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Original Article

Molecular regulation of autophagy related genes in papillary thyroid carcinoma

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Abstract: Background: Thyroid papillary carcinoma is the most common clinical thyroid malignancy. Autophagy is related to the occurrence and development of tumors. However, there is no report about the correlation between autophagy protein expression and biological characteristics of thyroid papillary carcinoma and lymph node metastasis. Therefore, the authors studied the relationship between autophagy protein expression and thyroid papillary carcinoma. Methods: In this study, we detected the mRNA and protein expressions of LC3, Beclin1, ATG2b, ATG3, ATG4a, ATG4b, ATG4c, ATG5, ATG7 and ATG10 in papillary thyroid carcinoma patients' blood. Results: We found that the expression of Beclin1 and LC3 in blood of the patients was significantly higher than that of the normal group, suggesting that the autophagic activity was enhanced in the thyroid tumor. Discussion: The results show that autophagy is associated with the occurrence and the progression of papillary thyroid carcinoma. The autophagy can become a new therapeutic target, especially to prevent the recurrence of postoperative cervical lymph node recurrence in papillary thyroid carcinoma.

Keywords: Papillary thyroid carcinoma, autophagy, mRNA expression, protein levels

Introduction

Thyroid papillary carcinoma is the most common clinical thyroid malignancy, often occurs in young women, and is prone to cervical lymph node metastasis [1]. Comprehensive treatments of thyroid papillary carcinoma include thyroid surgery, and thyroid hormone suppression therapy [2]. Many studies have shown that cervical lymph node metastasis also affects the prognosis of patients with papillary thyroid carcinoma [3]. For the transfer of lymph nodes with extracorporeal invasion, the patients has poor prognosis [4]. Therefore, revealing the occurrence and metastasis of papillary thyroid carcinoma, and preventing cervical lymph node recurrence have important clinical significances.

Autophagy is a metabolic process of cells [5, 6]. A large number of proteins are wrapped by the cell membrane, and organelle vacuoles appear in the cytoplasm [7]. Beclin1 is a key protein that can regulate autophagy and is a hallmark of initiation of autophagy [8]. It has been observed that the expression of Beclin1 protein in ovarian cancer, breast cancer and prostate cancer cells showed significant difference in normal breast tissue [9]. LC3 is a homologue of yeast ATG8 gene in mammals; in the process of autophagy formation, autophagy genes ATG3, ATG5, ATG7, ATG10 and LC3 are also involved [10]. LC3 is presented in two forms, LC3-1 and LC3-2. LC3-1 is presented in the cytoplasm and LC3-2 binds to the autophagic bubble, and when the cell is subjected to stress changes such as starvation, the cell undergoes autophagic changes. Once the autophagosome binds to the lysosome, LC3-2is degraded by hydrolytic enzymes in lysosomes. In different tissues and cells, the content of soluble LC3-I and membrane-bound LC3-II in cytoplasm vary greatly [11].

Autophagy has two effects, one is the promotion of cell survival, the other is the inhibition of the tumor growth. First, when the tumor cells with apoptosis function deficiency were stimulated, autophagy can exacerbate local inflammation by necrosis, promote tumor cell proliferation, and protect cells from death [12]. Second, autophagy not only protects tumor cells from stress changes, but also prevents the cell's genetic instability and thus inhibit

Table 1. List of mRNA sequences

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')
LC3	GGTTTCCCGTCACCAATTTTCC	TGTGGTTTCCAACGTAGAGGA
Beclin1	GCTGTCGTCAGGGCTGAAT	TCCACTGGAGACCTGCAACA
ATG2b	GCCATCAAGAATGTATCCGTTGC	CTTCACCCACAGGTTTAGGTTTT
ATG3	CAGTGGCTGTCCCTAGTAATTTT	AGAACTGCCATGAGTCCTACA
ATG4a	TTTGCTGGGGTTTACTTTGAGAA	AGGTCAGTCGGTTATATCCCTG
ATG4b	CCTATGCACACACCGTCAAGG	CAGGACACCGCTGATGATG
ATG4c	ACCGTCACCGAGTTGTCTTTT	GAACCCAGGTGGGGATCATAA
ATG5	TCAGCAGCGACTTGACCTAC	TGGGCAAAGTTACAGAAGCCG
ATG7	ACACCCTGGGCTCTATCATTT	CTTCTGACCGGCCATTTCCTC
ATG10	AGGGTTACTCAACTTCCCAGC	CAGCCCATACCACCTCCTG

Table 2. List of protein information

Protein name	Resource	Dilution factor	Molecular
LC3	Cell Signaling Technology (CST)	1:1000	14/16 kD
Beclin1	Cell Signaling Technology (CST)	1:1000	60 kD
ATG2b	Cell Signaling Technology (CST)	1:1000	232 kD
ATG3	Cell Signaling Technology (CST)	1:1000	35 kD
ATG4a	Cell Signaling Technology (CST)	1:1000	45 kD
ATG4b	Cell Signaling Technology (CST)	1:1000	44 kD
ATG4c	Cell Signaling Technology (CST)	1:1000	52 kD
ATG5	Cell Signaling Technology (CST)	1:1000	32 kD
ATG7	Cell Signaling Technology (CST)	1:1000	77 kD
ATG10	Cell Signaling Technology (CST)	1:1000	25 kD

the formation of tumor [13]. Recent studies have showed, the inhibition of autophagic genes or application of autophagic inhibitors can increase the chemosensitivity of tumor cells [14]. Therefore, autophagy enhances the formation and inhibits tumor progression, and the inhibition of autophagy can accelerate the reduction of tumor degeneration.

As described above, autophagy is related to the occurrence and development of tumors [15]. However, there is no correlation between autophagy protein expression and biological characteristics of thyroid papillary carcinoma and lymph node metastasis. Therefore, in this study, the authors studied the relationship between autophagy protein expression and thyroid papillary carcinoma, to provide effective targets and research basis for the development, lymph node metastasis and recurrence of papillary thyroid carcinoma and the development of new therapeutic methods.

Materials and methods

Blood collection

In this study, 10 patients with papillary thyroid carcinoma, 10 healthy people with routine examination of the clinic were chosen as the subjects. They were divided into control group and experimental group, with 10 cases in each group. All the patients have signed the informed consent. Blood was quickly collected using a syringe containing heparin.

Real-time RT-PCR

Total RNA was extracted from blood with Trizol reagent (Applied Biosystems, Invitrogen, USA). The total RNA was reverse transcribed (Prime Script RT Master Mix; Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Quantitative RT-PCR was performed using an ABI Prism 7500 (Life Technologies). Reactions were performed in 20 μL of reaction mixture containing 10 μL PCR master mix (SYBR Premix Ex Taq II; Takara Bio Inc.), 0.4 μL primer pairs, and 2 μL cDNA samples. After normalization with the expression of GAPDH, the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ methods. List of mRNA sequences in this study is showed in **Table 1**.

Western blot analysis

Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 15% gels. Separated proteins were then transferred to nitrocellulose membranes using tank transfer for 1.5 h at 200 mA in Tris-glycine buffer containing 20% methanol. The membranes were blocked with 5% skim milk for 18-24 h and incubated overnight at 4°C with diluted primary antibody. The resource and dilution factor of each antibody are showed in **Table 2**. The signal was detected using an enhanced chemiluminescence system (Cheml Scope5300, Clinx Science Instruments, Shanghai, China).

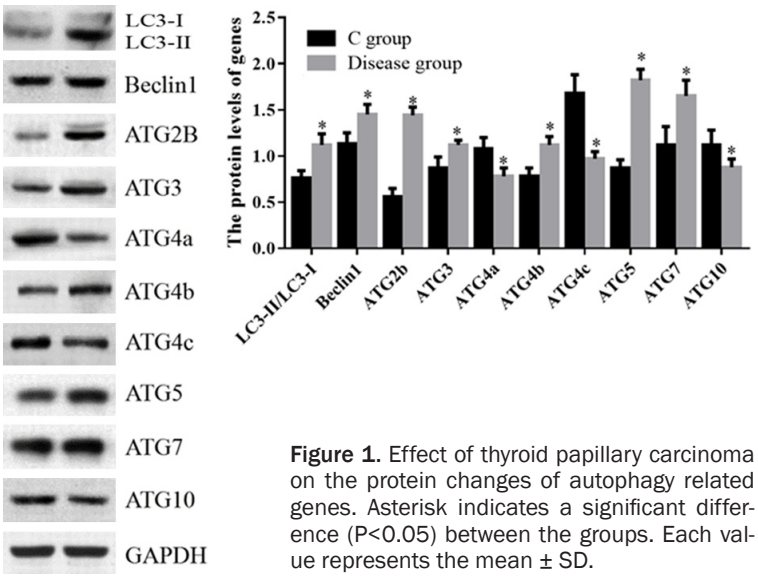

Figure 1. Effect of thyroid papillary carcinoma on the protein changes of autophagy related genes. Asterisk indicates a significant difference (P<0.05) between the groups. Each value represents the mean ± SD.

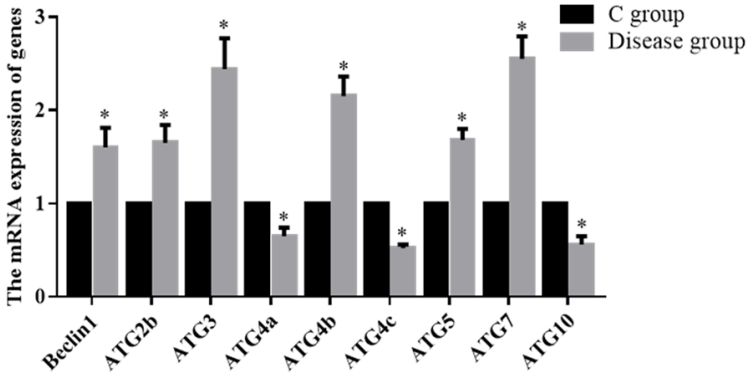

Figure 2. Effect of thyroid papillary carcinoma on the mRNA changes of autophagy related genes. Asterisk indicates a significant difference (P<0.05) between the groups. Each value represents the mean ± SD.

Statistical analysis

All statistical parameters were calculated using GraphPad Prism 7.0 software. Values were expressed as the mean ± SD. Comparisons of two groups were performed using Student's t-tests. P<0.05 was considered statistically significant. The Pearson's correlation coefficient was calculated for correlation analysis using the Statistics 6.0 program (version 19, SPSS Inc., Chicago, IL, USA).

Results

Effect of thyroid papillary carcinoma on the protein changes of autophagy related genes

The effect of thyroid papillary carcinoma on the protein changes of autophagy related genes is showed in **Figure 1**. In the present study, we examined the protein levels of LC3, Beclin1, ATG2b, ATG3, ATG4a, ATG4b, ATG4c, ATG5, ATG7 and ATG10. As the results showed, the protein levels of LC3-II/LC3-I, Beclin1, ATG2b, ATG3, ATG4b, ATG5 and ATG7 increased 147%, 128%, 257%, 128%, 143%, 209% and 147% compared to the C group (P<0.05). The protein level of ATG4a and ATG10 decreased to 72% and 78% compared to the C group (P<0.05).

Effect of thyroid papillary carcinoma on the mRNA changes of autophagy related genes

The effect of thyroid papillary carcinoma on the mRNA changes of autophagy related genes is showed in **Figure 2**. In the present study, we examined the mRNA levels of Beclin1, ATG2b, ATG3, ATG4a, ATG4b, ATG4c, ATG5, ATG7 and ATG10. As the results showed, the protein levels of LC3-II/LC3-I, Beclin1, ATG2b, ATG3, ATG4b, ATG5 and ATG7 increased 160%, 165%, 244%, 215%, 168% and 255% compared to the C group (P<0.05). The mRNA expressions of ATG4a and ATG10 decreased to 52% and 56% compared to the C group (P<0.05).

Correlation analysis

Pearson's correlation coefficient was calculated by correlation analysis. As shown in **Tables 3** and **4**, there was high correlation among each genes.

Discussion

Studies have suggested that autophagy activity in the central region of the tumor significantly enhances [16]. In this study, the protein levels of LC3 and Beclin1 increased in thyroid papillary carcinoma patients' blood, however, this couldn't indicate the whole change of autophagy in tumor. Studies have proved autophagy

Table 3. Correlation matrix

	LC3	Beclin1	ATG2b	ATG3	ATG4a	ATG4b	ATG4c	ATG5	ATG7	ATG10
LC3	1.00	0.99	1.00	0.75	-0.99	0.81	-0.98	0.95	0.88	-0.91
Beclin1	0.99	1.00	0.99	0.84	-1.00	0.88	-1.00	0.90	0.94	-0.96
ATG2b	1.00	0.99	1.00	0.75	-0.99	0.81	-0.98	0.95	0.88	-0.91
ATG3	0.75	0.84	0.75	1.00	-0.83	1.00	-0.88	0.51	0.97	-0.96
ATG4a	-0.99	-1.00	-0.99	-0.83	1.00	-0.87	1.00	-0.91	-0.93	0.95
ATG4b	0.81	0.88	0.81	1.00	-0.87	1.00	-0.92	0.59	0.99	-0.98
ATG4c	-0.98	-1.00	-0.98	-0.88	1.00	-0.92	1.00	-0.86	-0.96	0.98
ATG5	0.95	0.90	0.95	0.51	-0.91	0.59	-0.86	1.00	0.69	-0.74
ATG7	0.88	0.94	0.88	0.97	-0.93	0.99	-0.96	0.69	1.00	-1.00
ATG10	-0.91	-0.96	-0.91	-0.96	0.95	-0.98	0.98	-0.74	-1.00	1.00

Table 4. Rotating component matrix

Component	LC3	Beclin1	ATG2b	ATG3	ATG4a	ATG4b	ATG4c	ATG5	ATG7	ATG10
1	0.93	0.01	0.99	0.77	-0.77	0.58	-0.87	0.96	0.71	-0.76
2	0.34	0.95	0.12	0.54	-0.62	0.79	-0.49	0.14	0.70	-0.65

Note: Rotating convergence after three iterations.

can maintain the energy metabolism, promote the re-use of cell components in the cytoplasm and synthesis of autophagic dependent factors, and promote cell survival [17-19]. Thus, another role of autophagy in the papillary thyroid carcinoma may be the production of tumor cells, providing energy for rapid expansion of tumor cells in the early stages of tumor cancer formation [20].

When the tumor cells adapt to the stimulation in outside world, autophagic activity significantly enhances, inhibition of autophagy can cause tumor cell death [21]. On the other hand, for tumor cells with apoptotic defects, cell autophagy can cause tumor cell death, whether it is to inhibit or induce autophagy was based on the biological behavior of the tumor [22].

In this study, the expression of Beclin1 and LC3 in blood in experimental group was significantly higher than that in normal group, suggesting that the autophagic activity enhanced in the thyroid tumor. The difference in ATG2b, ATG3, ATG4a, ATG4b, ATG4c, ATG5, ATG7 and ATG10 also indicated the occurrence of autophagy in the thyroid papillary carcinoma. Autophagy can promote cell adaptation to changes in the internal environment to survive [23]. After the application of cytotoxic drugs, the performance of autophagic activity increased in part of the tumor cells, even cause autophagic death [24]. Autophagy plays a role in tumorigenesis, progression and lymph node metastasis [25]. Autophagy has a dual role in the inhibition of tumor growth, which in breast cancer, ovarian cancer and prostate cancer has been confirmed, however, in the tumor progression, especially the rapid tumor growth, central tumor ischemia, hypoxia, cell autophagy provides energy for tumor growth and allows tumor cells to survive [26]. In this experiment, the expression of Beclin1 and LC3 in the blood in experimental group was significantly higher than that in normal group, suggesting that autophagy enhancement was associated with thyroid papillary carcinoma.

Evidence is accumulating that autophagy also has a direct role in key aspects of tumor cell motility and invasion including through modulation of the tumor cell secretome, turnover of components inthe cell migration machinery and ECM proteins amongst other roles. An initial study in Drosophila demonstrated that autophagy was required for hemocyte migration during wound healing [27]. Specifically, the production of Rho1-induced cell protrusions and cell spreading of hemocytes, but not cortical actin dynamics, were dependent on both Atg1 (autophagy related gene 1) and the Drosophila homolog of cargo adapter p62/sqstm1 and inactivation of autophagy prevented blood cell migration to larval wound sites

[27]. Similarly, knockdown of Beclin1 prevented cell spreading in mouse macrophages, although the specific targets of autophagic degradation underlying the cell spreading defect in either cell type were not identified. Subsequently, a role for p62/Sqstm1 in targeting active RhoA and the mammalian homolog of Drosophila Rho1, to the autophagosome for degradation was shown [28]. Aberrant accumulation of RhoA at the cell mid-body, when autophagy was inhibited through ATG5 knockdown, resulted in cytokinesis defects, multinucleation and aneuploidy demonstrating novel consequences of autophagy deficiency in cancer cells [29].

In summary, autophagy happens in the progression of papillary thyroid carcinoma. Autophagy inhibitor can become a new therapeutic target, especially in preventing the recurrence of postoperative cervical lymph node recurrence in papillary thyroid carcinoma.

Disclosure of conflict of interest

None.

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