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An association of IgG anti-laminin-1 autoantibodies with endometriosis in infertile patients

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BACKGROUND: Laminin-1, a multifunctional glycoprotein of the basement membrane, is thought to be important in embryogenesis, embryonic implantation, and placentation. We recently showed that serum IgG anti-laminin-1 autoantibodies (auto-Abs) are associated with recurrent first-trimester miscarriages. The present study assessed the clinical significance of anti-laminin-1 Abs with infertility, accompanied with or without endometriosis. METHODS: Sixty-eight infertile patients who underwent laparoscopy or laparotomy and 39 healthy non-pregnant women were tested for IgG anti-laminin-1 Abs. The association between the Abs and endometriosis was analysed. The presence of laminin-1 mRNA was detected in endometriotic lesions. RESULTS: Twenty infertile patients were positive for anti-laminin-1 Abs. The Ab levels in those patients were significantly higher than those in healthy non-pregnant women ($P = 0.0005$). The presence of the Abs was significantly associated with endometriosis in those patients ($P = 0.0096$). The Abs recognized a particular domain, i.e., the laminin-α1 chain G domain. mRNA encoding laminin-α1, -β1, and -γ1 chains was expressed in 90% of endometriotic lesions. CONCLUSIONS: IgG anti-laminin-1 Abs were significantly associated with endometriosis in infertile patients. The Abs might be clinically important in the development of autoimmune-mediated reproductive failures and the assessment of the Abs may provide a novel non-invasive diagnosis of endometriosis.

Key words: anti-laminin-1 autoantibody/endometriosis/infertility/miscarriage

Introduction

Laminin, a major basement membrane glycoprotein consisting of three different subunits, α, β, and γ chains (Burgeson *et al*., 1994), is involved in diverse biological activities, including the promotion of cell adhesion, migration, proliferation, and differentiation (Colognato and Yurchenco, 2000). To date, at least 15 different isoforms of laminin have been identified. Laminin-1, composed of α1, β1, and γ1 chains, is the earliest synthesized network-forming component during embryogenesis and plays an important role in embryonic development (Miner *et al*., 1997; Aumailley *et al*., 2000; Colognato and Yurchenco, 2000).

Laminin-1 from the early human embryos increases type IV collagenase expression and is thought to enhance trophoblast adhesion to maternal matrix in the peri-implantation period (Turpeenniemi-Hujanen *et al*., 1992). In the blastocyst or early implanting mouse embryo, laminin-1 is localized in the inner cell mass and in trophectoderm basement membrane. As implantation proceeds, laminin-1 is expressed in chorionic basement membrane and in Reithert's membrane near the ectoplacental cone (Klaffky *et al*., 2001). In human first-trimester placenta, the laminin-α1 chain is detected in trophoblastic basement membrane that is in direct contact with extravillous trophoblastic cells. In the second-trimester placenta when extravillous trophoblast forms anchoring trophoblastic cell columns, the laminin-α1 chain is selectively found at site where the villous basement membrane is in contact with proliferating cells in trophoblastic islands or columns (Korhonen and Virtanen, 2001). Thus, laminin-1 may be required for the initial anchorage and migration of trophoblast cells into maternal decidua during implantation. Laminin-1 may also play a role in regulating trophoblast

proliferation and differentiation during implantation and placentation through an interaction with integrin receptors (Klaffky *et al*., 2001).

Anti-laminin-1 autoantibodies (auto-Abs) were first detected in the sera of monkeys with histories of reproductive failure, and their sera caused abnormalities in cultured rat embryos (Carey and Klein, 1989). It was further demonstrated that immunization of monkeys with mouse laminin-1 or with laminin-1 peptides caused sera embryotoxicity and infertility or spontaneous abortion (Weeks *et al*., 1989; Chambers *et al*., 1995). Passive immunization with rabbit anti-laminin-1 Abs in pregnant mice induced spontaneous abortion. Localization of the Abs, revealed them to be in Reichert's membrane and visceral yolk sac endoderm cells from the embryos (Foidart *et al*., 1983). Our recent clinical study showed that IgG anti-laminin-1 Abs in blood are significantly associated with the recurrent first-trimester miscarriages in humans (Inagaki *et al*., 2001). All of these observations suggest that anti-laminin-1 auto-Abs may be responsible for reproductive failure, interfering with the early stages of pregnancy.

In the present study, we examined the prevalence of IgG anti-laminin-1 auto-Abs in infertile patients to assess whether the Abs are associated with reproductive disorders, particularly during pre and peri-implantation stages. It was demonstrated that the presence of IgG anti-laminin-1 Abs was associated with endometriosis in infertile patients. The significant association between the Abs and endometriosis suggests that they may play a role in endometriosis-associated infertility.

Materials and methods

Subjects and plasma samples

Plasma samples were collected from 68 infertile patients (mean age: 33.7 years; range: 26–45) who underwent laparoscopy or laparotomy as part of their infertility investigation. These included 27 patients who later underwent IVF, and 39 healthy age-matched non-pregnant women (mean age: 29.6 years; range: 22–41) with normal menstrual cycles, no pelvic disorders and no history of autoimmune diseases, at Okayama University Hospital and at Nagoya City University Hospital.

Basal body temperatures were charted and hysterographies, examination of semen, and endocrine evaluations were performed in all these patients. Endometriosis was diagnosed by laparoscopic or laparotomic findings and by pathological analysis, and endometriosis was further classified according to the revised American Society for Reproductive Medicine (r-ASRM) Classification. Twenty-six out of the 68 infertile patients did not suffer from endometriosis. Of the 42 infertile patients with endometriosis, eight had stage I (minimal), six had stage II (mild), 16 had stage III (moderate), and 12 had stage IV (severe) of the disease.

We examined the association between the prevalence of the Abs and possible causes of infertility, including endometriosis, in the 68 patients. Informed consent was given by all subjects and the study was approved by the Institutional Review Boards both of Okayama University and of Nagoya City University.

Assay for IgG anti-laminin-1 Abs

Laminin-1 was prepared from the Engelbreth-Holm-Swarm tumour cells, as described previously (Timpl *et al*., 1979). Detection of IgG anti-laminin-1 Abs was performed using ELISA (Inagaki *et al*., 2001). Briefly, a polystyrene plate (Immulon 1B, Dynex Technologies, Inc., Chantilly, VA, USA) was coated with 10 μg/ml of laminin-1 per 50 μl/well with an overnight incubation at 4°C. After the plate was blocked with 10% fetal bovine serum, 100-fold diluted plasma samples (100 μl/well) were applied and then allowed to incubate for 1 h. Horseradish peroxidase-labelled anti-human IgG Abs were incubated for 1 h. *o*-Phenylenediamine solution containing H2O2 was reacted for 10 min and the reaction was terminated with 2 N H2SO4. Optical density (OD) was measured at 490 nm. Ab titres were calculated from the mean OD, after subtraction of nonspecific binding, with internal standards (Ab-positive controls). To study the association between the presence of anti-laminin-1 Ab and clinical manifestations, we adjusted a threshold value. The possible threshold values were first considered with a ROC curve from the patients' group and one to five standard deviations (1SD to 5SD) above the mean OD of 39 healthy non-pregnant controls. To assess the clinical significance of the possible threshold values, we calculated *P*-value using Fisher's exact test, odds ratio, and 95% confidence interval (CI) in each case and we finally chose 3SD above the mean OD of the controls as an appropriate cut-off value for diagnosis of endometriosis. One U/ml was defined as the OD that corresponds to 3SD above the mean OD of reference controls, to prevent confusion among future individual studies. This assay was reproducible with intra- and inter-assay coefficients of variation not exceeding 3.1 and 6.9% respectively.

Assay for IgG Ab to the G domain of laminin-α1 chain

Chinese hamster ovary cells, transfected with plasmids coding the G domain of laminin-α1 chain (amino acid residue: 2111–3060), was kindly provided by Dr Yasuo Kitagawa (Nagoya University). Cells were maintained in α-minimal essential medium containing 10% fetal calf serum and antibiotics. The recombinant G domain was purified from conditioned medium of cultured cells, using the Hitrap heparin-affinity column and the FPLC system (Amersham Pharmacia Biotech) (Yamaguchi *et al*., 2000). Binding of IgG anti-laminin-1 Abs to the G domain was tested with the protein coated polystyrene plates (Immulon 1B) (by incubating with 1 μg/50 μl/well of the protein for overnight at 4°C). After blocking, 100 μl/well of 25-fold diluted plasma samples were applied and then allowed to incubate for overnight at 27°C. Further steps were performed as described in the above subsection 'Assay for IgG anti-laminin-1 Abs'.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Endometriotic lesions were obtained from infertile patients with endometriosis undergoing laparoscopy or laparotomy at the time of surgery. The extraction of total RNA from the tissue samples was carried out with RNAzol™ B (Biotecx Laboratories, Inc., Friendswood, TX, USA). RT-PCR was performed using Gibco BRL, One-Step™ RT-PCR System (Life Technologies, Inc., Rockville, MD, USA), according to the manufacturer's protocol. cDNA synthesis and PCR amplifications were performed with 0.2 μg total RNA and the following primers. Primers for human laminin-α1 chain were sense 5′-GATGACAACAGATGGCACAGT-3′ and anti-sense 5′-ACTGGT-GGTATAGGCATCGAT-3′ at positions 6683 to 6703 and 7304 to 7324, respectively (a predicted product size: 642-bp). Primers for laminin-β1 chain were sense 5′-TGCCCCTGTGGATGGATTCAA-3′ and anti-sense 5′-GTCGTACTCCATGGAATATGG-3′ at positions 957 to 977 and 1930 to 1950 respectively (a predicted product size: 994-bp). Primers for laminin-γ1 chain were sense 5′-GCAAGA-CTGAACAGCAGACC-3′ and anti-sense 5′-TCCTATCAAGA-TCGCTGACC-3′ at positions 4241 to 4261 and 4909 to 4927, respectively (a predicted product size: 687-bp). RT-PCR conditions for laminin-α1 chain primers was at 50°C for 30 min and 94°C for

2 min, followed by 40 cycles at 94°C for 30 s, at 58°C for 1.5 min, at 72°C for 1.5 min, and final extension at 72°C for 10 min. The primers for laminin-β1 chain and laminin-γ1 chain were used under the same condition as laminin-α1 chain primers except for annealing temperature (56 and 60°C respectively). Positive control PCR reactions were performed using human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers provided with the RT-PCR kit (Toyobo Co., Ltd., Osaka, Japan). PCR products were analysed by electrophoresis on a 1.5% agarose gel.

Statistical analysis

Ab titres of anti-laminin-1 Abs in subpopulations of infertile patients were compared by the Student's *t*-test. The relationship between the prevalence of anti-laminin-1 Abs and causes of infertility was analysed by the Fisher's exact test. A P-value <0.05 was considered to be statistically significant. The Pearson's correlation coefficient test was used to assess the correlation between the Ab titres to the whole molecule and the α1 chain G domain of laminin-1.

Results

Prevalence of IgG anti-laminin-1 auto-Abs

IgG anti-laminin-1 Ab values in infertile patients who underwent laparoscopy or laparotomy (n = 68) were significantly higher than those in healthy non-pregnant women (n = 39) (P = 0.0005) (Figure 1). Twenty infertile patients (29%) were positive for the Abs.

We further analysed the association between the prevalence of anti-laminin-1 Abs and infertility factors in the infertile patients. There was a significant association between the presence of Abs and endometriosis (P = 0.0096). Seventeen of 42 infertile patients with endometriosis (40%) tested positive for anti-laminin-1 Abs. Other causes of infertility (tubal factor, hormonal abnormality, uterine anomaly, and unexplained) were not associated with the Abs (Table I). We also examined the association between the Abs and other factors in infertile patients without endometriosis, and it was observed that no other factors were associated with the presence of the Abs (Table II).

The values of anti-laminin-1 Abs were compared between infertile patients with and without endometriosis. Significantly elevated values of the Abs were observed in the 42 infertile patients with endometriosis (the mean value = 1.1 ± 1.2 U/ml) compared to those without endometriosis (0.46 ± 0.33 U/ml) (P = 0.015) (Figure 2).

When the threshold value was 1.0 U/ml, the diagnostic validity of this assay for the detection of endometriosis in infertile patients was 43% (sensitivity), 89% (specificity), 86% (positive predictive value), and 49 % (negative predictive value).

We further subdivided the 42 infertile patients with endometriosis into four stages as the severity of endometriosis. The mean values of the Abs at stage II (1.1 ± 0.77 U/ml, III (1.4 ± 1.7 U/ml), and IV (0.9 ± 0.81 U/ml) were significantly higher than those of 26 infertile patients without endometriosis (P = 0.0018, 0.014, and 0.022, respectively) (Figure 3). Stage I (0.81 ± 1.0 U/ml) did not differ from those without endometriosis in the study. The increase in the mean values from stage I through IV was not statistically significant. Two of eight infertile patients with stage I disease (25%), four out of six of those patients with stage II (67%), eight out of 16 of those patients with stage III (50%) and three out of 12 of those patients with stage IV (25%) were positive for anti-laminin-1 Abs.

Plasma samples from the infertile patients were also used to examine whether anti-laminin-1 Abs recognize the G domain of laminin-α1 chain. There was a good correlation between the Ab binding to the intact laminin-1 molecule and to laminin-α1 chain G domain (r^2 = 0.53, P < 0.0001). The data mean that a potential epitope for the Abs is located in the G domain.

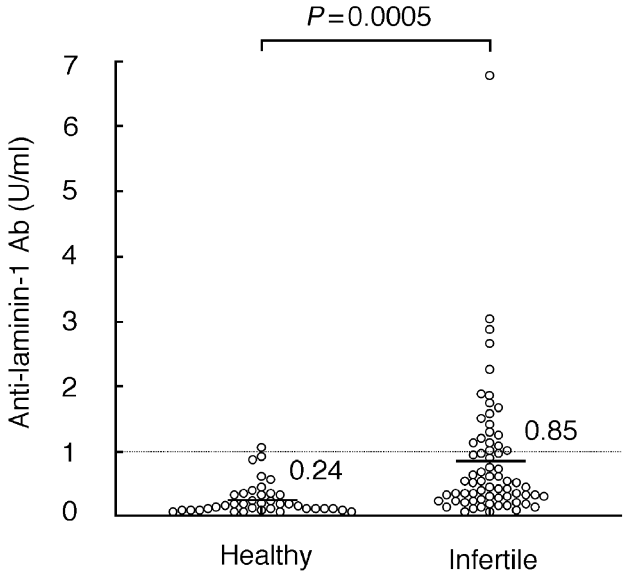

Figure 1. IgG values of anti-laminin-1 auto-Abs in 68 infertile patients who underwent laparoscopy or laparotomy. Anti-laminin-1 auto-Abs were detected using ELISA with a laminin-1-coated plate. The dotted line shows 1 U/ml (1 U/ml = the mean OD, + 3 SD of healthy non-pregnant women), as a threshold value of the Abs. Solid lines (and number) show the mean value.

Table I. Association between IgG anti-laminin-1 auto-Abs and possible causes of infertility in 68 infertile patients who underwent laparoscopy or laparotomy

Possible cause of infertility	Anti-laminin-1 Abs		*P*-value
	Positive (n = 20)	Negative (n = 48)	
Endometriosis			
+	17	25	0.0096
–	3	23	
Tubal factor			
+	5	17	NS
–	15	31	
Hormonal abnormality			
+	5	11	NS
–	15	37	
Uterine anomaly			
+	0	2	NS
–	20	46	
Unexplained			
+	1	10	NS
–	19	38	

P = Fisher's exact test; NS = not significant.

Table II. Association between IgG anti-laminin-1 auto-Abs and possible causes of infertility in 26 infertile patients without endometriosis

Possible cause of infertility	Anti-laminin-1 Abs		*P*-value
	Positive (n = 3)	Negative (n = 23)	
Tubal factor			
+	0	9	NS
–	3	14	
Hormonal abnormality			
+	2	5	NS
–	1	18	
Uterine anomaly			
+	0	2	NS
–	3	21	
Unexplained			
+	1	10	NS
–	2	13	

P = Fisher's exact test; NS = not significant.

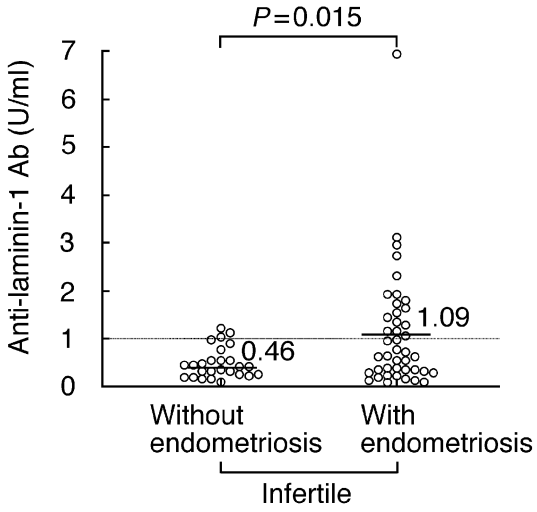

Figure 2. Comparison of IgG values of anti-laminin-1 auto-Abs between infertile patients with or without endometriosis. The dotted and solid lines show threshold and mean values of the Abs respectively.

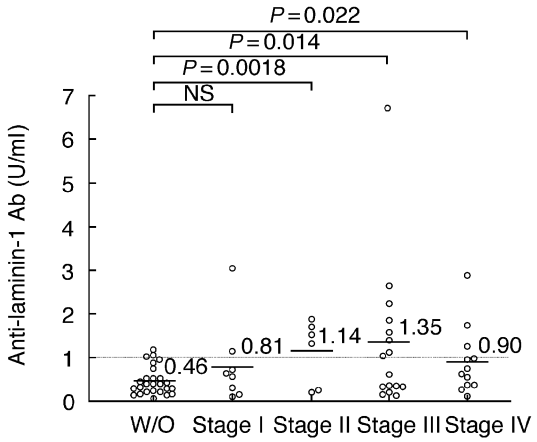

Figure 3. IgG values of anti-laminin-1 auto-Abs at different stages of endometriosis. The dotted and solid lines show threshold and mean values of the Abs respectively.

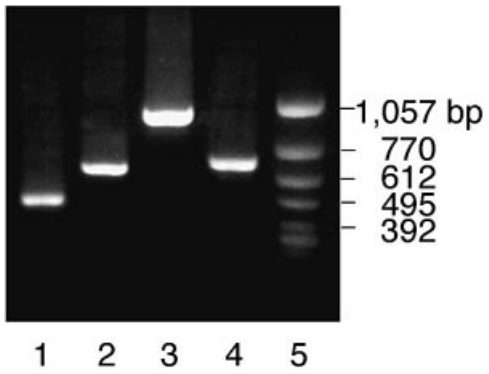

Figure 4. RT-PCR of mRNA laminin-1 chains in endometriotic lesion. Lane 1, GAPDH (452 bp); lane 2, laminin-α1 (642 bp); lane 3, laminin-β1 (994 bp); lane 4, laminin-γ1 (687 bp); lane 5, DNA size marker (φX174/Hinc II).

Expression of laminin-α1, -β1 and -γ1 mRNAs in endometriotic lesions

We investigated by RT-PCR whether mRNA encoding for the chains of laminin-1 was expressed in endometriotic lesions. Amplified products with the predicted size were detected with primers for laminin-α1 (642-bp), -β1 (994-bp) and -γ1 (687-bp) in endometriotic lesion (Figure 4). Laminin-1 (-α1, -β1, -γ1) mRNA was expressed in 37 of 41 tissue samples (90%). mRNA was detected in tissues at all four stages of endometriosis.

Discussion

In the present study, we showed that anti-laminin-1 Abs were strongly associated with infertility, especially when caused by endometriosis. These Abs recognized a particular domain, i.e. the laminin-α1 chain G domain. Further, we detected laminin-α1, -β1, and -γ1 mRNA in 90% of endometriotic lesions.

Laminin-1, the earliest synthesized basement membrane component during embryogenesis, is required for the initiation of basement membrane assembly at the peri-implantation stage. Laminin-1 from trophoblast plays an important role in the adhesion and migration of trophoblast cells into maternal decidua during implantation. Further, this protein may be involved in the regulation of trophoblastic proliferation and differentiation during implantation and placentation (Klaffky *et al*., 2001).

The Abs to laminin-1 are known to cause infertility, recurrent spontaneous abortions and sera embryotoxicity in animals (Carey and Klein, 1989; Weeks *et al*., 1989; Chambers *et al*., 1995). We previously reported that IgG anti-laminin-1 Abs are associated with recurrent first-trimester miscarriages in humans (Inagaki *et al*., 2001). In the present study, we demonstrated that infertile patients had significantly higher levels of the Abs. This result suggested that the Abs may be involved not only in recurrent first-trimester miscarriages but also in infertility in humans.

We also showed that the Abs were strongly associated with infertility, especially when caused by endometriosis. Despite a small sample number, the elevated Ab levels at stages II

through IV were significantly higher than infertile patients without endometriosis. However, the increase in the Ab values from stage I through IV was not statistically significant. It would be necessary to analyse an increased number of infertile patients and take into consideration effects of surgical or hormonal treatment in order to provide conclusive evidence for the relationships between Abs and different stages of endometriosis.

Endometriosis is a widely accepted cause of infertility. A number of studies indicate that infertile patients with endometriosis frequently have elevated levels of auto-Abs specific for endometrial, ovarian, nuclear antigens, and others (Mathur *et al*., 1982; Pillai *et al*., 1998; Mathur, 2000). Although the mechanism of the disease related to infertility is poorly understood, it has been suggested that an aberrant immunological mechanism including the production of auto-Abs might be involved in infertility. The presence of anti-laminin-1 Abs in infertile patients with endometriosis and the function of laminin-1 in implantation and plancentation suggest that anti-laminin-1 Abs may play a role in modulating very early reproductive processes and be responsible for endometriosis-associated infertility.

Endometriosis is thought to arise from the adhesion and migration of retrogradely shed menstrual endometrial cells to the extracellular matrix (ECM) of the peritoneum and ovary (Sampson, 1927). Although retrograde menstruation frequently occurs in most women, the majority has the ability to prevent adhesion of these tissues and endometriosis only develops in about 5–10% of the total population. It has been postulated that patients with endometriosis may have abnormalities in their ectopic cellular clearance mechanism. This would result in an inability to reject autologous endometrial cells, subsequently allowing endometriosis to develop after the adhesion, proliferation, and migration of ectopic endometrial implants. Various components of the ECM such as laminins and their specific integrins, are involved in this adhesion process. A recent study indicates that laminin-1 also plays a role in this process (Koks *et al*., 2000).

In the present study, laminin-1 mRNA was detected in endometriotic lesions at all stages. Immunohistochemical studies clarify that laminins including laminin-1 are located in the basement membrane surrounding glandular epithelial cells and vascular endothelial cells in both endometrium and endometriotic lesions (Beliard *et al*., 1997; Harrington *et al*., 1999). The evidence that laminin-1, auto-antigen of this Abs, exists in ectopic locations, provides support for an autoimmune response against laminin-1.

From our data, the anti-laminin-1 Abs in infertile patients with endometriosis clearly recognized the G-domain of the laminin-α1 chain. The G-domain contains the recognition sites of several integrin receptors, playing a role in various biological activities (Mercurio, 1995; Colognato and Yurchenco, 2000). It was previously shown that the direct inhibition of laminin-1 binding to integrin receptors and to other basement membrane components by anti-laminin-1 Abs impaired the formation of normal basement membranes and the epithelial morphogenesis (Kadoya *et al*., 1995). Therefore it is possible that the Abs may also directly interfere with the function of laminin-1 and disrupt early reproductive stages and be involved in the development of endometriosis. In a number of autoimmune diseases, the immune complexes are known to initiate inflammatory responses by activating the complement system that may be involved in disease pathogenesis. Thus, a histochemical study of the deposition of IgG in endometriotic lesions is necessary to ascertain whether the Abs form an immune complex that impairs cellular function and are involved in the pathogenesis of endometriosis.

Finally, the sera from infertile patients with endometriosis are frequently reported to be toxic to in-vitro embryo development. Treatment with a glucocorticoid reduced the embryotoxicity and promoted early embryo development (Simón *et al*., 1992). Moreover treatment with glucocorticoids during the IVF cycle improved pregnancy rates in auto-Ab-positive infertile patients with endometriosis (Kim *et al*., 1997). The immunosuppressive properties of glucocorticoids may facilitate implantation and pregnancy in infertile patients with endometriosis and are positive for IgG laminin-1 auto-Abs.

In light of these findings, the assessment of IgG anti-laminin-1 auto-Abs might prove useful for the diagnosis and medical treatment of endometriosis.

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