Relationship between Partial Uterine Cervical Tissue Excision and Preterm Birth: An Experimental Animal Study

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Am J Perinatol

Abstract

Objective To investigate whether the uterine cervix excision is associated with preterm birth in female mice.

Study Design Sexually mature female C57BL/6 mice ($n = 40$) were randomly divided into four groups (A, sham; B, cervical excision; C, lipopolysaccharide, 100 μg intrauterine injection; and D, cervical excision + lipopolysaccharide injection), with 10 mice per group. Three weeks after cervical excision, timed mating was performed. On gestational day 16, lipopolysaccharide was injected between the first and second horns of the right uterus near the cervix. The uterine cervix was obtained after delivery and was histologically analyzed.

Results The mean gestational period in group D was significantly lower than those in the other groups (17, 19.5, 19, and 18.2 days in groups D, A, B, and C, respectively; $p = 0.034$). The cervical length was shorter in the cervical excision groups ($p = 0.004$). The muscle-to-collagen ratio in the proximal cervix was higher in group D ($p = 0.037$).

Conclusion Prepregnancy cervical excision and subsequent lipopolysaccharide injection showed a high rate of preterm birth, which was higher than the known lipopolysaccharide injection related preterm birth rate. Prepregnancy cervical excision appears to have additive effects with inflammation in inducing preterm birth, which are associated with the relative muscular component amount.

Keywords
- uterine cervix
- excision
- infection
- preterm birth
- mouse model

Preterm birth occurs in 5 to 18% of pregnancies and is a leading cause of neonatal morbidity and mortality.[1] Although intrauterine infection is mostly studied as a causal factor for preterm labor, partial uterine cervical tissue excision procedures, such as conization, loop electrosurgical excision, and loop electrosurgical excision of the transformation zone, are known to be associated with spontaneous preterm labor.[2–6] The reason for measuring cervical length in the midtrimester of high-risk pregnant women who have undergone cervical tissue excision is based on the theoretical background that a defective cervix may be related to preterm birth. There is an abundance of clinical research data that support the association between cervical tissue excision and preterm birth;[2–6] however, the causal relationship between such variables is controversial. Conner et al[7] have questioned the notion that cervical tissue excision itself is

received
May 4, 2017
accepted
May 9, 2017

DOI https://doi.org/10.1055/s-0037-1603816.
ISSN 0735-1631.

an independent risk factor for spontaneous preterm labor. In their analysis, cervical dysplasia, even in the absence of cervical tissue excision, was independently associated with preterm birth. Although a conclusion can only be drawn from a prospective randomized controlled trial comparing cervical tissue excision and no cervical tissue excision in women with a normal nonpathological cervix, such a study design would undoubtedly present an ethical problem.

Therefore, we believed that only an animal experiment comparing cervical tissue excisions with no cervical tissue excisions could elucidate the potential relationship of this procedure with preterm birth. We also aimed to compare cervical tissue excision with the known inflammation-induced preterm birth animal model.

Materials and Methods

Animals and Experimental Design

Sexually mature female C57BL/6 mice were used for the experiment ($n = 40$). The mice were randomly assigned to one of the four following groups, with 10 mice per group: A, sham; B, partial cervical tissue excision; C, lipopolysaccharide (LPS); and D, partial cervical tissue excision + LPS. The study flow diagram is shown in ►**Fig. 1**. Partial cervical tissue excision was performed at 5 weeks of age. In our preliminary experiments, the earliest optimal time to mate the animals for conception was at approximately 3 weeks after cervical tissue excision. We found that even 3 weeks after cervical tissue excision, the conception rate did not increase any further; therefore, mating was performed at 8 weeks of age. The conception rate through mating at the same day 3 weeks after partial cervical tissue excision was 50% (40 out of 80 mice). The day that the mucus plug was found to be in the vagina was set as day 1. LPS (100 µg from *Escherichia coli* 055:B5 [L6959, Sigma-Aldrich, St. Louis, MO]) was used for the induction of inflammation. Body weight was measured preoperatively and during the postpartum period. The delivery was continuously monitored and was recorded using a camera with night vision. The mice were sacrificed 12 hours after they gave birth. The duration from the delivery of the first pup to the delivery of the last pup was approximately 2 hours. The uterine cervices were sampled for subsequent analyses. Five mice were allocated per cage before mating, and one mouse was allocated per cage after confirming the vaginal mucus plug for conception and delivery care. The experimental room was kept at a constant temperature (22–24°C) with a 12-hour light/night cycle. Food and water were supplied ad libitum. The animals were cared for in accordance with the 2000 guidelines for animal experiments (edited by the Korean Academy of Medical Sciences), which are consistent with the 1996 National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. All animal experiments were approved by the committee for the care and use of laboratory animals at Korea University (KUIACUC-2015–117).

Surgery

The mice were anesthetized with 2 to 4% isoflurane (Forane Sol., Choongwae, Seoul, South Korea) inhalation. For cervical tissue excision, the vaginal approach was used. The cervix was grasped using forceps, and a 1-mm-deep excision was made using a scalpel. The mean weight of the excised cervical tissue was 7 mg. The hemorrhage from the excision site was minimal, and the compression was sufficient for hemostasis. Based on our preliminary data, the length of the entire cervix of the mouse was 2.5 mm, and approximately 40% of the cervix (1 mm out of 2.5 mm) was excised. For standardization of the excised depth, a ruler was used during the operation. LPS was administered between the first and second horns of the right uterus near the cervix through intrauterine cavitary injection after an abdominal incision and the exteriorization of the uterus on day 16.[8] The sham animals underwent induction with anesthesia and no other procedures.

Histologic Studies

The cervical sample was obtained by cutting just above the junction of the uterine horns. The cervical tissue was fixed overnight in 4% neutral buffered formalin, processed and embedded in a paraffin block. Tissue sections (4 µm) were cut from each block, mounted on slides, deparaffinized in xylene, and rehydrated using graded ethanol. Each section was stained using hematoxylin (S2–4; Youngdong, Yongin, South Korea) and eosin (MA0101015, BBC Biochemical, Mt Vernon, WA) to quantify the cervical length. The Masson's trichrome staining kit (NovaUltra, Ellicott City, MD) was used to determine the percentage of the collagen and smooth muscle within the uterine cervix. The cervical lengths were measured microscopically and are described in ►**Fig. 2**. In the histological sections, the gradual transition of the endocervical mucosa to the endometrium in the isthmus or lower uterine segment was observed. In this region, the cells were intermediate in size between the larger endocervical and smaller endometrial lining cells and showed intermediate degrees of a pseudostratified

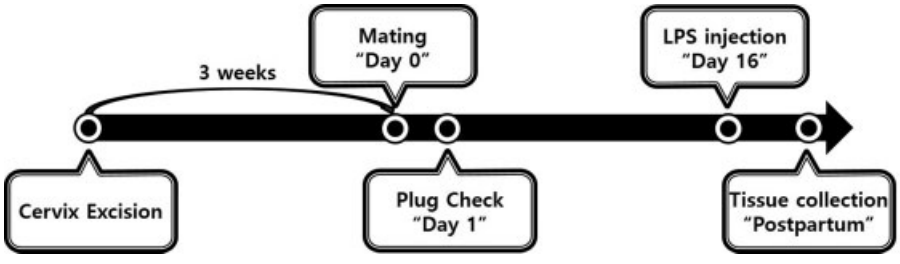

Fig. 1 Experimental design for the novel preterm birth animal model.

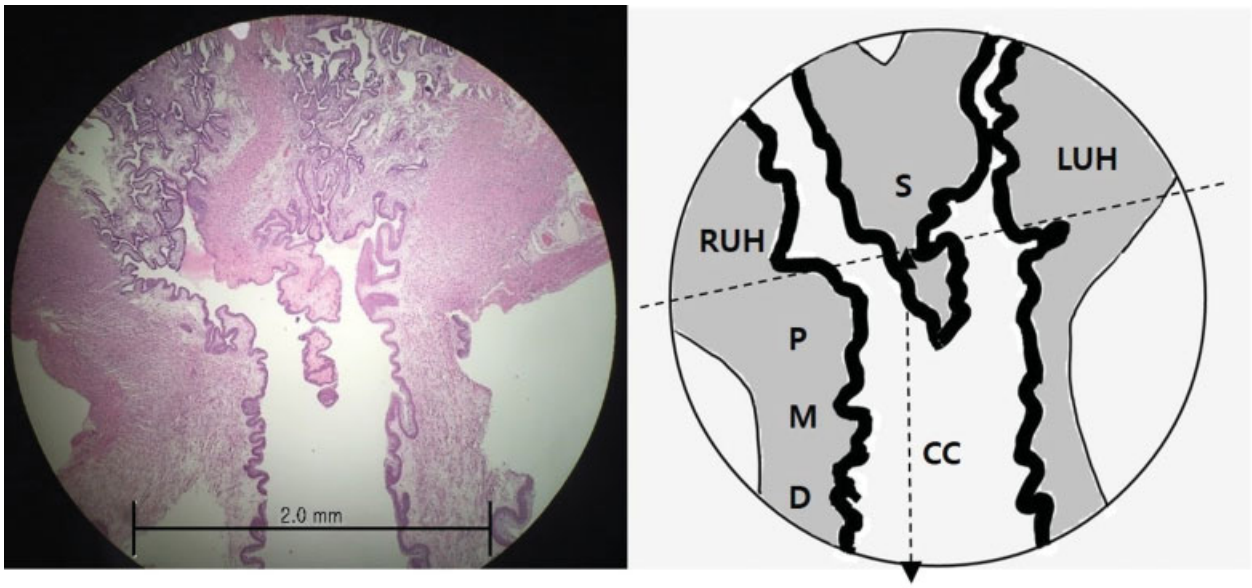

Fig. 2 Mouse cervix: the photograph clearly shows double uterine horns with a septum and a single cervix. Cervical length (dotted arrowed line) is measured between the transition point from the endocervix to the endometrium (dotted line) and external os of the cervix. 40× magnification. CC, cervical canal; D, distal cervix; LUH, left uterine horn; M, middle cervix; P, proximal cervix; RUH, right uterine horn; S, septum of the uterus.

architecture. The average percentages of the collagen and smooth muscle for three images in each proximal, middle, and distal cervical area were analyzed with a magnification of 200× using the Image J software (NIH, Bethesda, MD). Further, the three images of the cervical stroma were selected in a transversely outward manner from the endocervical canal to the exocervix. The smooth muscle-to-collagen ratios were calculated (►**Fig. 2**).

Statistical Analysis

All data were expressed as mean ± standard deviation. Analysis of variance was used to compare the data between the groups using Tukey's method for multiple comparisons. A p-value of <0.05 was considered statistically significant.

Results

The mean body weights on the mating day were not different between the study groups (►**Table 1**). The mean gestational period in group D was significantly lower than that in group A (19.5, 19, 18.2, and 17 in groups A, B, C, and D, respectively; $p = 0.034$; ►**Fig. 3**). Defining preterm birth as delivery within 24 hours after LPS injection or before day 18, the rates of preterm birth were 0, 30, 60, and 100% in groups A, B,

Table 1 Characteristics of the study subjects

	A	B	C	D	p-Value
	Sham	Cervical tissue excision	LPS	Cervical tissue excision + LPS	
Body weight (g) at mating day	18.4 ± 0.3	19.3 ± 0.7	18.7 ± 0.8	19.3 ± 0.7	0.226
Cervical length (μm)	6,050 ± 2,210	2,941.4 ± 1,703.3	7,438 ± 1,463	4,511 ± 1,332	0.002[a]
Gestational period (d)	19.5 ± 0.6	19 ± 1	18.2 ± 1.6	17 ± 0	0.034[a]
Number of pups	8.8 ± 0.5	8 ± 1	5 ± 3.7	5.3 ± 3.1	<0.001[a]
Number of gestational sac in the left uterus	5 ± 2.7	3.7 ± 2.1	3.2 ± 2.4	3.3 ± 2.1	0.031[a]
Number of gestational sac in the right uterus	3.8 ± 2.2	4.3 ± 1.5	4.6 ± 1.1	3.5 ± 1	<0.001[a]
Weight of the pups (g)	1.3 ± 0.05	1.3 ± 0.07	0.7 ± 0.5	0.8 ± 0.4	0.079
Height of the pups (cm)	2.8 ± 0.1	2.7 ± 0.2	2 ± 0.7	2 ± 0.6	0.078

Abbreviation: LPS, lipopolysaccharide.
Note: Data are presented as mean ± standard deviation.
[a]Statistically significant.

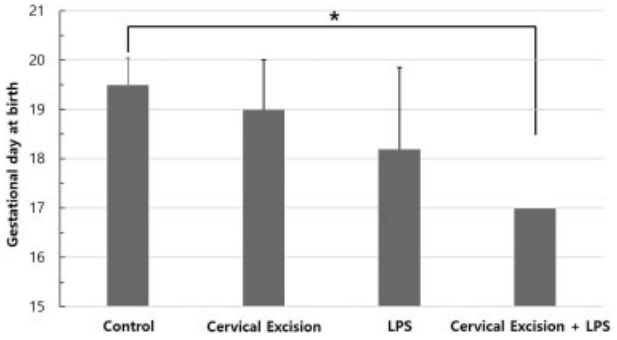

Fig. 3 Comparison of the gestational period among the study groups; LPS, lipopolysaccharide. *Statistically significant.

C, and D, respectively. The cervical length was shorter in the cervical tissue excision groups with and without LPS injection ($p = 0.002$). The postpartum cervical lengths were 2.4 times longer than the prepregnant lengths in group A (6.05/2.5 mm). The muscle-to-collagen ratio in the proximal cervix was relatively higher in group D (►**Table 2** and ►**Fig. 4**; $p = 0.037$). After cervical tissue excision, fibrosis was visible in the middle and distal portions of the cervix. Although fibrosis was noted in the middle and distal cervical areas, the muscle-to-collagen ratios were not statistically different in these areas versus those in the middle and distal cervical areas of the cervices that were not excised. Fewer pups were born in the LPS groups with and without cervical tissue excision than in the other groups (►**Table 1**; $p < 0.001$), and the weight and height of the pups tended to be lower in the LPS groups. The low birth weight after an infection, irrespective of a prior cervical tissue excision, was as expected. Therefore, this result has not been included in the following section. There was also no maternal mortality.

Discussion

Our data show that prepregnancy partial cervical tissue excision has only a minimal effect on spontaneous preterm delivery; however, prepregnancy cervical tissue excision and subsequent intrauterine infection can lead to a high spontaneous preterm delivery rate. This rate is likely to be even higher than the preterm delivery rate in a group with only intrauterine infection. The effect of prior cervical tissue excision alone on gestational duration was not greater than that of intrauterine infection; further, intrauterine infection alone did not have a stronger effect than that exerted by prior cervical tissue excision with intrauterine infection. The combination of prior cervical tissue excision with a subsequent intrauterine infection induced preterm deliveries in all experimental setting cases. These results suggest that a prior cervical tissue excision has an additive effect on infection-associated spontaneous preterm birth.

This interesting and important result is consistent with the clinical data from several previous studies that have shown a positive relationship between cervical tissue excision and risk of spontaneous preterm birth.[2–6] A recent large cohort study by Miller et al[6] investigated whether dysplasia alone or a prior excisional procedure is associated with preterm birth. Of 18,528 women, the frequency of preterm birth in women with prior cervical tissue excision was higher than that in women with a short cervix (<2.5 cm) between 18 and 24 weeks of gestation and a history of cervical dysplasia alone (8.4 vs. 6.4 and 6.5%, respectively; $p < 0.001$). In conclusion, prior excision, irrespective of the cervical length, was believed to increase the risk of preterm birth.

More recently, Castanon et al[5] presented data from a nested case–control study (frequency matched to maternal age at delivery, parity, and study site) suggesting that the risk of preterm birth with a prior small excision (<10 mm) was not different from that with a diagnostic punch biopsy. Instead, a larger excision (≥15 or 2.66 cm^3) was associated with a higher risk of preterm birth as the volume excised increased. In our experiment, the excised depth was 1 mm out of a 2.5-mm mouse cervix, representing 40% of the entire cervix. The depth of the experimental excision was the maximum depth that we could excise using the naked eye. Considering that the prepregnancy human cervix is 2 to 3 cm long,[9] a 1-mm depth in a mouse cervix would be equivalent to a 1-cm depth in humans. In the study by Castanon et al,[5] the risks of preterm birth were 15.3 and 18% with 15- to 19-mm and ≥20-mm excisions, respectively. Considering that the rate of preterm birth after cervical tissue excision with a 1-mm depth was 30% (3/10), the rate of preterm birth associated with cervical tissue excision might be higher in mice than in humans.

The human cervical stroma is known to be composed of 85% extracellular matrix, which is mainly collagen, and 15% smooth muscle.[10,11] A recent study shows that the area of the internal os contains 50 to 60% smooth muscle cells, and the external os contains 10% smooth muscle cells.[12] This is

Table 2 Comparison of the muscle-to-collagen ratios in the postpartum cervix between the study groups

	A	B	C	D	*p*-Value
	Sham	Cervical tissue excision	LPS	Cervical tissue excision + LPS	
Proximal cervix	0.9 ± 0.5	0.8 ± 0.6	0.6 ± 0.3	1.3 ± 0.6	0.037[a]
Middle cervix	0.9 ± 0.7	0.7 ± 0.4	0.5 ± 0.2	1 ± 0.7	0.229
Distal cervix	0.8 ± 0.5	0.7 ± 0.4	0.4 ± 0.2	1.1 ± 0.6	0.269

Abbreviation: LPS, lipopolysaccharide.
Note: Data are presented as mean ± standard deviation.
[a]Statistically significant.

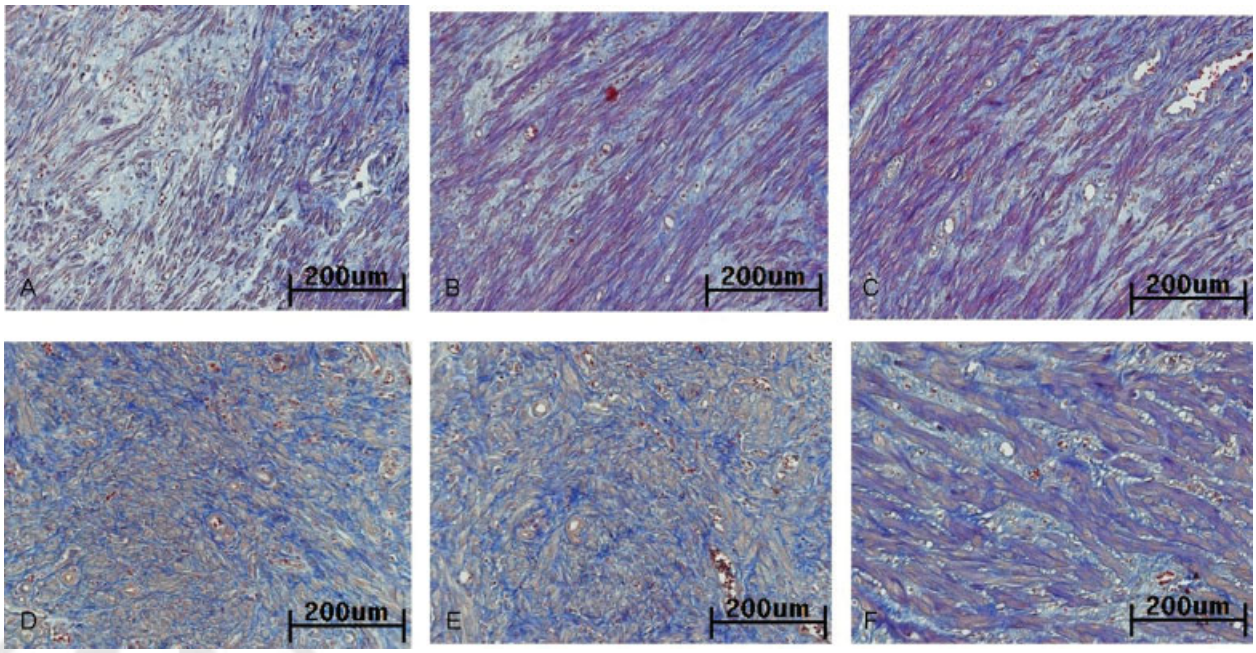

Fig. 4 Histological differences of the uterine cervix between the sham and cervical tissue excision + lipopolysaccharide (LPS) groups as observed using the Masson's trichrome stain in 200× magnification. (**A**) Distal portion of the cervix in the nonexcised sham group. (**B**) Middle portion of the cervix in the sham group. (**C**) Proximal portion of the cervix in the sham group. (**D**) Distal portion of the cervix in the cervical tissue excision + LPS group. (**E**) Middle portion of the cervix in the cervical tissue excision + LPS group. (**F**) Proximal portion of the cervix in the cervical tissue excision + LPS group.

consistent with the data in our experiments; the proximal and middle cervix tended to have a higher percentage of muscle than the distal cervical stroma (►**Table 2**). The percentage of muscle in the distal portions of the mouse cervix was higher than that in the human cervix by almost 40 to 50%, as described in ►**Table 2** and ►**Fig. 4**. Interestingly, when cervical tissue excision and subsequent intrauterine infection occurred, the muscular portion of the cervix increased up to 65%, especially in the proximal cervix. If the increased proportion of the muscle in the cervix in the excision and LPS groups is related to a higher rate of preterm birth in the same group, as demonstrated in our results, a combination of excision and intrauterine infection might induce the formation of a higher number of muscle cells in the proximal cervix. This idea is supported by the "muscular cervix" concept as the potential cause of a weak internal cervical os.[13,14] This pathological cervical insufficiency with increased smooth muscle cells can induce preterm cervical ripening, labor, and delivery through a variety of inflammatory and structural responses, including the upregulation of cytokines, prostaglandins, and matrix metalloproteinases, as well as decreased collagen concentration and elastic fiber content.[15–19] Considering the fibrosis in the middle and distal cervical areas in our study, the muscle-to-collagen ratios were not different between the middle and distal cervical areas in the excision group and those in the nonexcision group. The muscle portion of a normal uterine cervix gradually decreases from the proximal to the distal part.[12] Even though the middle and distal cervix had a fibrosis after excision, the level of the distal cervix in the excision group was the level of the middle cervix in the nonexcision group, which has greater muscle portions than those in the distal cervix. This would be the reason why the muscle-to-collagen ratios of the distal cervices were not different for the cervical tissue excision and nonexcision groups.

Our results provide clinically meaningful information and may nearly confirm the relationship between cervical tissue excision and spontaneous preterm birth. The current results are important to physicians who manage women who have undergone or plan to undergo cervical tissue excision. Women with a prior excision who want a successful pregnancy should be closely followed up through periodic ultrasonographic examinations to monitor cervical changes during pregnancy. During excision, physicians should minimize the damage to a normal cervix in women who desire a future pregnancy.

As described by Elovitz and Mrinalini[20] and Phillips et al,[21] development of a new model for preterm birth research is important. Our excision plus inflammation model shows the highest rate of preterm births among those observed with prior models. The inflammation concept was originally based on the classical model by Elovitz et al.[8] However, in our experience, a 250-μg per mouse dose of LPS would be lethal to the mother, and subsequently, the rate of complete preterm birth modeling would not be that high. For the safety of the model, the dose was reduced to 100 μg per mouse, and as mentioned earlier, there were no cases of maternal mortality. This point has been addressed by other studies.[22] Our model of cervical tissue excision plus low-dose LPS injection can provide maternal safety as well as high efficacy.

Nonetheless, the present study has limitations. First, our model cannot address fetal safety because all mice that had preterm births gave birth to dead fetuses, except for two mice (one from group C and another from group D). In mice that did not have preterm births, all fetuses were born alive. Preterm birth itself was considered as the main cause of stillbirth in mice. Second, our results alone cannot explain why an excision aggravates an infection-associated spontaneous preterm birth. The immunological, structural, and microbiomic roles of the cervix need to be elucidated through comparisons among the groups in our study design. Third, regarding the analysis of relative muscle amounts, a comparison should be made in each experimental group at a given time point before delivery, and the quantification using specific antibodies to the smooth muscles and collagens would be informative to determine how muscle-to-collagen ratios change quantitatively in the perinatal period.

In conclusion, prepregnancy cervical tissue excision had an additive effect to inflammation in inducing preterm birth and was associated with the relative amount of the muscular component. Further investigations on the mechanism of the cervical changes can improve our understanding of spontaneous preterm labor.

Note
This paper was partially presented at the 37th Annual Meeting of the Society for Maternal-Fetal Medicine, Las Vegas, NE, January 23–28, 2017.

Funding
This research was financially supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by the Ministry of Science, ICT, and Future Planning (2014R1A1A1002300) and a grant from Korea University Medical Center and Anam Hospital, Seoul, Republic of Korea (grant no. K1620211). The funding source did not affect the results of this study.

Conflict of Interest
None.

Acknowledgment
The authors acknowledge Professor Youngseok Lee, Department of Pathology, for his valuable comments and suggestions. This research was technically supported by the Core Laboratory for Convergent Translational Research in College of Medicine, Korea University, Seoul, South Korea.

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