had undetectable THSB2 mRNA. A significant correlation between microvessel density and expression of thrombospondin-2 was found (R=0.677; p=0.009). In samples with HVD, 17 of 33 cases (51%), hypermethylation of THBS2 occurred, however, methylation pattern had no significant influence on microvessel density.

Table 1. Selected clinical and pathological features of studied tumors and methylation status of THBS2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hypermethylated THBS2 n (%)</th>
<th>Unmethylated THBS2 n (%)</th>
<th>Lack of THBS2 mRNA n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients (%)</td>
<td>35 (50)</td>
<td>59 (84)</td>
<td>27 (38)</td>
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<table>
<thead>
<tr>
<th>Menopausal status</th>
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<tbody>
<tr>
<td>Before</td>
<td>n=31</td>
<td>19 (61)</td>
<td>28 (90)</td>
</tr>
<tr>
<td>After</td>
<td>n=39</td>
<td>16 (41)</td>
<td>29 (78)</td>
</tr>
<tr>
<td>χ²; p value</td>
<td>χ²=2.8; p=0.09</td>
<td>χ²=1.53; p=0.21</td>
<td>χ²=0.26; p=0.6</td>
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<th>Tumor grade</th>
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<tr>
<td>1+2</td>
<td>n=32</td>
<td>14 (43)</td>
<td>30 (93)</td>
</tr>
<tr>
<td>3</td>
<td>n=38</td>
<td>21 (55)</td>
<td>29 (76)</td>
</tr>
<tr>
<td>χ²; p value</td>
<td>χ²=0.92; p=0.33</td>
<td>χ²=3.9; p=0.04</td>
<td>χ²=3.2; p=0.07</td>
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<th>FIGO stage</th>
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<tr>
<td>I+II</td>
<td>n=15</td>
<td>9 (60)</td>
<td>11 (73)</td>
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<tr>
<td>III+IV</td>
<td>n=55</td>
<td>26 (47)</td>
<td>48 (87)</td>
</tr>
<tr>
<td>χ²; p value</td>
<td>χ²=0.76; p=0.38</td>
<td>χ²=0.05; p=0.81</td>
<td>χ²=6.35; p=0.01</td>
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<th>Histological type</th>
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<tr>
<td>Serous</td>
<td>n=31</td>
<td>14 (45)</td>
<td>30 (96)</td>
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<tr>
<td>Nonserous</td>
<td>n=39</td>
<td>21 (5)</td>
<td>29 (74)</td>
</tr>
<tr>
<td>χ²; p value</td>
<td>χ²=1.24; p=0.26</td>
<td>χ²=6.55; p=0.01</td>
<td>χ²=0.93; p=0.33</td>
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<th>Microvessel density</th>
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<tbody>
<tr>
<td>AVD</td>
<td>n=37</td>
<td>18 (48)</td>
<td>33 (89)</td>
</tr>
<tr>
<td>HVD</td>
<td>n=33</td>
<td>17 (51)</td>
<td>26 (78)</td>
</tr>
<tr>
<td>χ²; p value</td>
<td>χ²=0.05; p=0.84</td>
<td>χ²=1.4; p=0.2</td>
<td>χ²=6.77; p=0.009</td>
</tr>
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Abbreviations: n-number of patients in clinicopathological groups; FIGO- indicates International Federation of Gynecology and Obstetrics; AVD- average microvessel density; HVD- high microvessel density.
Aberrant methylation in promoter region of various genes is acquired during tumorigenesis but is not normally present in ovarian tissue. In this study, we examined expression of thrombospondin-2 and its mechanisms of regulation in ovarian cancer and its relationship with tumor angiogenesis. We discovered that thrombospondin-2 gene was silenced in a subset of malignant ovarian tumors (38%). Interestingly, alterations in the expression of the thrombospondin-2 gene were more often seen in early rather than in late stage tumors. Conversely in most of the advanced stage tumors THBS2 mRNA was detected. Probably, thrombospondin-2 expression may be associated with an aggressive phenotype in this class of malignancies. Previous data has shown that thrombospondin-2 may possess inhibitory functions similar to thrombospondin-1 tumors in patients with advanced epithelial ovarian carcinoma. On the other hand, thrombospondin may play a role as a potent stimulator of tumor progression and angiogenesis, as it modulates proteolytic enzyme levels in endothelial cells (13, 30). Kodama et al. (31) found that the expression of thrombospondin-2 was detected in only 24% of epithelial malignant ovarian tumors. Interestingly, women exhibiting thrombospondin-2 expression demonstrated a markedly poorer prognosis than those with downregulated expression. The role played by thrombospondin-2 in the regulation of angiogenesis is not clear. Positive and negative effects of thrombospondins on angiogenesis have been reported. Alvarez et al. (32) suggested that the reduction of thrombospondin expression may be coupled with the development of a pro-angiogenic environment and a malignant phenotype. Our current results are in agreement with this hypothesis. We have found that in tumors with a high number of microvessels, altered expression of THBS2 is more often. It has been postulated that thrombospondins are responsible for the proteolytic degradation of the extracellular matrix, which is one of key processes in early angiogenesis (33). The exact mechanism of thrombospondin’s inhibitory effects is not known. Bornstein et al. have found that thrombospondin-2-null mice, generated by targeted disruption of the THBS2 gene, display a complex phenotype that was at least in part characterized by a variety of connective tissue abnormalities and increased vascular density in skin and subcutaneous tissues (34). These authors suggested that the possible role of thrombospondin-2 was the regulation of cell function by modulating cell-matrix interactions, rather than acting as an integral component of the matrix. In a subsequent study by Bornstein et al. (17), it was demonstrated that this protein had a potent influence on tumor cell adhesion, interfered with fibronectin-mediated extracellular matrix interactions and altered proteases activity. Kasper et al. (35) studied the expression of thrombospondin-1 in relation to angiogenic activity in pancreatic tumors. In their study, tumors with a high expression of thrombospondin had also higher MVD and this correlation was highly significant. The authors concluded that as a modulator of angiogenesis, thrombospondin-1 is strongly expressed in most pancreatic adenocarcinomas and is likely to contribute to the extensive neovascularization and spread of this highly aggressive tumor.
One of the postulated explanations of gene expression regulation may be the promoter-associated CpG island hypermethylation that also appears to play a role in the post-transcriptional inactivation of THBS2 (27). To determine whether aberrant methylation-associated inactivation is involved in down-regulating thrombospondin-2 expression in ovarian cancer, we analysed the methylation status of THBS2. In our present study we found that hypermethylation of THBS2 occurred in 50% of malignant tumor samples. In most of these cases (81%) with altered THBS2 expression, hypermethylation of the promoter region of gene occurred. A strong correlation between these variables suggests that in ovarian cancer an aberrant methylation process might be responsible for downregulation of THBS2. Our results are in line with the observations published by Whitcomb et al. who investigated methylation status of THBS2 in endometrial adenocarcinoma (27). These authors detected hypermethylation of THBS2 in 15 of 24 (62.5%) endometrial tumors and in 2 of 5 (40%) studied cancer cell lines. The authors suggested that promoter methylation of THBS2 is a common molecular event in endometrial cancer. Moreover, it is likely that post-transcriptional inactivation of thrombospondin-2 by aberrant DNA methylation of the promoter region may participate also in ovarian carcinogenesis through THBS2 down-regulation.

Little is currently known about the role of methylation in ovarian cancer. Previous studies by Stradhee et al. demonstrated that hypermethylation of the CpG islands is a frequent event in ovarian cancers (24). The majority of studies to date have focused on the p16 gene, however, the loss of p16 expression is seen in a substantial proportion of ovarian tumors, conflicting results have been obtained as to whether or not methylation plays a role in this event (36-38). In our previous study we had demonstrated the significant role of tumor suppressor methylation in the inactivation of OPCML gene and in abnormal expression of oncogene SNCG in ovarian tumorigenesis (39, 40). We have found that abnormal methylation pattern has occurred in 75% of the ovarian cancer with altered OPCML expression. Our results also indicate that upregulation of SNCG expression is very common in ovarian cancer (77%) and is correlated with demethylation of SNCG. It is possible that ovarian cancer has a more complex pattern of methylation, since not all genes that exhibit hypermethylation appear commonly methylated in the same tumor (24).

Detection of the promoter CpG island hypermethylation may offer several advantages compared with other DNA alterations in ovarian cancer. Abnormal DNA methylation represents a chemically and biologically stable tumor-specific marker that can be readily detected, independent of the level of gene expression (41). The mechanisms controlling the expression of thrombospondin-2 are not completely understood. Previous observations emphasized the complex nature of THBS2 regulation, and indicated that both transcriptional and post-transcriptional mechanisms could be involved. Putative transcription factor binding sites that might be significant for THBS2 regulation included p53, NF-kappaB, Spl, Myc-CF1, NF-Y, CF1, API, and GATA sites (19, 42). Adolph et al. (42) found
differences in the lengths of transcription start sites which were tissue-specific and probably determined differences in expression of the THBS2. These results suggested that a tissue-specific gene could be determined, at least in part, by a selection of the transcription start site and result in differences in the 5’ untranslated region. The expression of thrombospondin-2 homologue, thrombospondin-1 may also be regulated by hypermethylation. Oue et al. (26) measured the expression of this gene using quantitative RT-PCR in gastric cancer samples and in the surrounding non-neoplastic mucosae. The authors examined the correlation between thrombospondin-1 mRNA expression and the promoter methylation status of THBS1, as well as with the p53 mutation status. The results of the study indicated that expression levels of thrombospondin-1 were associated with promoter hypermethylation of THBS1, however, the expression was not associated with the p53 mutation status. Similar results were obtained by Yang et al. who investigated patterns of expression and the mechanisms of regulation of thrombospondin-1 in neuroblastoma (43). THBS1 was silenced in a subset of undifferentiated, advanced-stage tumors and cancer cell lines and the transcriptional silencing of thrombospondin-1 was caused by aberrant methylation. Moreover, disrupting methylation with 5-Aza-dC led to a significant inhibition of neuroblastoma growth in vivo and re-expression of thrombospondin-1 in a subset of neuroblastoma xenografts. The results of this study suggest that demethylating agents may prove to be effective candidates for the treatment of neuroblastoma in children.

In summary, promoter region hypermethylation might be responsible for altered expression of the THBS2 gene in ovarian cancer. It is likely that post-transcriptional inactivation of thrombospondin-2 by aberrant DNA methylation may also participate in angiogenesis of malignant ovarian tumors through THBS2 down-regulation. The relative importance of various mechanisms inhibiting tumor growth by thrombospondin-2 is probably not the same in different types of malignancies. A complete understanding of the THBS2 role in tumor progression may eventually permit the design of therapies that could specifically target these mechanisms.

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