Biomarkers, Genomics, Proteomics, and Gene Regulation

Increased Immunoreactivity to SLIT/ROBO1 in Ovarian Endometriomas

A Likely Constituent Biomarker for Recurrence

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While surgery is currently the treatment of choice for endometriosis, recurrence remains a serious problem, and its prevention is an unmet clinical need. SLIT, a secreted protein that functions through the Roundabout (ROBO) receptor as a repellent for axon guidance and neuronal migration, has been recently found to induce tumor angiogenesis. We investigated the potential role of SLIT/ROBO1 in ovarian endometriomas and examined their predictive value in recurrence based on tissue samples from 43 patients with recurrence and 45 without recurrence. Microvascular density counts were evaluated by CD34 immunohistochemistry, and statistical analyses were performed to evaluate the effect of SLIT/Robo1 on recurrence risk after adjustment for other risk factors. We found that SLIT expression was positively correlated with microvascular density in ectopic endometrium and that its expression was higher in ectopic endometrium than control endometrium. Both SLIT and Robo1 expression were higher in recurrent cases than in non-recurrent cases. Higher immunoreactivity to SLIT, along with the presence of adhesion, PR-B, and nuclear factor- κ B, was identified to be a risk factor for recurrence, with a sensitivity of 86% and a specificity of 87%. Therefore, increased SLIT immunoreactivity is likely an important constituent factor for recurrence of ovar-

ian endometriomas, possibly through promoting angiogenesis in ectopic endometrium. Thus, the SLIT/ROBO1 system may be a potential target for reducing the risk of recurrence. (Am J Pathol 2009, 175:479–488; DOI: 10.2353/ajpath.2009.090024)

Endometriosis is a common gynecological disorder and a leading cause of disability and loss of productivity in women of reproductive age, and is associated with dysmenorrhea, pelvic pain, and subfertility.¹ Due to either poorly documented or limited, short-term, efficacy of medical treatment alone, surgery is currently the treatment of choice for the management of endometriosis.² However, recurrence still remains a serious problem: 40% to 45% of patients have a relapse of the disease 5 years after the primary surgery and require further surgeries.^{2,3} However, repeated surgeries are positively associated with increased morbidity and health care costs and, in endometriosis, with damage to ovarian reserve.^{4–8} Clearly, prevention of recurrence is an unmet clinical need that has not been adequately addressed.

The exact causes for recurrence are still poorly understood. Several clinical studies suggest that the recurring endometriotic lesions arise from residual lesions or cells not completely removed during the primary surgery.^{9–11} Some other studies suggest, however, that recurrence may originate from *de novo* lesions derived from endometrium through retrograde menstruation.¹² The possibility that lymph node involvement by endometriotic foci and lymphovascular invasion could be responsible for recur-

Supported by grants 30872759 (S.W.G.) and 03030401/30571952 (X.S.L.) from the National Science Foundation of China, grant 074119517 from the Shanghai Science and Technology Commission (S.W.G.), and by the NIH grant CA126897 (J.G.G.).

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Accepted for publication April 23, 2009.

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rence also has been proposed,¹³ since lymph node involvement in some rare forms of endometriosis has been reported.^{14–17}

Regardless of its possible causes, angiogenesis is of paramount importance in the growth and survival of endometriotic lesions and thus in recurrence, since it must be an absolute requirement for any lesion to become clinically relevant. As in tumor metastases, endometriotic lesions require nutritional supply and thus neovascularization to maintain proliferation and to invade into ectopic sites within the host¹⁸; hence, angiogenesis has been recognized as an attractive target for novel medical therapeutics.^{19,20}

In endometriosis, the involvement of vascular endothelial cell growth factor (VEGF) and other angiogenic mediators has long been recognized.^{21–24} Preclinical studies have demonstrated the promising potential of anti-angiogenic therapies for endometriosis.^{25–27} Since endometriotic angiogenesis involves several pathways and the blockade of just a single pathway may not effectively suppress angiogenesis,²⁸ the identification of all possible angiogenic molecules and pathways would help devise more effective strategies to suppress angiogenesis in endometriosis. In addition, the role of angiogenesis in the recurrence of endometriosis so far has received scant attention.

SLIT is a secretory glycoprotein family consisting of three members, SLIT1, SLIT2, and SLIT3, which were originally identified as secreted repellents in axon guidance and neuronal migration.^{29–31} It has been shown to be an endogenously available inhibitor of leukocyte chemotaxis.³² The receptors for SLIT are the transmembrane protein family Roundabout (ROBO), which currently consists of four members: ROBO1–4.³³ Wang et al demonstrate that SLIT is secreted by various cancer cells and ROBO1 is expressed in vascular endothelial cells, where SLIT can attract vascular endothelial cells *in vitro* and promote tumor-induced angiogenesis in a xenograft model of human malignant melanoma cells.³⁴ Consistent with this finding, SLIT-ROBO4 is reported to function as a chemoattractant to recruit vascular endothelial cells to sites for vasculogenesis.^{35,36} More recently, it has been reported that, in a chemically-induced squamous cell carcinoma model in the hamster buccal pouch, increased SLIT expression was associated with higher tumor angiogenesis as reflected by increased VEGF expression and microvessel density (MVD).³⁷ More remarkably, treatment with a monoclonal antibody against ROBO1 that interrupts the SLIT-ROBO interaction inhibited tumor angiogenesis and growth, indicating that SLIT-mediated tumor angiogenesis is a critical process in the development of chemical-induced squamous cell carcinoma.

We hypothesized that SLIT/ROBO1 may also be involved in endometriotic angiogenesis and that their expression levels may thus be indicative of the propensity for recurrence. Therefore, we sought to investigate the expression and localization of SLIT, ROBO1, and CD34, which is considered to be a marker for MVD and thus a measure of angiogenesis in normal and pathological endometrium,³⁸ in women with recurrent and non-re-

current ovarian endometrioma and in control endometrium. We also sought to correlate SLIT/ROBO1 immunoreactivity with MVD and some putative prognostic variables. Finally, we sought to determine the prognostic value, if any, of SLIT, ROBO1, and MVD, along with other possible prognostic variables, in predicting recurrence of endometriosis.

Materials and Methods

Patients

The 109 originally recruited patients, their tissue samples and their use in the identification of biomarkers for recurrence of ovarian endometriomas have been reported previously.^{39,40} Briefly, patients with ovarian endometriomas were included for this study who underwent conservative or semiradical surgery via either laparotomy or laparoscopy at Shanghai OB/GYN Hospital, Fudan University Shanghai Medical College from 2003 to 2004. All diagnoses were confirmed histologically by experienced pathologists.

For this study, 21 patients were excluded because their tissue samples were used up, leaving 88 patients. Among them, 43 of them had recurrence within 30 months after surgery while the other 45 had not had recurrence at least 32.2 months after surgery. The two groups were remarkably similar in age, rAFS stage, and menstrual phase.

The recurrence of endometrioma, two months after the surgery, was defined as 1) the presence of ovarian cysts of ≥ 3 cm in diameter, along with characteristic echoes as detected by transvaginal ultrasonography for two consecutive menstrual cycles,⁴¹ coupled with or without the recurrence of dysmenorrhea or pelvic pain requiring medical intervention, or as 2) the presence of *de novo* ovarian endometrioma as confirmed by histology following a second surgery. Excepting four patients in the recurrence group, the determination of recurrence, or lack thereof, was made by ultrasound.

The time, in months, between surgery and the onset of recurrence was recorded as time-to-recurrence if it occurred, and the time between surgery and the last month of follow-up was recorded as censored time-to-recurrence if there was no recurrence. For the recurrence group, the time to recurrence ranged from 2 to 29.2 months, with a median of 11.8 months. For the non-recurrence group, the length of follow-up ranged from 32.2 to 54.2 months, with a median of 37.4 months. We note that the shortest length of follow-up in the non-recurrence group exceeded the longest recurrence-free period of the recurrence group, ensuring that the former group had enough opportunity for recurrence yet none occurred. We also note that the shortest length of follow-up in the non-recurrence group was over 30 months, which is beyond the stage in which the recurrence of ovarian endometriomas is reported to be purely stochastic.⁴²

For each patient, information was collected through reading medical charts and interviewing patients on age

at surgery, body mass index at surgery, results of pelvic exams, type of surgery, mode of surgery (conservative or semiradical), complaint of dysmenorrhea or infertility, presence of adenomyosis or myoma, if any, laterality of endometrioma, size of endometrioma (defined to be the diameter in centimeter of the largest cyst), presence of adhesion or not, rAFS scores and stage, postoperative medication use, improvement of symptomology, and time to recurrence. The menstrual phase at which the patient was in at the time of surgery also was determined based on the number of days elapsed since the last period.

As controls, we also included endometrial tissue samples from 30 women with tubal infertility or surgically

diagnosed benign ovarian cysts, but none had endometriosis, adenomyosis, or myoma. The age of women in this control group ranged from 23 to 46 years, with a mean age of 35.3 (SD = 6.9) years. Among the 30 women, 3, 10, and 5 were in the early, mid, or late proliferative phase, respectively, and the other 5, 4, and 3 were in the early, mid, or late secretory phase, respectively. In other words, 18 were in the proliferative phase and the other 12 were in the secretory phase. There is no difference in age or menstrual phase between the control and the recurrence or non-recurrence groups (all *P* values >0.09).

This study was approved by the institutional ethics review board of Shanghai OB/GYN Hospital.

Table 1. Characteristics of the Recurrence and Non-Recurrence Groups, and Statistical Significance (*P* value) of the Difference between the Two Groups

Variable name	Recurrence group (n = 43)	Non-recurrence group (n = 45)	<i>P</i> value
Age at surgery (in years)	32.9 ± 7.1 (18 to 47), median = 32	33.8 ± 6.2 (22 to 45), median = 33.0	0.53
Length of follow-up (in months)	12.6 ± 6.4 (2 to 29.2), median = 11.8	38.0 ± 4.6 (32.2 to 54.2), median = 37.4	<0.00001
Menstrual phase (n) when surgery was performed			0.80
Proliferative	17 (39.5%)	19 (42.2%)	
Secretory	26 (60.5%)	26 (57.8%)	
rAFS stage (n)			0.56
I	0 (0.0%)	0 (0.0%)	
II	5 (11.6%)	6 (13.3%)	
III	24 (55.8%)	27 (60.0%)	
IV	14 (32.6%)	12 (26.7%)	
Body mass index	20.9 ± 2.8 (17.1 to 32.3), median = 20.3	21.2 ± 2.3 (16.9 to 26.8), median = 20.7	0.27
Complaint of Infertility (n)			0.21
No	34 (79.1%)	40 (88.9%)	
Yes	9 (20.9%)	5 (11.1%)	
Complaint of dysmenorrheal			0.06
No	10 (23.3%)	19 (42.2%)	
Yes	33 (76.7%)	26 (57.8%)	
Previous endometriosis-related surgery (n)			0.76
No	37 (86.0%)	40 (88.9%)	
Yes	6 (14.0%)	5 (11.1%)	
Previous use of endometriosis-related medication			0.08
No	32 (74.4%)	40 (88.9%)	
Yes	11 (25.6%)	5 (11.1%)	
Presence of adenomyosis and/or myoma (n)			0.55
No	33 (76.7%)	32 (71.1%)	
Yes	10 (23.3%)	13 (28.9%)	
Presence of adhesion (n)			0.03
No	15 (34.9%)	26 (57.8%)	
Yes	28 (65.1%)	19 (42.2%)	
Mode of surgery (n)			0.67
Laparotomy	23 (53.5%)	22 (48.9%)	
Laparoscopy	20 (46.5%)	23 (51.1%)	
Method of surgery (n)			0.36
Conservative	33 (76.7%)	38 (84.4%)	
Semi-radical	10 (23.3%)	7 (15.6%)	
Size			0.34
<5 cm	15 (34.9%)	13 (28.9%)	
5 to 10 cm	27 (62.8%)	28 (62.2%)	
>10 cm	1 (2.3%)	4 (8.9%)	
Laterality (n)			0.23
Left ovary only	17 (39.5%)	20 (44.4%)	
Right ovary only	7 (16.3%)	13 (28.9%)	
Bilateral	19 (44.2%)	12 (26.7%)	
Post-surgery medication (n)			0.40
No	20 (46.5%)	23 (51.1%)	
Yes	23 (53.5%)	22 (48.9%)	

Tissue Sample, Antibodies, and Immunohistochemistry

Archived, formalin-fixed, paraffin-embedded tissue blocks were retrieved from the Department of Pathology, Shanghai OB/GYN Hospital. Serial 4- μ m sections were obtained from each block, with the first resultant slide being stained for H&E to confirm pathological diagnosis, and the subsequent slides stained for pan-SLIT, ROBO1, and CD34. Routine deparaffinization and rehydration procedures were performed.

Antibodies to ROBO1 and pan-SLIT were prepared and characterized as reported previously.³⁴ Mouse monoclonal anti-CD34 (M-0117) was purchased from Changdao Bio-Reagent Company (Shanghai, China). Mouse pre-immune IgG antibody was purchased from Biofriendship Company (Shanghai, China). The concentration used for the pan-SLIT and ROBO1 antibody was both 5 μ g/ml, that for CD-34, 1 μ g/ml. For antigen retrieval, the slides were heated at 98°C in an EDTA buffer (pH 9.0) for a total of 45 minutes and cooled naturally to the room temperature. Sections were then incubated with the primary antibody overnight at 4°C. After slides were rinsed the horseradish peroxidase-labeled secondary

antibody, Supervision Universal (Anti-Mouse/Rabbit) Detection Reagent (Shanghai Gene Tech Company, Shanghai; Cat. No: GK500705), was incubated at room temperature for 30 minutes. The bound antibody complexes were stained for 3 to 5 minutes or until appropriate for microscopic examination with diaminobenzidine and then counterstained with hematoxylin (30 seconds) and mounted.

Each staining run incorporated a positive control slide from an endometrial carcinoma tissue sample. A negative control was also incorporated using pre-immune IgG instead of the primary antibody. The scoring of the immunoreactivity to SLIT and ROBO1 was evaluated by Image-Pro Plus version 6.0 without knowledge of the recurrence status of the patient being evaluated.

The PR-B, NF- κ B, and COX-2 immunoreactivity data that were reported previously^{39,40} were also used in our study.

Quantification of Angiogenesis

MVD was assessed on CD34 stained slides by light microscopy in the areas having the highest numbers of capillaries and small venules (neovascular hot spots).

Figure 1. SLIT immunoreactivity. **A:** SLIT staining in an endometrial carcinoma tissue, serving as a positive control. **B:** SLIT staining in an ovarian endometrioma tissue, using pre-immune IgG antibody instead of the primary antibody, which serves as a negative control. **C and D:** SLIT staining in epithelial cells of ectopic endometrium in non-recurrence and recurrence patients. **E:** SLIT staining in epithelial cells from a control eutopic endometrium. All magnifications were $\times 400$ (An arrow indicates the expression of SLIT on glandular epithelial cells).

Then microvessel counting followed on five chosen 400× fields of the “hot plot” by the same investigator without knowledge of the recurrence status of the patient being evaluated. Endothelial cells or cell cluster clearly separated from adjacent microvessels, ectopic endometrial cells, and other connective tissue elements were taken into account for microvessel counting. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. The MVD was defined to be the mean of the vessel counts obtained in these fields, as reported previously.³⁸

Statistical Analysis

For descriptive statistics, we used boxplot⁴³ to graphically depict groups of immunoreactivity data, in which the bottom and top of the box represent the lower and upper quartiles, respectively, the band near the middle of the box represents the median, and the ends of the whiskers represent

the smallest and the largest non-outlier observations. The difference in frequency between the recurrence and non-recurrence groups was evaluated using Fisher's exact test. The comparison of distributions between the two or more groups was made using the Wilcoxon's test and Kruskal's test. To evaluate the effect of SLIT, ROBO1, and CD34 immunoreactivity level and other possible factors on the risk of recurrence, a logistic regression model was used. The laterality of ovarian cysts was coded by a dummy variable *b* taking value of 1 if the patient has bilateral cysts or 0 otherwise.

The postoperative use of medication, if ever, was lumped into one category. Since estimates of sensitivity and specificity based on the same data that was used for fitting are usually too optimistic, leaving-one-out cross-validation was used to estimate sensitivity, specificity, and positive and negative predictive values.

P values of less than 0.05 were considered statistically significant. All computations were made with R 2.8.0⁴⁴ (www.r-project.org).

Figure 2. ROBO1 immunoreactivity. **A:** ROBO1 staining in an endometrial carcinoma tissue, serving as a positive control. **B:** ROBO1 staining in an ovarian endometrioma tissue, using pre-immune IgG, instead of the primary, antibody, which serves as a negative control. **C** and **D:** ROBO1 staining in epithelial cells of ectopic endometrium from non-recurrence and recurrence patients. **E:** ROBO1 staining in epithelial cells from a control eutopic endometrium. **F:** ROBO1 staining in endothelial cells of ectopic endometrium. All magnifications were × 400. An arrow indicates the expression of ROBO1 on glandular epithelial cells (**A–E**) and on endothelial cells (**F**).

Results

The characteristics of patients in the recurrence and non-recurrence groups are listed in Table 1.

SLIT and ROBO1 Immunoreactivity

Representative immunostaining for SLIT and ROBO1 in ectopic and control endometrium is shown in Figures 1, A–E and 2, A–F. As can be seen from these figures, SLIT immunoreactivity was seen mostly in glandular epithelial cells and was localized both in the cytoplasm and membrane (Figure 1, A–E). The immunoreactivity to ROBO1 was seen mostly in vascular endothelial cells, as well as in glandular epithelial cells, and was localized both in the cytoplasm and membrane in ectopic endometrium (Figures 2, C, D, and F). In control endometrium, in contrast, ROBO1 immunostaining was seen exclusively in glandular epithelial cells (Figure 2E).

In control endometrium, no difference in SLIT or ROBO1 immunoreactivity levels was found between proliferative and secretory phases ($P = 0.48$ and $P = 0.17$, respectively). In ectopic endometrium, no such difference was found either ($P = 0.81$ and $P = 0.47$, respectively). There was no discernable pattern in either SLIT or ROBO1 immunoreactivity levels as a function of menstrual phase in control endometrium (data not shown).

The correlation coefficient between SLIT and ROBO1 immunoreactivity levels was 0.11 ($P = 0.56$). However, after removing three apparent outliers that had a ROBO1 staining level of 0, the correlation coefficient was elevated to 0.65 ($P = 0.0002$). In ectopic endometrium, the correlation coefficient was 0.34 ($P = 0.001$).

The immunoreactivity levels of both SLIT and ROBO1 were significantly higher in ectopic endometrium than in the control endometrium ($P = 0.03$ and $P = 0.0002$, respectively). In particular, the immunoreactivity levels of both SLIT and ROBO1 were significantly higher in the recurrence group than those of the non-recurrence group ($P = 0.009$ and $P = 0.03$, respectively; Figures 3 and 4), with the mean and SD of the SLIT staining score in the two groups being 0.032 (0.038) and 0.061 (0.055), respectively, and that of ROBO1, 0.032 (0.034) and 0.063 (0.071), respectively. The mean (and SD) of SLIT staining scores in the control group was 0.023 (0.027), which was significantly lower than the recurrence group but statistically not different from the non-recurrence group ($P = 2.8 \times 10^{-5}$ and $P = 0.46$, respectively). The mean (and SD) of ROBO1 staining scores in the control group was 0.012 (0.010), significantly lower than either the recurrent or non-recurrence group ($P = 3.9 \times 10^{-5}$ and $P = 0.008$, respectively).

MVD in Control and Ectopic Endometrium

CD34 immunostaining was seen mostly in vascular endothelial cells in both control and ectopic endometrium (Figure 5, A–B). While neither SLIT nor ROBO1 immunoreactivity levels showed any pattern as a function of menstrual phase in control endometrium, MVD, as mea-

Figure 3. Boxplot of SLIT immunoreactivity levels in control, non-recurrence, and recurrence ectopic endometrium.

sured by CD34 immunoreactivity, increased as the menstrual phase approaching to ovulation and then declines (Figure 6). In control endometrium, the ROBO1 immunoreactivity level correlated positively with the MVD level ($r = 0.38$, $P = 0.036$), but not SLIT level ($r = 0.06$, $P = 0.75$). In ectopic endometrium, the results were reversed: the correlation coefficient between SLIT level and MVD was 0.32 ($P = 0.03$) while that between ROBO1 and MVD was 0.18 ($P = 0.09$).

The MVD value in ectopic endometrium was over twice as higher as in the control endometrium (32.4 ± 2.3 vs. 14.3 ± 5.6 , $P = 3.4 \times 10^{-14}$), suggesting that angiogenesis was higher in ectopic endometrium as compared with control endometrium. The MVD value was higher in recurrence group than the non-recurrence group, but the difference did not reach statistical significance ($P = 0.069$, Figure 7).

Figure 4. Boxplot of ROBO1 immunoreactivity levels in control, non-recurrence, and recurrence ectopic endometrium.

Figure 5. CD34 immunoreactivity (A) and (B) Immunohistochemical staining of CD34 in vascular endothelial cell of an ovarian endometrioma tissue and in a control eutopic endometrium, respectively (An arrow indicates the expression of CD34 on endothelial cells). Magnification: $\times 400$.

The Relationship between SLIT/ROBO1 Immunoreactivity and Lesion Characteristics

We examined the relationship, if any, between SLIT/ROBO1 immunoreactivity and certain characteristics of the women with ovarian endometriomas and of lesions. With the only exception of ROBO1 and rAFS score ($r = 0.26$, $P = 0.016$), we found no relationship between SLIT/ROBO1 immunoreactivity levels and rAFS stage, rAFS score, laterality of lesions, dysmenorrhea or not, presence or absence of adenomyosis, presence or absence of adhesion, infertility or not and size (all P values > 0.2).

Since COX-2, NF- κ B/p65, and PR-B have been previously identified by us to be potential biomarkers for recurrence based on almost the identical groups of patients,^{39,40} we also examined the relationship between the immunoreactivity levels of SLIT/ROBO1, and CD34 and COX-2, NF- κ B/p65, and PR-B, but no significant correlation was found (all P values > 0.2).

Figure 6. Boxplot of MVD in normal endometrium as a function of menstrual phase. EP, MP, and LP mean early-, mid-, and late-proliferative phase, respectively, while ES, MS, and LS denotes early-, mid-, and late-secretory phase, respectively.

Multivariable Analysis

Given the difference in SLIT/ROBO1 immunoreactivity levels between recurrence and non-recurrence groups, we performed a multiple logistic regression analysis to identify variables that are associated with the recurrence status, which included 14 variables: age at surgery, menstrual phase, previous medical treatment of endometriosis, previous endometriosis-related surgery, presence of adenomyosis or not, bilateral endometrioma or not, presence of adhesion, complaint of dysmenorrhea, size of the largest cyst, mode of surgery, rAFS score, CD34, SLIT, ROBO1 and the interaction terms of the menstrual phase and SLIT/ROBO1. The stepwise procedure identified the presence of adhesion ($P = 0.026$) and SLIT immunoreactivity level ($P = 0.007$) as the only two variables that are associated with the recurrence status.

Figure 7. Boxplot of MVD levels in control, non-recurrence, and recurrence ectopic endometrium.

Table 2. Parameter Estimates of the Final Logistic Regression Model that Identified PR-B, NF- κ B/p65, SLIT Immunoreactivity Levels and the Presence of Adhesion as Risk Factors for Recurrence of Endometriosis

Variable	Estimate	Standard Error	P value	Odds ratio (95% Confidence interval)
PR-B	-0.652	0.219	0.003	0.52 (0.34, 0.80)
NF- κ B/p65	1.241	0.286	1.4×10^{-5}	3.46 (1.97, 6.06)
SLIT	20.426	7.133	0.004	0.74×10^9 (629.75, 8.76×10^{14})
Presence of adhesion	1.653	0.660	0.012	5.22 (1.43, 19.04)

Interestingly, if the three variables, COX-2, NF- κ B/p65, and PR-B, that were reported previously to be associated with the recurrence risk were included in the logistic regression model along with SLIT, ROBO1, CD34, and the presence of adhesion, we found that only NF- κ B/p65, PR-B, SLIT, and the presence of adhesion were significant predictors (Table 2), indicating that SLIT immunoreactivity is a predictor of recurrence risk independent of NF- κ B/p65, PR-B, and of the presence of adhesion.

Based on the regression model, a classification rule can be constructed that classifies patients as recurrent if the logistic regression model gives a probability of 0.5 or greater and as non-recurrent otherwise. The leave-one-out cross-validation based on this rule yielded a sensitivity of 86% and a specificity of 87%, an odds ratio of 36.1, and positive and negative predictive values (PPV and NPV) of 0.86 and 0.86, respectively, as compared with the corresponding values of 81%, 82%, 18.6, 0.82, and 0.81, respectively, if SLIT was dropped from the model. Hence the inclusion of SLIT in the regression model represents a considerable improvement in both sensitivity and specificity when classifying the patients.

Discussion

We have found in this study that SLIT and ROBO1 immunoreactivity are both elevated in ectopic endometrium as compared with the control endometrium. In addition, SLIT expression levels correlated positively with MVD in ectopic endometrium, suggesting the involvement of SLIT/ROBO1 system in angiogenesis in endometriosis. Higher SLIT/ROBO1 immunoreactivity in the recurrent than the non-recurrence group indicates that SLIT/ROBO1 mediated angiogenesis plays a role in recurrence. Finally, SLIT immunoreactivity, along with three previously identified putative biomarkers for recurrence of ovarian endometriomas (PR-B, NF- κ B, and the presence of adhesion), jointly constitute a classification rule with a sensitivity of 86% and a specificity of 87%, a small but meaningful improvement over the one with only PR-B, NF- κ B, and the presence of adhesion.³⁹ This supports the role of SLIT/ROBO1 in conferring recurrence risk in ovarian endometriomas. Our finding lends further support for the notion that there are identifiable molecular differences intrinsic to the ovarian endometriomas that confer recurrence risk differential.³⁹

The increase in predictive power, as measured by both sensitivity and specificity, in classifying recurrent versus non-recurrent patients as a result of addition of SLIT immunoreactivity to three previously identified risk factors for recurrence, coupled with apparently no discernable

relationship between SLIT and PR-B and NF- κ B/p65 immunoreactivity, strongly suggests that SLIT is a biomarker for recurrence independent of PR-B and NF- κ B/p65 in conferring recurrence risk. This also suggests that recurrence is a multifaceted event, involving not only angiogenesis but also invasion, inflammation, proliferation (as PR-B and NF- κ B/p65 are involved) and the extensiveness of the disease (with the presence of adhesion as a proxy). This implies that biomarkers for recurrence of endometriosis that are of practical use likely consist of a panel of constituent biomarkers each measuring different aspect of recurrence propensity of a patient.

Our MVD measurements as a function of menstrual phase in control endometrium are consistent with previous reports⁴⁵ and with the role of steroid hormones in regulating endometrial angiogenesis.⁴⁶ The lack of correlation between MVD and SLIT that we observed in control endometrium could mean that SLIT may not be the principal force in regulating endometrial angiogenesis in normal circumstances. Alternatively, increased SLIT expression may precede the increase in MVD, but due to the design of our study we could only have snapshot observations of their relationship. In addition, the use of an anti-pan SLIT antibody may have obscured the principle SLIT and its relationship with endometrial angiogenesis. Future research should clarify these issues.

As the SLIT2 protein is reported to attract endothelial cells and promote tube formation in a ROBO1- and phosphatidylinositol kinase-dependent manner, and since the neutralization of ROBO1 reduces the MVD and the tumor mass *in vivo*,³⁴ cross talk, mediated by the SLIT2-ROBO1 system, appears to occur between tumor cells and vascular endothelial cells. The difference in ROBO1 immunostaining locations between control and ectopic endometrium as we observed in this study seems to suggest that such SLIT-ROBO1-mediated cross-talk may also exist in ovarian endometriomas. This cross-talk may facilitate angiogenesis, ensuring the blood supply to growing ectopic endometrium, and thus increasing the risk of recurrence. In addition, since one principal indication of recurrence is the return of pain (of various forms), and since the SLIT-ROBO1 system is known to play a role in neural development, increased SLIT expression may also be related with increased activities of pain mediators, yet to be demonstrated. After all, the vascular and the nervous system share similar signaling pathways and cross talk and, when deregulated, contribute to medically relevant diseases.⁴⁷

Our study has several strengths. First, our sample size of 88 is moderately large, providing adequate statistical power and at the same time being likely to be represen-

tative of our entire patient pool. Second, as all patients received care from a single hospital with a more or less standardized treatment protocol, any heterogeneity in treatment has been minimized. Finally, our study design was consistent with a newly proposed design, termed prospective-specimen-collection, retrospective-blinded-evaluation (PRoBE), for pivotal evaluation of the accuracy of biomarkers used for classification or prediction,⁴⁸ thus should minimize various biases that plagued many biomarker-discovery studies in cancers.⁴⁹

Of course, our study also has limitations. First, since we used a pan-anti-SLIT antibody for immunohistochemistry, our study could not specify which one of three members of the SLIT family may be chiefly and causally responsible for recurrence. This, of course, does not compromise our ability in any way to identify patients with an elevated recurrence risk, but to elucidate the mechanisms underlying recurrence, this should be determined in future studies. Our preliminary investigation suggests that SLIT2 is over-expressed in endometriotic cells and its expression can be inhibited by PI3K inhibitors (Zhao et al, unpublished data). Further work is warranted to specify which SLIT is chiefly responsible for elevated recurrence risk and the underlying mechanisms.

Second, we did not evaluate several molecules known to be involved in angiogenesis in endometriosis, such as VEGF and FGF,²⁴ and thus we were unable to evaluate the importance in predicting recurrence relative to SLIT. Regardless of these deficiencies, however, the observed close correlation of immunoreactivity levels between SLIT and ROBO1 is consistent with the structural finding,⁵⁰ and the similar relationship between SLIT and MVD in ectopic endometrium and between ROBO1 and MVD in control endometrium strongly supports the notion that the SLIT-ROBO1 signaling is involved in endometriotic angiogenesis.

In conclusion, we found that higher SLIT and ROBO1 immunoreactivity in women with recurrent ovarian endometriomas than those without recurrence, and that SLIT expression levels correlated closely with endometriotic MVD. Finally, we found that SLIT immunoreactivity, along with three previously identified putative biomarkers, are predictive of recurrence with estimated sensitivity of 86% and specificity of 87%. These results suggest that increased SLIT expression may induce increased endometriotic angiogenesis, and thus conferring higher recurrence risk. Thus, targeting the SLIT-ROBO1 signaling, either through suppression of the PI3K/Akt pathway or otherwise, along or in combination with other therapeutic agents, may hold promises in reducing recurrence risk of endometriosis and perhaps also for treatment purpose as well.

Acknowledgments

We thank the two anonymous reviewers for their helpful comments.

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