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Original Article

Prune 2 immunohistochemical expression in uterine smooth muscle tumors

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Abstract: The histologic classification of uterine smooth muscle tumors regarding their malignant potential is often challenging dilemma. Prune 2 has been reported to have a diagnostic utility in differentiating leiomyosarcomas from gastrointestinal stromal tumors. However, its diagnostic value in leiomyosarcoma versus other uterine smooth muscle tumors has not been elucidated yet. The aim of this study was to assess the value of Prune 2 immunohistochemical expression in distinguishing uterine smooth muscle tumors including leiomyomas (LM), Leiomyomas with bizarre nuclei (LBN), smooth muscle tumors of uncertain malignant potential (STUMP), and leiomyosarcomas (LMS) and to investigate its prognostic role in LMS. Immunohistochemical expression of Prune 2 was investigated in 94 uterine smooth muscle tumors including; 38 LM, 12 LBN, 6 STUMP and 38 LMS. Prune 2 immunostaining was scored based on the extent and intensity of staining. All 94 uterine smooth muscle tumors showed at least focal weak Prune 2 expression. The difference between LMS group and the combined non-sarcomatous groups (LM, LBN, and STUMP) was insignificant (P=0.101). On comparing LMS with LM, LBN, and STUMP individually, the only significant difference was noted upon comparing LMS with LBN (P=0.013). A highly significant difference was observed considering different subtypes of leiomyomas (P<0.001). Moreover, stage I LMS exhibited high Prune 2 expression as compared to higher stages (P=0.012). In conclusion; Prune 2 immunohistochemical expression in uterine smooth muscle tumors has a limited diagnostic value in discriminating these tumors. However, Prune 2 could act as a potential prognostic marker in uterine leiomyosarcomas.

Keywords: Prune 2, uterine smooth muscle tumors, leiomyosarcoma, STUMP, leiomyoma, immunohistochemistry

Introduction

Uterine smooth muscle tumors are the most common neoplasms in female genital tract [1]. Three basic morphologic features represent significant predictors for malignant potential of uterine smooth muscle tumors including; cytological atypia, mitotic index, and coagulative tumor cell necrosis [2]. Based on these features; uterine smooth muscle tumors are classified into five categories (1) leiomyoma (LM), (2) mitotically active leiomyoma (a leiomyoma with more than 10 mf/10 hpf) (3) leiomyomas with bizarre nuclei (LBN), (4) leiomyosarcoma (LMS), and (5) smooth muscle tumor of uncertain malignant potential (STUMP). A diagnostic strategy has been proposed for their diagnosis on the basis of routine hematoxylin & eosin level [3].

However, due to the overlapping features between malignant and benign smooth muscle tumors, the differential diagnosis can be problematic and challenging in some cases. Sometimes, it is difficult to differentiate LMS from certain variants of leiomyomas including LBN, mitotically active leiomyoma and STUMP [4]. Another diagnostic pitfall; is that some clinically aggressive tumors may have bland cytological features on examination by hematoxylin & eosin [2, 5]. Accordingly, the use of immunohistochemistry may help in reaching to the final diagnosis when the type of tumor is in question. Many markers were examined for this diagnostic purpose including p16, p53, IMP3, Ki-67, fascin [6-11], stathmin 1 [12] and PHH3; yet, these markers often have a specific drawback that limits their utility [4, 13].

Prune homolog 2 (Drosophila) (Prune 2) is a protein encoded by the Prune 2 gene [14], and is known to be a susceptibility gene for Alzheimer disease [15]. The level of Prune 2 expression in

Table 1. Main characteristics of leiomyosarcoma patients (n=38)

	No (%)
Age (years)	
<50	16 (42.1%)
≥50	22 (57.9%)
Mean age ± SD	54±10.3
Tumor size	
<10 cm	24 (63.2%)
≥10 cm	14 (36.8%)
Clinical Stage	
Stage I	30 (78.9%)
Stage II	4 (10.5%)
Stage III	2 (5.3%)
Stage IV	2 (5.3%)
Histopathologic subtype	
Spindle	32 (84.2%)
Epithelioid	6 (15.8%)

adult nerve tissue was found to be higher than immature nerve tissue indicating its role in maintenance of mature nerve cells. High Prune 2 mRNA expression was detected in dorsal root ganglion, whole brain, spinal cord, prostate and uterus. Hypothetical Prune 2 gene consists of C9orf65 and BMCC1/BNIPXL, both of which are malignant tumor associated genes [16].

In prostate cancer cells, it acts as a tumor suppressor gene and its expression is regulated by prostate cancer antigen 3 [17]. In a functional study, Prune 2 suppresses Ras homolog family member A (RhoA) activity through its BNIP-2 and Cdc42GAP homology (BCH) domain by interfering with binding between RhoA and A-kinase anchor protein 13 (Lbc), a Rho-specific guanine exchange factor; this interference results in reduced stress fiber formation and suppression of oncogenic cellular transformation [18]. On the other hand; Prune 2 was proved to be implicated in pathogenesis of certain tumors including neuroblastoma [19], parathyroid & prostatic carcinomas [17, 20] and possibly leiomyosarcoma [21].

However, little is known about the role of Prune 2 in LMS which was found to have a high diagnostic utility in differentiating LMS from GIST in 99% of cases [22, 23]. To our knowledge, the value of Prune 2 expression in leiomyosarcoma versus other uterine smooth muscle tumors has not been elucidated yet. This concept stimulates us to investigate whether Prune 2 immunohistochemical expression has a role in differentiating leiomyosarcoma versus other uterine smooth muscle tumors. In addition, we assess its prognostic value in leiomyosarcoma.

Materials and methods

Tissue collection

This retrospective study was conducted on 94 cases of randomly selected uterine smooth muscle tumors (38 leiomyomas (LM), 12 leiomyomas with bizarre nuclei (LBN) (previously termed atypical leiomyoma), 6 smooth muscle tumors of uncertain malignant potential (STUMP), and 38 leiomyosarcomas (LMS)). The subtypes of 38 cases of LM included 18 conventional, 8 mitotically active, 4 for each cellular & epithelioid types and 2 for each hydropic & lipomatous types. All cases were retrieved from the archives of the Early Cancer Detection Unit, Ain Shams Obstetrics and Gynecology Hospital, Cairo, Egypt during the period 2010-2015. The cases were reviewed and the histopathological diagnosis was assigned based on the 2014 World Health Organization classification of tumors of female reproductive organs [24]. The available clinicopathologic features for LMS cases, including age, tumor size, and histopathological subtype were collected. In addition, their pathologic staging according to FIGO classification of uterine sarcomas was evaluated [24]. The study was carried out with full local ethics approval.

Immunohistochemical staining procedure

Four-micron thick sections of the formalin-fixed and paraffin-embedded tissue blocks of all studied 94 uterine smooth muscle tumors cases were prepared. Immunohistochemical staining using a rabbit polyclonal anti-Prune homolog 2 antibody (Abcam Company, Cambridge, UK, Cat. # ab80262, diluted 1:200) was performed applying a labelled streptavidin- biotin-peroxidase complex technique. Briefly, tissue sections were deparaffinized in xylene and rehydrated in descending grades of alcohol. After rinsing in PBS, antigen retrieval was done by treating the tissue sections with citrate buffer (pH 6.0) for 10 minutes in a 700-W microwave oven. The endogenous peroxidase activity was inhibited by incubating the sections in

Table 2. Prune 2 immunohistochemical expression in uterine smooth muscle tumors (n=94)

Tumor type	Prune 2 expression	
	Low expression	High expression
Non-sarcomatous tumors (n=56)	12/56 (21.4%)	44/56 (78.6%)
Leiomyoma (n=38)	12/38 (31.6%)	26/38 (68.4%)
Leiomyomas with bizarre nuclei (n=12)	0/12 (0%)	12/12 (100%)
STUMP* (n=6)	0/6 (0%)	6/6 (100%)
Leiomyosarcoma (n=38)	14/38 (36.8%)	24/38 (63.2%)

*STUMP = smooth muscle tumors of uncertain malignant potential.

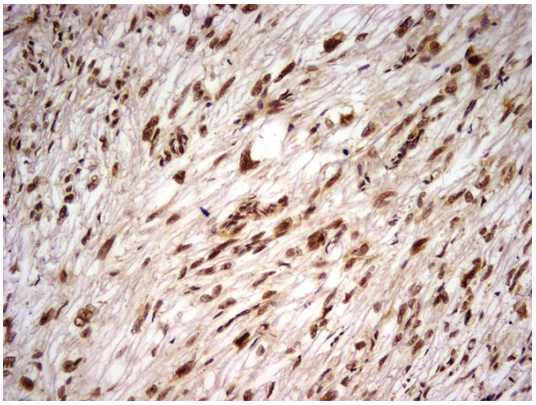

Figure 1. Leiomyoma with bizarre nuclei (LBN) showed high Prune 2 expression; immuoperoxidase, original magnification ×200.

3% hydrogen peroxide for 10 minutes, and then washed in buffer. This is followed by incubation with the primary antibody (Prune homolog 2) overnight at room temperature. The antibody reaction was detected with the avidin-biotin detection kit using diaminobenzidine as chromogen. Sections were counterstained with Harris Hematoxylin then mounted in Canada balsam. Proper positive and negative controls were performed.

Immunohistochemical analysis

Prune 2 immunostaining was scored based on the extent and intensity of staining. Ten random high-power fields, each containing approximately 100 cells, were observed under the microscope. First, staining extent was scored according to the proportion of positive tumor cells: 0% (score 0), ≤10% (score 1), 11% to 25% (score 2), 26% to 50% (score 3), 51% to 75% (score 4), and >75% (score 5). Second, staining intensity was scored as follow: no staining (score 0), weak (score 1), moderate (score 2), and marked (score 3). Final scores were calculated by adding the scores of extent and intensity. For final scores of 0-4 were regarded as low expression, while for final scores >4 were regarded as high expression [21].

Statistical analysis

IBM SPSS statistics (V. 23.0, IBM Corp., USA, 2015) was used for data analysis. Data were expressed as mean ± SD for quantitative parametric measures in addition to both number and percentage for categorized data. Student t test was used to compare between two independent mean groups for parametric data. Chi-square test was also done to study the association between each two variables or comparison between 2 independent groups as regards the categorized data. The probability of error less than 0.05 was considered statistically significant, while less than 0.01 and 0.001 were highly significant.

Results

Among the studied 94 uterine smooth muscle tumors, the mean age ± SD of patients with LM, LBN, STUMP and LMS were 46.2±13.6, 47.2±10.5, 45±5, and 54±10.3 years respectively. The main characteristics of LMS patients (n=38) are summarized in **Table 1**.

The immunohistochemical expression of Prune 2 was predominantly cytoplasmic while both cytoplasmic and nuclear staining was noted in some of the cases. The expression of Prune 2 in uterine smooth muscle tumors are shown in **Table 2** and **Figures 1**-**6**.

The difference between the LMS group on one hand and the combined three non-sarcomatous groups (LM, LBN, and STUMP) on the other hand, regarding Prune 2 expression was assessed and proved to be non-significant (x^2= 2.688, P=0.101). On comparing LMS with LM, LBN, and STUMP individually, the only significant difference as regards Prune 2 expression was noted upon comparing LMS with LBN (X^2=6.140, P=0.013), while comparing LMS with STUMP did not reach statistical significance (X^2=3.242, p=0.072).

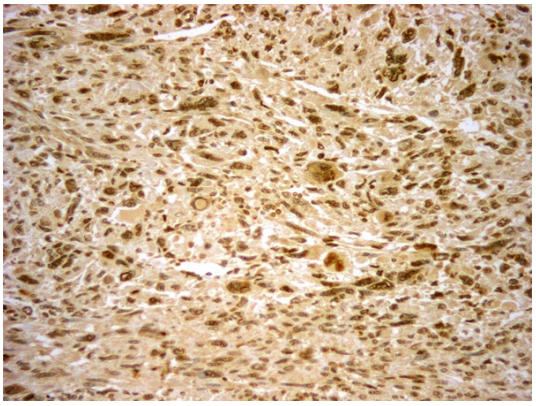

Figure 2. Smooth muscle tumors of uncertain malignant potential (STUMP) with high Prune 2 expression; immuoperoxidase, original magnification ×200.

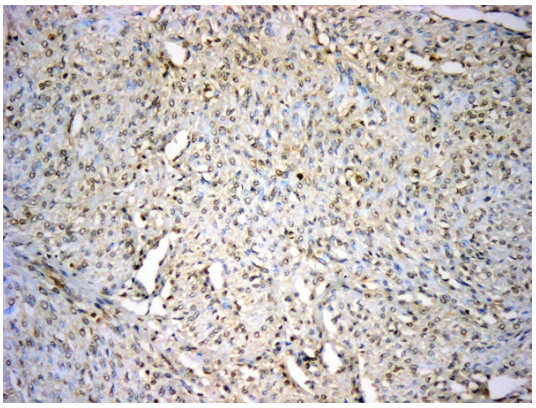

Figure 3. Mitotically-active leiomyoma with low Prune 2 expression; immuoperoxidase, original magnification ×200.

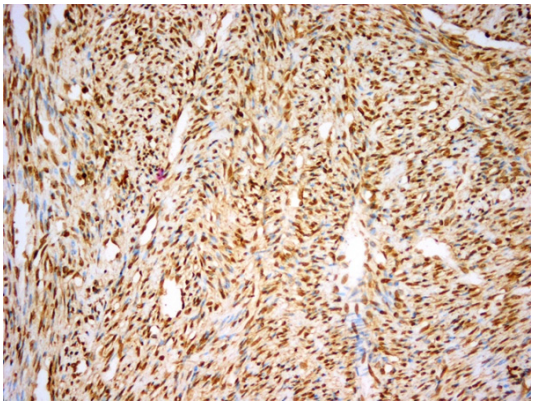

Figure 4. Convensional leiomyoma with high Prune 2 expression; immuoperoxidase, original magnification ×200.

A highly statistical significant difference in Prune 2 expression was noted considering different types of studied leiomyomas (X^2=38.00, p<0.001), see **Table 3**. It was remarkable that all the cellular and mitotically-active leiomyomas exhibited low Prune 2 expression, while all the conventional leiomyomas and the remaining other subtypes demonstrated high expression.

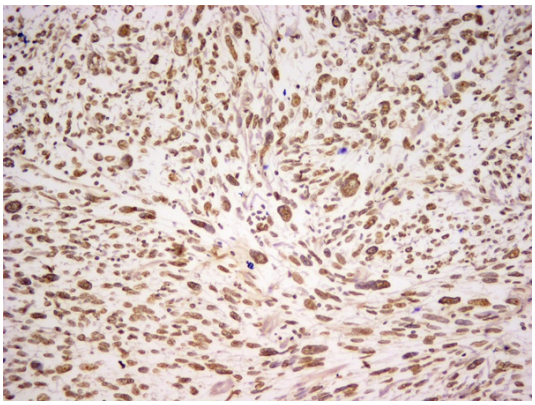

Figure 5. Leiomyosarcoma (stage I) showed high Prune 2 expression; immuoperoxidase, original magnification ×200.

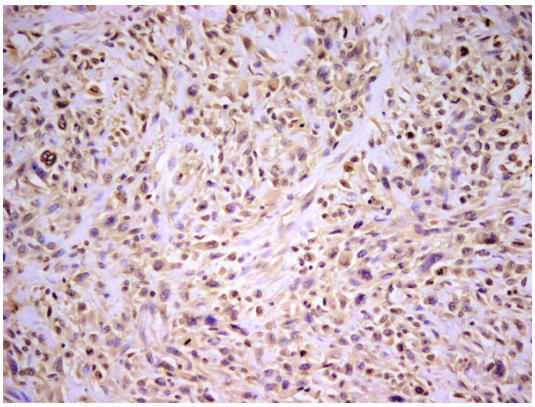

Figure 6. Leiomyosarcoma (stage III) showed low Prune 2 expression; immuoperoxidase, original magnification ×200.

Among the LMS cases, the correlation between Prune 2 protein expression and the clinicopathologic characteristics was studied, see **Table 4**. The higher clinical stages of LMS (Stages II+III+IV) demonstrated low Prune 2 expression when compared to stage I tumors and this observation reached statistical significance (X^2=6.341, p=0.012). No significant relationship was found between Prune 2 expression and patient's age (p=0.943), tumor size (p=0.557), and histopathological subtypes (P=0.846).

Table 3. Prune 2 immunohistochemical expression in leiomyoma cases (n=38)

Leiomyoma subtype	Prune 2 expression		X^{2*}	p*
	Low expression	High expression		
ConventionI leiomyoma (n=18)	0/18 (0%)	18/18 (100%)	38.00	<0.001
Mitotically active leiomyoma (n=8)	8/8 (100%)	0/8 (0%)		
Cellular leiomyoma (n=4)	4/4 (100%)	0/4 (0%)		
Epithelioid leiomyoma (n=4)	0/4 (0%)	4/4 (100%)		
Hydropic leiomyoma (n=2)	0/2 (0%)	2/2 (100%)		
Lipomatous leiomyoma (n=2)	0/2 (0%)	2/2 (100%)		

*Pearson Chi-Square test.

Table 4. Relationship between Prune 2 expression and clinicopathologic characteristics of LMS cases (n=38)

Characteristic	Prune 2 expression No (%)		X^2	p
	Low expression	High expression		
Age (years)				
<50 (n=16)	6 (37.5%)	10 (62.5%)	0.005	0.943
≥50 (n=22)	8 (36.4%)	14 (63.6%)		
Tumor size				
<10 cm (n=24)	8 (33.3%)	16 (66.7%)	0.345	0.557
≥10 cm (n=14)	6 (42.9%)	8 (57.1%)		
Clinical Stage				
I (n=30)	8 (26.7%)	22 (73.3%)	6.341	0.012
II+III+IV (n=8)	6 (75%)	2 (25%)		
Histopathologic subtype				
Spindle (n=32)	12 (37.5%)	20 (62.5%)	0.038	0.846
Epithelioid (n=6)	2 (33.3)	4 (66.7%)		

Discussion

Prune homolog 2 (Drosophilia) (Prune 2) with its different isoforms has been linked to several cellular processes including cellular transformation [18] and apoptosis [25]. The first report to associate Prune 2 to leiomyosarcomas was published by Price et al. [22]. They reported that Prune 2 could serve as a biomarker for LMS to accurately distinguish such sarcomas from gastrointestinal stromal tumors (GIST) using RT-PCR gene classifier. Subsequently, Yang et al. [23] also emphasized the higher Prune 2 mRNA expression among 31 LMS compared to 37 cases of GIST and they added that Prune 2 is correlated with the survival of leiomyosarcoma patients. Zhao et al. [21] have studied Prune 2 protein immunohistochemical expression among two cohorts of LMS cases from two different cancer centers for possible prognostic role and conflicting results were yielded in these two cohorts.

Therefore, this study was conducted to evaluate-for the first time-the expression of Prune 2 in the full spectrum of uterine smooth muscle tumors including LM, ALM, STUMP, and LMS and to determine whether it might be of diagnostic value in uterine smooth muscle tumors or not. We also further investigated whether Prune 2 protein expression might be of prognostic value among LMS.

This study revealed that Prune 2 expression in all examined uterine smooth muscle tumors was at least focal and weak. Prune 2 expression was not significant in differentiating LMS from the three non-sarcomatous studied groups and therefore has limited diagnostic utility in problematic cases of uterine smooth muscle tumors. The diagnostic utility of Prune 2 as a biomarker for LMS in its differential with GIST is also restricted by the fact that its expression is not yet studied in other sarcomas [22, 23].

To predict the possible role of Prune 2 in the pathogenesis of uterine smooth muscle tumors, its differential expression among variants of leiomyomas and also its correlation to different clinicopathologic parameters among leiomyosarcomas were investigated. It was interesting that all leiomyoma subtypes in addition to all cases of LBN showed high Prune 2 expression with the exception of cellular and mitotically active LM. The latter two subtypes consistently showed low Prune 2 expression. This finding might be attributed to the pro-apoptotic role of Prune 2 isoform BNIP2 and Cdc42GAP homology (BCH) motif-containing molecule at

the carboxyl terminal region 1 (BMCC1) reported in neuronal cells [14]. We assume that Prune 2 might have the same role as a pro-apoptotic protein in leiomyomas and hence its low expression leads to the balance loss between cell division and cell death, with consecutive high cellularity and elevated mitotic rate among this benign subset of smooth muscle tumors - the leiomyomas. It is worthy to note that all LBN showed high Prune 2 expression similar to conventional LM and therefore its expression is not related to the pathogenesis of this peculiar leiomyoma subtype.

The similar high expression of Prune 2 in all STUMP cases and in 73.3% of Stage I LMS to conventional leiomyomas might indicate that this protein is not directly related to the early steps of malignant switch and early carcinogenesis in uterine smooth muscle tumors.

Regarding Prune 2 expression among the established cases of LMS, this study could not establish a significant relationship between Prune 2 expression and either patient's age or tumor size. *Zhao* et al. [21] have found no relationship between Prune 2 expression and patient's age among leiomyosarcomas, yet they reported a significant relationship between high Prune 2 expression and smaller tumor size group (<10 cm). They assumed that the Prune 2 has a regulating role in programming cell apoptosis and thus the larger LMS tumor size might have worse survival. We also noted that higher stages of LMS showed low expression of Prune 2 upon comparison with Stage I LMS cases (P=0.012). This finding might mark the possible favorable prognostic value of high Prune 2 expression in leiomyosarcomas. This finding is in agreement with *Zhao* et al. [21], as these researchers although found no relationship between Prune 2 expression and tumor stage (stage I&II vs. III&IV), yet they reported that Prune 2 protein expression showed a significant positive correlation with overall survival. This possible favorable prognostic value of Prune 2 has been noted in neuroblastomas [14] and Prostatic carcinomas [23].

This apoptotic role that furnishes positive prognostic value for Prune 2 in these tumors was attributed to the interaction of BMCC1/Prune 2 with RhoA and RhoC (members of the Ras superfamily) with overexpression of the BMCC1 reduces active RhoA levels while knockdown has the reverse effect [18]. On the contrary to these findings, Prune 2 was found to infer a negative prognosis among breast carcinomas and was reported to be correlated with advanced nodal status and distant metastases suggesting its use as a marker for identification of more aggressive breast cancer [26]. They refer these findings to the possible role of Prune 2 in promoting cellular motility stimulating expression of genes involved in metastatic pathways namely inhibition of antimetastasis function of nm23-H1 [27, 28].

In conclusion, the present study has proved that Prune 2 is expressed by all uterine smooth muscle tumors and thus has limited diagnostic utility in the clinical practice to help establishing an accurate diagnosis in problematic cases of these tumors. Yet, Prune 2 could act as a potential prognostic marker among leiomyosarcomas with its low expression identifies the leiomyosarcomas that will be possibly associated with higher stages. The real impact of Prune 2 expression on overall survival and on the clinical outcome among leiomyosarcomas in addition to its probable interaction with proliferation, apoptosis and metastatic pathways among these sarcomas awaits further investigations.

Disclosure of conflict of interest

None.

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