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P16 and P21 are involved in the pathogenesis of endometrial thinning
A cross-sectional study

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Abstract

P16 plays a role in the negative regulation of cell proliferation, regulating cell apoptosis to control the growth of tumor cells. P21 is a nuclear protein that suppresses DNA synthesis and inhibits cell division. This study aimed to examine the expression and roles of P16 and P21 in endometrial thinning. Thirty cases of endometrial biopsy diagnosed as endometrial thinning were assessed by p16 and p21 immunohistochemistry from March 2014 to August 2020 in Huazhong University of Science and Technology Union Shenzhen Hospital. Another thirty cases of normal endometrium in the same period were assessed as controls. The specimens underwent histological analysis, and P16 and P21 were assessed by immunohistochemistry. There were no statistically significant differences in age, menstrual cycle, BMI, sex hormone levels, gravidity and parity between the two groups (all $P > .05$). In the endometrial thinning group, P16 was expressed in the endometrial adenoid nucleus, cytolymph and interstitial cell nucleus. In the normal group, P16 was mainly expressed in the endometrial adenoid nucleus, with some P16 signals detected in the endometrial interstitial nucleus. P21 expression was mainly detected in the endometrial adenoid nucleus. P16 and P21 amounts in endometrial thinning cases were significantly lower than those of the normal endometrial group. There was no correlation between p16 and p21 amounts. This study revealed aberrant expression of P16 and P21 in the endometrium might be due to a compensatory effect of the thin endometrium to increase cell proliferation and suppress cell apoptosis. However, the pathological roles of P16 and P21 in endometrial thinning and the contribution of cell senescence deserve further investigation.

Abbreviation: CDK = cellular nuclear protein-dependent kinase.

Keywords: aging cells, endometrial thinning, expression, P16, P21

1. Introduction

The infertility rate is 8%~12% in reproductive-aged couples worldwide.[1] Factors that prevent normal implantation and pregnancy in female infertility mostly involve the endometrium.[2] Endometrial receptivity refers to the capacity of the endometrium to accept the invading embryo, which is the premise of successful embryo implantation.[3] The endometrium includes the basal layer and the functional layer, which is the site for embryo implantation and experiences periodic changes in the menstrual, proliferation and secretion periods, being subject to changes of sex hormones.[4] The basal layer regenerates after menstruation to the endometrial wound, reforming the endometrial functional layer. An appropriate endometrial thickness is the key factor in assuring the success of embryonic implantation. Indeed, the embryo is accepted only with an endometrial thickness in the medium luteal phase reaching about 10 mm.[5,6] A thin endometrium has an endometrial thickness of <7 mm in the medium luteum phase (6–10 days after ovulation).[7] In patients with a thin endometrium, embryo receptivity is affected, which may directly lead to infertility.[8]

A "thin" endometrium is characterized by high blood flow impedance of the uterine radial artery (RA), poor epithelial growth, decreased vascular endothelial growth factor expression, and poor vascular development.[9] Morphologically,

endometrium thinning is characterized by sparse blood vessels in hysteroscopy and sparse cells with lightly stained nuclei and cytoplasm, and histologically by deficient spiral artery.[10] There are many factors causing endometrial thinning, which can be divided into three categories, including organic, functional and idiopathic parameters. For example, temporary thinning of the lining can be caused by certain drugs, which could be restored after drug discontinuation. However, the pathogenesis of endometrial thinning is unclear, and the possible mechanisms include: increased impedance of uterine blood flow, with the resistance index values of uterine and radiative arteries significantly higher in endometrial thinning compared with the normal endometrium throughout the menstrual cycle[11]; impaired vascular development and poor growth of the glandular epithelium, with decreased expression of vascular endothelial growth factor, leukemia inhibitory factor and integrin β3, among others, in the late stage of hyperplasia and the middle stage of secretion,[12] as well as reduced amounts of blood vessels and CD38, a duct endothelial cell marker[9]; transcriptomic alterations, with aberrantly activated inflammatory environment associated with the downregulation of multiple genes in response to oxidative stress[13]; decreased estrogen receptor expression in glandular and interstitial cells in both proliferative and secretory phases[14]; estrogen receptor gene polymorphisms.[15]

On the molecular level, plasma containing abundant growth factors significantly increases the growth and proliferation of endometrial mesenchymal stem cells and increase the pregnancy rate.[16] Another research revealed that G-CSF significantly improves endometrial thickness in a rat model with endometrial thinning, reducing endometrial apoptotic cells.[17] Furthermore, metformin promotes the regeneration of damaged endometrium by inhibiting apoptosis.[18] Clinically, endometrial thinning is treated by hysteroscopic adhesiolysis, hormonal manipulation by estrogen and GnRH-agonists, vasoactive products (e.g., aspirin, vitamin E, pentoxifylline, l-arginine, and sildenafil), intra-uterine infusion of G-CSF and regenerative medicine, although most of these treatments only provide minor benefits.[19] Investigating genes that regulate endometrial cell proliferation and apoptosis may provide an efficient option for treating endometrial thinning.

P16 is a cellular nuclear protein-dependent kinase (CDK) inhibitory protein, which competitively binds to CDK4 against Cyclin Dl and plays a role in the negative regulation of cell proliferation, regulating cell apoptosis to control tumor cell growth.[20] P16 upregulation is considered an important marker of senescence in mammalian cells.[21] In addition, increased expression of P16 was detected in the mouse endometrium during embryonic implantation,[22] responding to hormone regulation and prompting endometrial apoptosis to participate in the formation of a "receptive endometrium". On the other hand, P21 is a nuclear protein that binds to the CDK complex and inactivates multiple kinases, thereby suppressing DNA synthesis and inhibiting cell division.[23] In addition, the amounts of apoptotic cells in substrate and functional glandular cells are highly positively correlated with the number of p21 positive cells.[24]

Considering the key contributions of P16 and P21 in regulating cell proliferation and apoptosis in the endometrium, these proteins may play important roles in the formation of appropriate endometrial thickness, further promoting the formation of a "receptive endometrium". Therefore, the present study aimed to explore the pathogenetic mechanism of endometrial thinning by assessing P16 and P21 levels in the thin endometrium.

2. Material and Methods

2.1. Study design and grouping

This was a cross-sectional study. A total of 30 cases with endometrial thinning diagnosed by Doppler ultrasound and hysteroscopy at the Gynecology department of Huazhong University of Science and Technology Union Shenzhen Hospital from March 2014 to August 2020 were selected for further confirmation by biopsy. Totally 30 cases of endometrial curettage were assigned to the control group, with samples collected prior to other gynecology surgeries and confirmed by a pathologist as endometrial tissue at the normal proliferative stage. There were 30 cases each in the endometrial thinning and control groups.

Inclusion criteria were: 18 to 44 years of age, no history of hormone use within 3 months before examination, and normal sexual hormone levels 2 to 5 days after menstruation. Other inclusion criteria in the endometrial thinning group were: need for artificial assisted reproduction due to infertility; hysteroscopy revealing smooth and thin endometrium on the 15th to 25th day of the menstrual cycle; no adhesion in the uterine cavity, and clearly visible opening of the fallopian tube; endometrial thickness measured by transvaginal color Doppler ultrasound of <7 mm in the medium luteal phase. The additional inclusion criterion in the normal control group was endometrial thickness ≥7 mm, confirmed by a pathologist as endometrial tissue in the normal proliferative phase.

In both groups, individuals with medical disorders such as tuberculosis, diabetes, thyroid and adrenal gland pathologies, serious infections, organ diseases and/or tumors were excluded. This study was approved by the Medical Ethics Committee of Shenzhen Nanshan District People's Hospital (No. 072652).

2.2. Immunohistochemistry

Anti-P16 (MAB-0673) and anti-P21 (MAB-0235) primary antibodies and ready-to-use secondary antibodies were purchased from Fuzhou Maixin Biotechnology Co., Ltd. (China) and applied in the immunohistochemical SP method, strictly in accordance with the manufacturer's instructions. Positive cells were detected as brown or brown-yellow spots in the cytoplasm and/or nucleus. Endometrial glands and stromal areas in 10 high-power fields were observed under a light microscope at 400×, counting 100 cells/field. The average value was determined according to the proportion and distribution of positive cells.

2.3. H&E staining

Slices were incubated a 50°C for 2 hours, followed by xylene dewaxing 3 times for 10 minutes each. After successive incubations with 100% 90%, 80%, and 70% alcohol for 2 minutes each, the samples were rinsed with tap water for 3 minutes, before incubation with hematoxylin staining solution for 10 minutes. The samples were rinsed with tap water for 3 minutes, and 1% hydrochloric acid alcohol (prepared with 70% alcohol) was added for 6 seconds. After rinsing with tap water for 3 minutes, saturated lithium carbonate reverse blue was added for 10 seconds, followed by rinsing with tap water for 3 minutes. Next, 5% eosin staining solution was added for 5 seconds, which was followed by dehydration with ethanol gradient (70%, 80%, 90%, and 100%). Finally, two xylene incubations were performed (5 min each) before mounting with neutral gum.

2.4. Statistical methods

SPSS for Windows 20.0 (SPSS) was used for data analysis. Data are mean ± standard deviation. The Shapiro–Wilks test was performed to confirm that the continuous data had normal distribution, and the Student's t test was used to assess between group differences. Correlation analysis was performed to assess the association of P16 and P21 amounts. $P < .05$ indicated statistical significance.

3. Results

3.1. Clinical data

All specimens were examined by a pathologist as well as by transvaginal ultrasound and hysteroscopy. There were no statistically significant differences in age, menstrual cycle, BMI, sex hormone levels, gravidity and parity between the two groups (all $P > .05$). Endometrial thickness was 4.56 ± 0.75 mm in the endometrial thinning group, indicating a significantly lower value compared with the control group (8.76 ± 1.69 mm; $P < .05$). All clinical data are shown in Table 1.

3.2. Histological and hysteroscopic observations

Figure 1 shows the gross structures of the endometrium in the normal and endometrial thinning groups, observed by hysteroscopy. In the normal endometrium, H&E staining showed cuboidal epithelium, loose stroma, and large distributions of glands and blood vessels; in addition, the glands were tubular, and epithelial cells were cylindrical or cubic on the surface of glands (Fig. 2A). In the endometrial thinning group, cells were sparse, with lighter nucleus and cytoplasm, and no obvious spiral artery and other vessels (Fig. 2B).

3.3. P16 and p21 levels

P16 expression in the normal group was mainly detected in the nucleus of endometrial glandular cells, with light staining in the endometrial interstitial nucleus (Fig. 3A). In the endometrial thinning group, P16 was detected in the nucleus and cytoplasm of endometrial glandular cells, as well as in the nucleus of interstitial cells (Fig. 3A). P21 was mainly detected in the nucleus of endometrial glandular cells in both groups (Fig. 4A).

Both P16 and P21 amounts were reduced in the endometrial thinning group compared with the normal group (Figs. 3B and 4B, and Table 2). However, there was no correlation between P16 and P21 amounts in the two groups (Table 3).

4. Discussion

The present clinical study demonstrated that P16 and P21 expression patterns are altered in endometrial thinning. It is well known that defective receptive endometrium is a cause of recurrent pregnancy loss and endometrium thinning.[25] A previous report showed that decreased number of p16 -positive, senescent cells in the human endometrium is a potential marker of miscarriage[26,27] and increased P16 expression in the endometrium was detected during "embryonic implantation" in mice.

The P16 protein is localized within the nucleus and plays an important role in the cell cycle.[28] Interstitial p16 levels may reflect p16 -induced cellular aging, which has been confirmed in a variety of benign interstitial tumors.[28] As shown above, P16 was mainly expressed in the nucleus of endometrial adenocytes, with reduced amounts in the normal group; meanwhile, P16 was expressed in the nucleus and cytoplasm of endometrial adenoid cells, as well as in the nucleus of interstitial cells in the endometrial thinning group. Overall, our data revealed that P16

Table 1

clinical data of 2 groups($\bar{x} \pm s$).

|	Thin group	Normal group	t	P
Age (yr)	32.27 ± 10.27	40.07 ± 12.26	−1.96	.08
Menstrual cycle (d)	26.82 ± 3.76	25.72 ± 4.35	1.37	.17
Endometrial thickness (mm)	4.56 ± 0.75	8.76 ± 1.69	−2.01	.03
BMI (kg/m²)	23.48 ± 1.69	24.18 ± 1.53	−0.71	.35
Gravidity	3.02 ± 0.83	2.98 ± 1.02	0.37	.68
Parity	2.12 ± 1.23	56.33 ± 31.31	0.26	.81
E2 (pmol/L)	69.36 ± 36.36	1.21 ± 0.52	0.38	.65
P (nmol/L)	1.02 ± 0.26	8.35 ± 1.66	−0.63	.42
FSH (IU/L)	9.29 ± 2.76	5.92 ± 1.77	0.24	.83
LH (IU/L)	6.38 ± 1.22	5.92 ± 1.77	0.36	.72
PRL (nmol/L)	15.32 ± 7.12	13.11 ± 6.12	0.42	.62
T (nmol/L)	0.45 ± 0.21	0.48 ± 0.16	0.26	.73
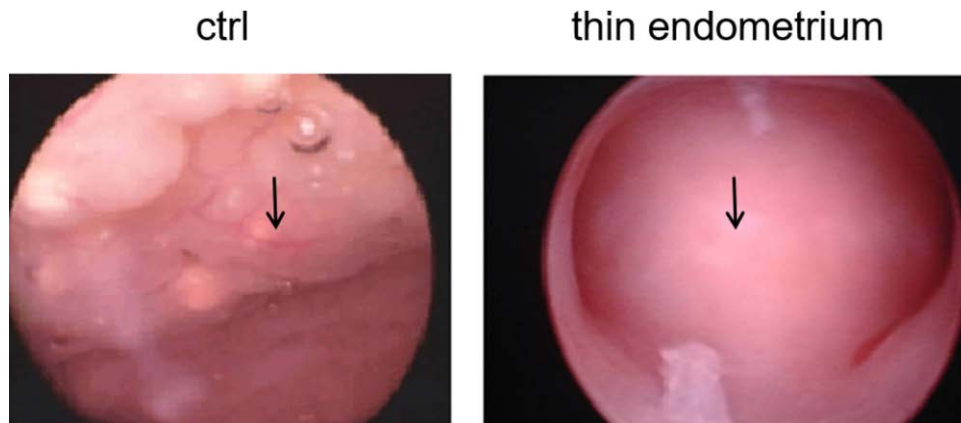
Figure 1. Endometrial structure obtained by hysteroscopy. Representative images of the endometrium in the normal and endometrial thinning groups are shown.
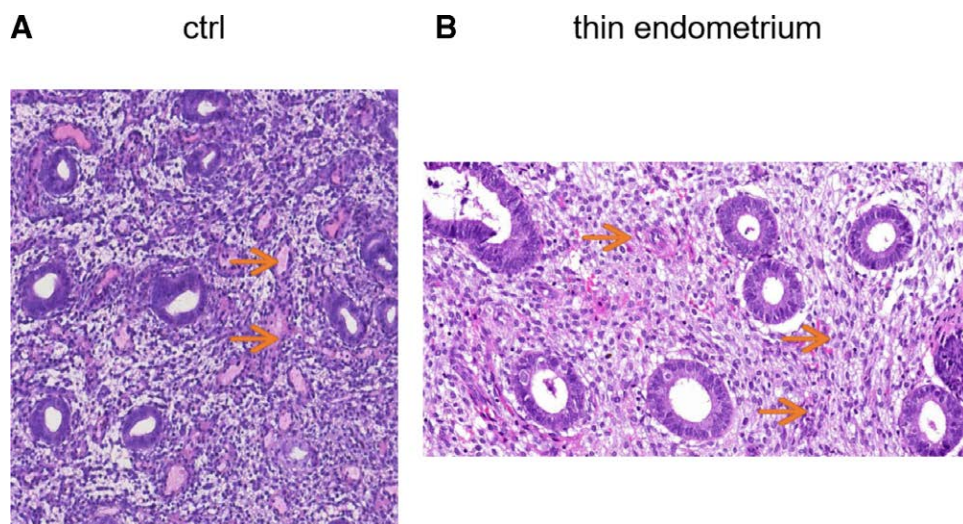
Figure 2. Histological properties of the endometrium. Hematoxylin and eosin staining of endometrial samples was performed, and representative images in the normal and endometrial thinning groups are shown (400×). Figure 2A (normal endometrium): HE staining showed dwarf cubic epithelium, loose stromal, and large distribution of glands and blood vessels. The glands were tubular, and epithelial cells were cylindrical and cubic on the surface of the glands. Figure 2B (thin endometrium): cells were sparse, with lighter nucleus and cytoplasm, and no obvious spiral artery and other vessels were seen.
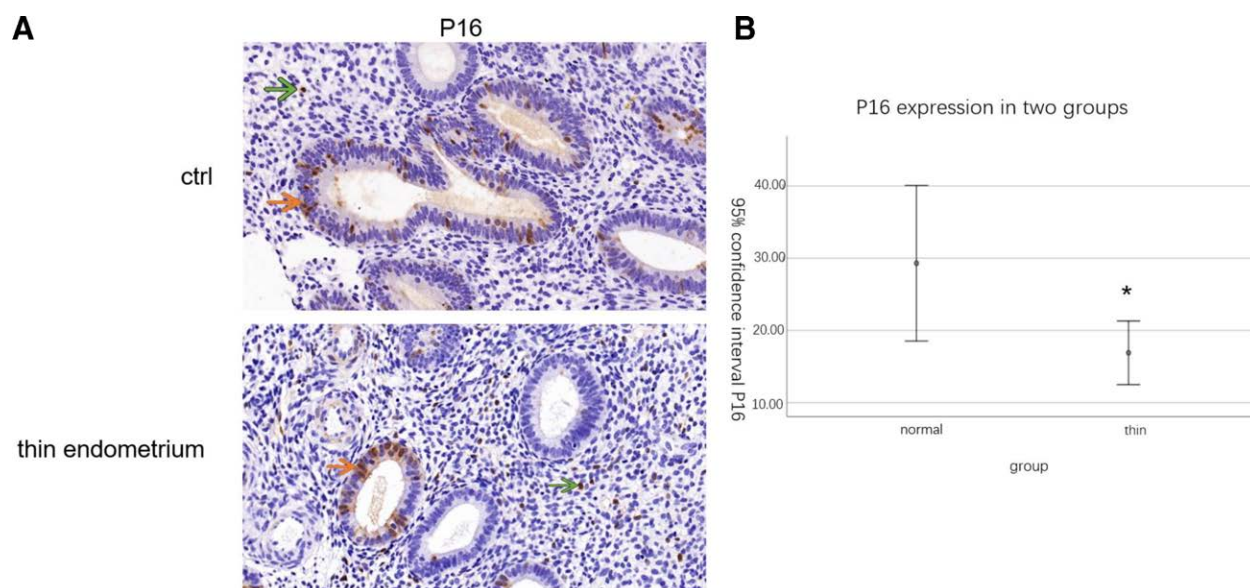

Figure 3. Endometrial P16 amounts are reduced in endometrial thinning. Immunohistochemical signals are shown in Figure 3A for the normal and endometrial thinning groups (400×). P16 was expressed in the nuclei of endometrial glandular cells (green arrow) and endometrial stromal cells (orange arrow) in the normal group. In the endometrial thinning group, P16 was expressed in endometrial glandular cell nuclei and cell organelles (orange arrow) and endometrial stromal cell nuclei (green arow). Quantitation is shown in Figure 3B.

expression was lower in the endometrial thinning group than in the control group. These findings indicate that increased endometrial cell proliferation in endometrium thinning while suppressing cell aging/apoptosis of endometrial cells to compensate for the thin endometrium.

Overexpression of p21 eventually suppresses DNA synthesis and prevents cell proliferation; in addition, the number of apoptotic cells in substrate and functional glandular cells is highly positively correlated with the number of p21-positive cells.[24] P21 signals in the normal and endometrial thinning groups were mainly localized in the nucleus of endometrial adenocytes. In addition, our results revealed decreased P21 expression in the endometrium thinning group compared with control samples, indicating reduced apoptosis in the endometrium may increase endometrial thickness to accommodate embryonic implantation.

Previous findings showed that P16 and P21 jointly regulate fibroblastic growth.[29] However, our results showed no correlation between P16 and P21 in either group. Further studies are required to explore the underlying mechanism.

The above findings demonstrate critical roles for P21 and P16 in endometrial thinning, indicating that cellular senescence may be critical in this disorder. In addition, the altered expression of P21 and P16 suggests that these two proteins may constitute biomarkers or therapeutic targets in endometrial thinning.

The limitations of this study should be mentioned. This was a cross-sectional study, and it was impossible to clearly demonstrate a causal relationship between these proteins and endometrial thinning. In addition, it had a relatively small sample size, which might reduce the statistical power. Therefore, further investigation is required to further unveil
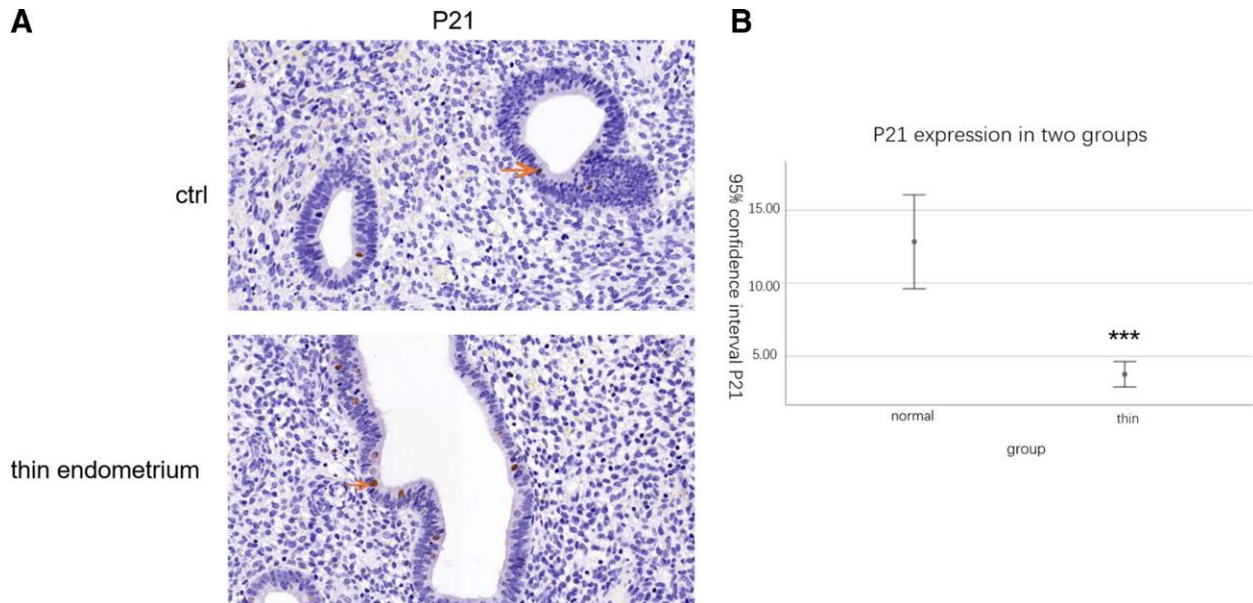
Figure 4. Endometrial P21 amounts are reduced in endometrial thinning. Immunohistochemical signals are shown in Figure 4A for the normal and endometrial thinning groups (400×). P21 was expressed in endometrial glandular cell nuclei (orange arrow) in both groups. Quantitation is shown in Figure 4B.

Table 2

Comparison of p16, p21 expression in two groups($\bar{x} \pm s$).

	N(case)	P16	P21
Thin group	30	16.90 ± 11.82	3.76 ± 2.33
Normal group	30	29.30 ± 28.84	12.83 ± 8.62
P		.033	.000

Table 3

The correlation between p16 and p21 in the two groups.

	Pearson	P
Thin group	0.118	.534
Normal group	−0.041	.829
Two groups	0.143	.277

the exact pathological mechanism of endometrial thinning, which might help solve the problem of female infertility around the world.

5. Conclusion

In conclusion, this study revealed aberrant expression of P16 and P21 in the endometrium might be due to a compensatory effect of the thin endometrium to increase cell proliferation and suppress cell apoptosis. However, the pathological roles of P16 and P21 in endometrial thinning and the contribution of cell senescence deserve further investigation.

Author contributions

Aiwen Le carried out the assays, participated in data collection, and drafted the manuscript. Qifeng Li and Xianchan Zheng participated in data acquisition, analysis, and interpretation. Huan Yang participated in manuscript review and proofreading. All authors read and approved the final manuscript.

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Writing – original draft: Aiwen Le.
Writing – review & editing: Huan Yang.

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