Role for Autophagy-Related Markers Beclin-1 and LC3 in Endometriosis

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Research Article

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Abstract

Background Endometriosis is a common and challenging disease in women of childbearing age with high personal and social costs. Many molecular differences between endometriosis lesions and the eutopic endometrium present difficulties in the development of new drug therapies and therapies. Autophagy is a response to stress and has recently been studied in human cancers. Two important autophagy genes, Beclin-1 and LC3, have been reported in several human cancers. However, the reports of Beclin-1 and LC3 in endometriosis are limited and controversial.

Methods & Results In the present study, we investigated the expression of Beclin-1 and LC3, and found their downregulation in endometriosis and negatively correlated with the stage of endometriosis.

Conclusions Abnormal expression of Beclin-1 and LC3 may be related to the occurrence and development of endometriosis.

Background

Endometriosis is a common, chronic inflammatory disease defined by the presence of hormone-dependent endometrial cells including glandular cells and stromal cells that grow in ectopic sites. It is reported that endometriosis affects approximately 10% of women worldwide, and 50–60% of endometriosis women have pelvic pain[1]. Moreover, up to 50% of infertility in women is caused by endometriosis. Costs for endometriosis are nearly $12,000 per patient placing a huge burden on society and the economy[2]. However, mechanism remains unclear.

Autophagy is an evolutionarily conserved and highly regulated lysosomal degradative pathway that degrades macromolecules and organelles inside the cell. Autophagosome formation and maturation are regulated by several core autophagy gene (*Atg*) proteins in a highly controlled manner. Thirty specific genes have been identified in yeast that regulate autophagy, 16 are homologous to humans[3]. The gene encoding Beclin-1 (molecular weight, 60 kDa is located on chromosome 17q21 and is a mammalian cell homolog of the yeast autophagy-related gene *Atg6.* It mediates the initiation stage of autophagy [4].

Microtubule-associated protein light chain 3 (LC3) is a key protein in autophagy, encoded by a mammalian ortholog of yeast *Atg8* to produce a soluble protein (approximately 17 kDa) that is ubiquitous in mammalian tissues and cultured cells. LC3 in its two forms (LC3-I and LC3II) plays a major role in the cytoplasm of autophagosomes and autologous lysosomes.[5]. Studies on autophagy and endometriosis are rarely reported, and the results have been inconsistent. One study showed that autophagy promoted the transition of endometrial epithelial-mesenchymal cells, contributing to the development of endometriosis, and that LC3 was elevated in ectopic endometrium[6]. Conversely, another study showed that the autophagy process inhibited the development of endometriosis, and expression of LC3 was reduced in the ectopic endometrium[7].

In this study, immunohistochemistry was used to describe the expression of autophagy-related indicators Beclin-1 and LC3 in ectopic endometrial tissues of patients with endometriosis of different stages and normal endometrium.

Methods

Sample Collection

84 women with endometriosis who underwent surgery in the Gynecology Department of Nanhai District Maternal and Child Health Hospital of Foshan City from November 2016 to October 2018 (Table 1) were provided informed consent and the study was approved by the Ethics Committee of the hospital. The patients ranged from 26 to 49 years (average, 34 ± 6.9 years). All patients underwent laparoscopic ovarian endometriosis cystectomy.

The control group comprised 32 patients, aged 22–48 years (average, 39.2 ± 7.0

years) from the same hospital who underwent endometrial curettage or total hysterectomy during the same time period because of tubal infertility and uterine fibroids. All of the study patients underwent surgery in the proliferative phase of the menstrual cycle, following 3–7 days after menstruation. None of the patients took steroids, were pregnant, or in lactation 6 months before any of the surgeries

All subjects were asked for a detailed medical history, which included information

on vaccinations, menstruation, marriages, and family. They received a detailed physical examination, laboratory and functional examinations, including hematuria, coagulation, vaginal secretions, liver and kidney function, thyroid function, electrocardiogram, detailed pelvic abdominal B ultrasound, and chest X-ray, to rule out the presence of any other endocrine diseases.

Immunohistochemistry

Paraffin-embedded tissues were archived. Each block was serially sectioned at 4-μm thickness for immunohistochemical staining. Baked at 60°C for 2 hours, deparaffinized and rehydrated for high-pressure, heat-mediated antigen retrieval for 5 minutes. The sheets were blocked endogenous peroxidase activity by using 3% hydrogen peroxide in 10 minutes, followed by washes (5 minutes each) with 0.01 M phosphate-buffered saline (PBS), and then blocked with the same serum as the secondary antibody source for 20 minutes at room temperature. The working solution for the primary detection antibodies, rabbit anti-Beclin-1(Abcam, ab55878, Cambridge, UK) and rabbit anti-LC3A/B (Abcam, ab48394, Cambridge, UK, was prepared in accordance with the primary antibody specification. The primary detection antibodies and the tablets were placed in a wet box and transferred to a 4°C refrigerator overnight, then washed three times with 0.01 M PBS (5 minutes each). Using the instructions for the horse radish peroxidase-labeled secondary antibody, the concentration of the secondary antibody was

determined, and incubated with the homologous primary antibody in the wet box for 1 hour at room temperature. The sheets were washed three times with 0.01 M PBS, incubated with streptavidin-biotin complex in a wet box for 30 minutes at room temperature, and washed another three times with 0.01 M PBS (5 minutes each). Immunolabeling was visualized by dropwise addition of 3,3'-diaminobenzidine color staining solution for 5 to 10 minutes. The sheets were washed with water, and the tablets were stained with hematoxylin, then dehydrated and finally sealed with a neutral gum.

The sections were independently reviewed by two pathologists using a double-blind method. Beclin-1 was mainly expressed in the membrane and cytoplasm. LC3 was mainly expressed in the cytoplasm. Some nuclei and stroma were visible when strongly positive.

We observed five 40 × 10 high power fields at random; 100 cells were counted, and the color depth of the cells was observed. Ultimately, the ratio of cells and the depth of staining were assessed comprehensively. Beclin-1 and LC3 have the same scoring criteria.

1)Positive staining cell score: 1, positively stained cells accounting for ≤ 10% of the total cells; 2, positively stained cells accounting for 11–50% of the total cells; and 3, positively stained cells accounting for 51–75% of the total cells.

2)Dyeing depth score: 0, no staining; 1, light yellow; 2, yellow; and 3, dark yellow to brown.

3)Total score: positive staining cell ratio score × staining intensity score; ≤ 3 is negative, > 3 is positive.

Grading And Staging Of Endometriosis

The original records and surgical images of the endometriosis group were reviewed. Using the records of the location, number, size and adhesion of the endometriotic lesions, the American Society for Reproductive Medicine classification was used to correct the endometriosis staging method [3].

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software. Expression of Beclin-1 and LC3 protein in both groups, and in subgroups at different stages of endometriosis, were analyzed by the chi-square test. Spearman correlation analysis was used to analyze the correlation. $P < 0.05$ was used to indicate a statistically significant difference.

Results

Expression of Beclin-1 and LC3 Immunohistochemistry

Beclin-1 protein was expressed in both groups (Fig. 1A-E); however, the rate of positivity for Beclin-1 protein staining in the endometriosis group (67.9%; 62/84), was significantly lower than that in the control

group (96.9%; 31/32; P < 0.05). LC3 was mainly expressed in cytoplasm, and was also expressed in some cell stroma and part of the nuclei in cells that were strongly positive (Fig. 2). The rate of positivity for LC3 staining in the endometriosis group was 76.1% (64/84), which was significantly lower than control group (93.75%; 30/32; P < 0.05) (**Table 2**).

Relationship Between Beclin1 And Lc3 And Clinical Stage Of Endometriosis

For subgroup analysis, because of the small sample size, the single patient in stage I endometriosis (score, 4 points), was combined with the phase II patients for analysis. There were 32 cases of stage I and II endometriosis in the endometriosis group, and the rate of Beclin-1 positivity was 93.8% (30/32). There were 19 cases of stage III and 33 cases of stage IV endometriosis, with rates of Beclin-1 positivity of 68.4% (13/19) and 57.6% (19/33), respectively. The difference was statistically significant (**Table 3**).

The rate of LC3 positivity in the 32 cases of stage I and II endometriosis was 96.9% (31/32), 78.9% (15/19) in the 19 cases of stage III, and 54.5% (18/33) in the 33 cases of stage IV endometriosis. The difference was statistically significant (**Table 4**).

The chi-square segmentation method was used to analyze differences in autophagy-associated markers between stage III and stage IV endometriosis. The results showed that there was no significant difference for Beclin-1 expression or LC3 expression between stage III and stage IV. Therefore, these two sets of data were combined and compared with those for stage I and stage II.

Among stage III and IV endometriosis, Beclin-1 positivity was 61.5%. LC3 positivity in the stage III and IV was 63.5%, a statistically significant difference (**Table 5**).

Correlation Analysis Of Beclin-1 And Lc3 Expression

In the endometriosis group, there were 59 cases of Beclin-1 and LC3 co-positivity, 17 cases of co-negativity, three cases of Beclin-1 positivity and LC3 negativity, and five cases of Beclin-1 negativity and LC3 positivity. Spearman correlation analysis showed a positive correlation for the expression of both markers in the ectopic endometrium (Table 6).

Discussion

Autophagy is a homeostasis process involving the self-digestion of cells to provide energy. It plays a negligible role in cell development and differentiation in processes that include cancer, infectious diseases, metabolic disease, aging, and neurodegenerative disease [8–12].

Beclin-1 is necessary for the formation of autophagosomes. Without Beclin-1, the flux of autophagy will be blocked, causing malformation of autophagosomes[13]. As a key autophagosome regulatory factor,

an adequate concentration of Beclin-1 is an integral part of start-up process, allowing it to participate in multiple physiological and pathological processes, including the adjustment to pressure, the development of the organism, the formation of cancer, immune diseases, heart disease and neurodegenerative disease[14]. Research by Ren et al. has revealed that the expression level of Beclin-1 falls in both eutopic endometrium and foci of adenomyosis [15]. In our research, we have found that Beclin-1 mainly locates in the cell membrane and the cytoplasm. In the experimental group, the rate of positive Beclin-1 expression was prominently below that of the control group.

These results indicate that: 1) When the expression of Beclin-1 falls, or when the gene is mutated or missing, the autophagy of Beclin-1-mediated ectopic endometrial cells is reduced, which leads to decreases in the autophagy-dependent proteins, the degradation of organelles, and autophagosome formation. The ectopic endometrial cells are then able to escape autophagy, adhere to the extracellular matrix of other cells, invade into tissues, and grow on the outside of endocardium, thus causing endometriosis. 2) If the concentration of Beclin-1 is reduced, the number of mature autophagosomes will be reduced as well. Thus the apoptosis of normal cells can be influenced, which could lead to the survival of ectopic endometrium. 3) The decrease and inactivation of Beclin-1 will cause the loss of regulatory control of some of the genes in the Bcl-2 family, which can reduce the autophagy power in cells and the programmed cell death, thereby allowing the survival of ectopic endometrial cells. The deposition of ectopic endometrium stimulates the activation of local and general inflammation, including increases in chemokines and other proinflammatory cytokines, which can accelerate the occurrence of endometriosis.

LC3 and Beclin-1 proteins participate in the construction of autophagy, reflecting the number of autophagosomes, and act as specific markers of autophagy[5]. LC3 is mainly localized in pre-autophagic vacuoles and at the membrane surface of autophagic vacuoles as its active constituent lipo-LC3-II, with the effect of being selectively incorporated into the autophagic substrate and enhancing coupling of the expansion and closure of the autophagic membrane[15]. LC3 is also capable of giant cell ingestion and antigen presentation[16]. Our research has shown that the rate of LC3 positivity was reduced compared with that in the control group. Possible mechanisms to explain this result include: 1) Ectopic endometrial cells produce less autophagosmes or smaller autophagosmes, thus diminishing the efficiency and function of autophagosome formation, reducing the exercise ability of autophagy, failing to combine with lysosomes, decreasing the formation of autophagic lysosomes and autophagic cell death, which allow the ectopic endometrial cells to escape autophagy and grow ectopically. 2) The reduction of LC3 depresses the ability to digest ectopic endometrial cells, debris and residue; this failure to eliminate the tissue triggers inflammatory and immune mechanisms, resulting in local and systematic stimulation thereby activating inflammation. Previous research has demonstrated that interleukin (IL)-8 can stimulate the proliferation of ectopic endometrial cells and enhance endometrial cell adherence to fibronectin, thus promoting the incidence of endometriosis[17].

Our research indicates that the rate of positive expression of LC3 and Beclin-1 decreases with increasing clinical stages of endometriosis. Furthermore, the positive correlation between endometriosis and Beclin-1 and LC3 expression suggests that the reduction in autophagic ability and autophagic activity of ectopic

endometrial cells, and the concomitant decrease in autophagosome formation, leads to the occurrence and development of endometriosis. The ability to digest ectopic endometrial cells through autophagic mechanisms is depressed and, because of local inflammatory infiltration and dysimmunity, ectopic endometrial cells exhibit a stronger capacity for angiogenesis. Proliferation and migration of ectopic endometrial cells activated by angiogenic factors could mutually promote both the occurrence and development of endometriosis, in a manner similar to that described by Wu [18]et al., who reported correlations between LC3 and Beclin-1 expression and the development and progression of colorectal cancer.

Conclusions

Beclin-1 and LC3 are downregulated in endometriosis and negatively correlated with clinical stage of endometriosis, and might be involved in the occurrence and development of endometriosis. Interactions between these two autophagy-related factors may boost the development of endometriosis. Further research should combine multiple autophagy regulatory factors with human epididymal protein 4 and cancer antigen 125 to scrutinize the effect of Beclin-1 and LC3 on the occurrence and development of endometriosis. It would also be useful to explore the mechanistic relationships between ovarian hormones, their receptors and autophagy in endometriosis. Autophagy regulation could become a new approach in the diagnosis and treatment of endometriosis.

Declarations

Ethics approval and consent to participate

The experiments involved in this study were permitted by Ethics committee of Nanhai Maternal and Child Heath Hospital. All patients had agreed and signed a contract to use their samples experimentally.

Consent for publication

Not applicable.

Availability of data and material

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZWK did all the experiment and write the manuscript. TTY designed the experiment and revised the manuscript. all authors read and approved the final version of the manuscript

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Tables

Tables 1 to 6 are available in the Supplementary Files section

Figures

Figure 1

Expression of Beclin-1 in normal endometrium and ectopic endometrium

(A) Expression in normal endometrial tissue.

(B) Expression in phase I endometriosis.

(C) Expression in phase II endometriosis.

(D) Expression in phase III endometriosis.

(E) Expression in phase IV endometriosis.

Figure 2

Expression of LC3 in normal endometrium and ectopic endometrium.

(A)Expression in normal endometrial tissue.

(B)Expression in phase I endometriosis.

(C) Expression in phase II endometriosis.

(D) Expression in phase III endometriosis.

(E) Expression in phase IV endometriosis.

Supplementary Files

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- Table2.xls
- Table4.xls
- Table5.xls
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