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Expressions of Epidermal Growth Factor Receptors Atg5 and p62 in Papillary Thyroid Carcinoma Patients Complicated with Cervical Lymph Node Metastasis

Hua Pan[1,2], Lei Wang[1], Shengyong Liu[1], Junehua Zha[2], Yong Jiang[1*] and Hongwei Peng[2*]

[1]The Third Affiliated Hospital of Soochow University, Changzhou First People's Hospital, Changzhou 213003, Jiangsu Province, China
[2]Liyang People's Hospital, Liyang 213300, Jiangsu Province, China

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ABSTRACT The aim of this study was to analyse the expressions of epidermal growth factor receptors (EGFR) Atg5 and p62 in thyroid carcinoma patients complicated with cervical lymph node metastasis. Cancer and normal paracancerous tissue specimens were collected from 80 patients diagnosed as papillary thyroid carcinoma (PTC) from March 2013 to February 2015. Compared with normal tissue, the expression rates of EGFR and p62 in PTC and metastatic lymph node tissues were significantly higher, but those of Atg5 were lower ($P<0.05$). Atg5 and p62 expressions were negatively correlated in PTC and metastatic lymph node tissues ($P<0.01$). The 3-year survival rate of patients with high EGFR and p62 expressions in PTC tissues was lower than that of patients with low expressions, but higher in patients with high Atg5 expression ($P<0.05$). TNM stage, lymph node metastasis, EGFR and p62 were independent risk factors for PTC prognosis, while Atg5 was a protective factor.

INTRODUCTION

Thyroid carcinomas are the most common type of head and neck tumour in human body, accounting for about one percent of all malignant tumours, with the incidence rate rising annually at approximately four percent worldwide (Du et al. 2018). Among them, papillary thyroid carcinoma (PTC) has the highest incidence rate, that is, eighty-five percent, but its pathogenesis has not been completely clarified yet (To et al. 2018). Regardless of great progress on the methods of diagnosis and treatment for this disease in recent years, the prognosis of PTC patients is still poor due to its proneness to lymph node metastasis (Sun et al. 2015; Mao et al. 2020).

Autophagy is an active process during which cells adapt to the changes of internal and external environments, and a cyclic process in which some cytoplasm, proteins and degenerated and necrotic organelles fuse with lysosomes in autophagic vacuoles to form autolysosomes and to generate amino acids and nucleotides through degradation for cell reutilisation (Hu et al. 2020). Therefore, autophagy is also known as type II programmed cell death, as a normal cell function that is highly conserved during evolution (Vegliante et al. 2016; Li et al. 2020). Autophagy plays various roles in the growth, development, maturation and death of tumour cells (Nyfeler and Eng 2016; Mowers et al. 2018). Epidermal growth factor receptor (EGFR) is closely associated with the generation and development of tumour cells and abnormally highly expressed in multiple solid tumours. Besides, EGFR is involved in cell autophagy by regulating several signal transduction pathways (Kim et al. 2018). Currently, whether autophagy interacts with EGFR to jointly promote the progression of PTC complicated with cervical lymph node metastasis remains unclear.

Objectives

Therefore, the aim of this study was to analyse the expressions of EGFR and autophagy-related proteins (Atg5 and p62) in PTC tissues and metastatic cervical lymph nodes, and to explore their clinical significance.

MATERIAL AND METHODS

Baseline Clinical Data

This study has been approved by the ethics committee of the hospital, and written informed

Address for correspondence:
Yong Jiang
E-mail: ean48935@163.com
Hongwei Peng
E-mail: penghwlph@foxmail.com

consent has been obtained from all patients. Carcinoma and normal paracancerous tissue specimens were collected from 80 patients who were admitted to the hospital from March 2013 to February 2015 and pathologically diagnosed as PTC. Meanwhile, 45 cases of tissue specimens were obtained from PTC patients complicated with cervical lymph node metastasis. All patients enrolled received no chemotherapy, radiotherapy or other treatments before operation and had complete 3-year follow-up data. There were 28 males and 52 females aged 40-79 years old, with an average of (48.32 ± 12.23) years old. According to the 2009 UICC tumour-node-metastasis (TNM) staging criteria, 39 patients were in stage I + II, and 41 patients were in stage III + IV. The tumour diameter was less than 1 cm in 35 patients and more than or equal to 1 cm in 45 patients. As for the histological grade, 26 patients had well-differentiated PTC, 32 patients had moderately differentiated PTC, and 22 patients had poorly differentiated PTC. All the tissue specimens were fixed in ten percent neutral formalin and then routinely embedded in paraffin for subsequent immunohistochemistry. PTC patients were followed up through telephone interviews and outpatient visits, and the overall survival was the duration from the start of operation to the end of follow-up or the death of patients.

Main Reagents and Apparatus

Rabbit anti-human polyclonal antibody against EGFR, rabbit anti-human monoclonal antibody against Atg5 and anti-p62 monoclonal antibody were purchased from Beijing Aviva Systems Biology Co. Ltd. (China). Streptavidin-peroxidase kit and diaminobenzidine (DAB) coloration kit were provided by Abcam (USA) and Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (China), respectively.

Paraffin microtome was purchased from Leica (Germany). Incubator was bought from Shanghai Chengshun Instrument Co. Ltd. (China). Optical microscope was obtained from Olympus (Japan). ScanScope CS system was provided by Aperio (USA).

Immunohistochemical Assay

The paraffin-embedded tissue specimens were sliced into sections, put into forty percent ethanol solution and spread in a water bath at 40°C for 4 minutes, followed by baking in an oven at 60°C. Then the sections were deparaffinised in xylene for 20 minutes, and xylene was removed by difference concentrations (70%, 90% and 100%) of ethanol solutions, 2 minutes for each concentration. Subsequently, the sections were reacted with three percent hydrogen peroxide for 10 minutes at room temperature to block the activity of endogenous peroxidase. Afterwards, tissue repair was performed for EGFR, Atg5 and p62 proteins at high temperature and pressure, and the sections were rinsed using PBS and incubated with five percent goat serum for 30 minutes. After removal of blocking buffer, the sections were added with primary antibodies and placed in a refrigerator at 4°C overnight, with PBS as the negative control. On the next day, the sections were taken out, washed with PBS and incubated with secondary antibodies at room temperature for 30 minutes, followed by rinsing with PBS 5 times (3 minutes each time). Finally, colour development was terminated by adding DAB and washing with PBS, and the sections were rinsed in water, differentiated by hydrochloric acid-ethanol solution, dehydrated with ethanol solutions, transparentised with xylene, mounted with neutral resin and observed under a microscope (Awi et al. 2021).

Determination Criteria of EGFR-, Atg 5- and p62-Positive Cells

The prepared sections were observed under a light microscope, and yellowish-brown granules were visible in the cells with positive expression. Subsequently, the sections were evaluated independently by two pathologists using the double-blind method according to a previous literature (Sun et al. 2021). Specifically, 5 high-power fields (×400) were randomly selected from each section and observed under the light microscope. Positive expression was determined by the total score of staining intensity and number of positive cells. The staining intensity is scored as 0 point (negative), 1 point (weakly positive) and 2 points (positive). The scores of number of positive cells include 0 point (no positive cells), 1 point (5-25% of positive cells), 2 points (25-50% of positive cells), 3 points (50-75% of positive cells) and 4 points (>75% of

positive cells). The product of the two scores more than 3 indicates positive expression, while that of less than or equal to 3 suggests negative expression.

All data were statistically analysed by SPSS 16.0 software. The categorical data were expressed as percentage and subjected to the $\chi 2$ test. The correlations between rank variables were subjected to the Spearman's analysis. Survival was tested by the Kaplan-Meier method. The Cox's proportional hazards regression model was used to assess the prognosis. Two-tailed $P<0.05$ was considered statistically significant.

RESULTS

EGFR, Atg5 and p62 Expressions in Different Tissues

The expression rates of EGFR and p62 were 81.25 percent and 72.50 percent in PTC tissues, and 75.56 percent and 75.56 percent in metastatic lymph node tissues, respectively, which were significantly higher than those in normal paracancerous tissues ($P<0.05$). The expression rates of Atg5 in PTC tissues (21.25 percent) and metastatic lymph node tissues (33.33%) declined significantly compared with that in normal paracancerous tissues ($P<0.05$) (Table 1).

Associations of EGFR, Atg5 and p62 Expressions with PTC Clinical and Pathological Parameters

The positive expression rate of EGFR had no significant correlations with the clinicopathological parameters of PTC patients ($P>0.05$), and the expressions of Atg5 and p62 were of no significant differences in patients with different genders, ages and tumour diameters ($P>0.05$). Atg5 expression decreased with rising degree of tumour differentiation ($P<0.05$). TNM stage III + IV group had significantly lower Atg5 expression (19.51%) than that of I + II group (48.72%) ($P<0.05$). The expression of Atg5 declined significantly in lymph node metastasis group (22.22%) in comparison with that in non-lymph node metastasis group (48.57%) ($P<0.05$). However, the expression of p62 showed the opposite trends (Table 2).

Correlations between EGFR, Atg5 and p62 Expressions

Spearman's correlation analysis showed that Atg5 and p62 expressions were significantly negatively correlated in PTC tissues ($r=-0.656$, $P<0.01$), while EGFR had no significant correlation with Atg5 or p62 ($r=0.042$, $r=0.116$, $P>0.05$). There was a significantly negative correlation between Atg5 and p62 expressions in metastatic lymph node tissues ($r=-0.562$, $P<0.01$), whereas EGFR was not significantly correlated with Atg5 or p62 ($r=0.071$, $r=0.136$, $P>0.05$).

Correlations of EGFR, Atg5 and p62 Expressions in PTC Tissue with Overall Survival

Among the 80 patients followed up for 3 years, 22 patients died, with a 3-year survival rate of 72.50 percent (58 out of 80). The 3-year survival rates of patients with high expressions of EGFR and p62 in PTC tissues (67.69% and 65.52%) were lower than those of patients with low EGFR and p62 expressions (93.33% and 90.91%), but it was higher in patients with high

Table 1: EGFR, Atg5 and p62 expressions in different tissues [n (%)]

	EGFR		*Atg5*		*p62*	
	-	+	-	+	-	+
PTC tissue (n=80)	15 (18.75)	65 (81.25)	63 (78.75)	17 (21.25)	22 (27.50)	58 (72.50)
Paracancerous tissue (n=80)	70 (87.5)	10 (12.50)	12 (15.00)	68 (85.00)	62 (77.50)	18 (22.50)
Metastatic lymph node tissue (n=45)	11 (24.44)	34 (75.56)	30 (66.67)	15 (33.33)	11 (24.44)	34 (75.56)
$\div^2$	87.533	70.570	51.333			
P	<0.001	<0.001	<0.001			

EGFR: Epidermal growth factor receptor; PTC: papillary thyroid carcinoma.

Table 2: Associations of EGFR, Atg5 and p62 expressions with PTC clinical and pathological parameters

Characteristic	*n*	*EGFR*		χ^2	*P*	*Atg5*		χ^2	*P*	*p62*		χ^2	*P*
		-	+			-	+			-	+		
Gender				1.931	0.165			0.014	0.906			0.027	0.87
Male	28	13(46.43)	15(53.57)			12(42.86)	16(57.14			14(50.00)	14(50.00)		
Female	52	25(48.08)	27(51.92)			23(44.23	29(55.77)			27(51.92)	25(48.08)		
Age (year)				0.33	0.566			0.139	0.709			0.313	0.576
<45	32	10(31.25)	22(68.75)			20(62.50)	12(37.50)			14(43.75)	18(56.25)		
≥45	48	18(37.50)	30(62.50)			28(58.33)	20(41.67)			22(45.83)	26(54.17)		
Tumor Diameter				0.001	0.978			0.098	0.755			0.02	0.887
<1 cm	35	18(51.43)	17(48.57)			19(54.29)	16(45.71)			15(42.86)	20(57.14)		
≥1 cm	45	23(51.11)	22(48.89)			25(55.56)	20(44.44)			19(42.22)	26(57.78)		
Differentiation Degree				0.136	0.934			6.742	0.034			9.7995	0.007
High	26	12(46.15)	14(53.85)			20(76.92)	6(23.08)			5(19.23)	21(80.77)		
Moderate	32	16(50.00)	16(50.00)			17(53.13)	15(46.88)			13(40.63)	19(59.38)		
Low	22	10(45.45)	12(54.55)			9(40.91)	13(59.09)			14(63.64)	8(36.36)		
TNM Stage				0.328	0.567			7.625	0.006			5.598	0.018
I+ II	39	11(28.21)	28(71.79)			20(51.28)	19(48.72)			13(33.33)	23(58.97)		
III + IV	41	14(34.15)	27(65.85)			33(80.49)	8(19.51)			7(17.07)	34(82.93)		
Lymph Node Metastasis				0.013	0.91			6.113	0.013			61.863	<0.001
Yes	45	20(44.44)	25(55.56)			35(77.78)	10(22.22)			8(17.78)	37(82.22)		
No	35	16(45.71)	19(54.29)			18(51.43)	17(48.57)			15(42.86)	20(57.14)		

EGFR: Epidermal growth factor receptor; PTC: papillary thyroid carcinoma; TNM: tumor-node-metastasis.

Atg5 expression (94.12%) than that of patients with low expression (66.67%) (P<0.05) (Table 3).

Table 3: Correlations of EGFR, Atg5 and p62 expressions in PTC tissue with overall survival

Index	*n*	*3-Year survival rate/[n (%)]*
EGFR		
+	65	44(67.69)
-	15	14(93.33)
$\div^2$		5.616
P		0.018
Atg5		
+	17	16(94.12)
-	63	42(66.67)
$\div^2$		5.060
P		0.024
p62		
+	58	38(65.52)
-	22	20(90.91)
$\div^2$		5.158
P		0.023

EGFR: Epidermal growth factor receptor; PTC: papillary thyroid carcinoma.

Factors Affecting Prognosis of PTC Patients

The analysis results of Cox's proportional hazards regression model indicated that TNM stage, lymph node metastasis, EGFR and p62 were independent risk factors for PTC prognosis (P<0.05), while Atg5 was a protective factor (P<0.001) (Table 4).

DISCUSSION

PTC, the most common type of thyroid malignancies, is more common in young and middle-aged women and prone to metastasising to lymph nodes in the early stage, severely threatening the life of patients (Cordioli et al. 2017). The onset and progression of PTC involve many oncogenes and tumour suppressor genes, so studying the expressions of relevant proteins is beneficial to further clarifying the pathogenesis of tumour, enhancing the accuracy of molecular diagnosis and improving the prognosis of patients. There are 3 types of cell death, that is, type I (apoptosis), type II (autophagy) and type III (necrosis) (Klöditz and Fadeel 2019). Autophagy plays a key role in suppressing inflammation or tumour growth (Yang et al. 2017). However, the damage to autophagic activity to facilitate the progression of cytopathy and tumour has been proven (Jiang et al. 2018).

Atg family proteins have abnormal expressions in tumour cells (Cheung et al. 2020). Atg5 first binds Atg12 to form an Atg5-Atg12 ubiquitin-like connection system. Then the system is connected to the outer membrane, and the autophagosomal membrane is extended. Next, autophagosomes are formed. Phagosomes cannot be fused with lysosomes due to the lack of Atg5 in macrophages (Ye et al. 2018). Atg5 has been reported to participate in the onset and progression of various tumours, with reduced protein expressions in gastric, colon and ovarian cancer tissues due to gene mutations (Görgülü et al. 2019). In this study, the expression rate of Atg5 was lower in PTC and metastatic lymph node tissues than that in normal paracancerous tissues, suggesting that Atg5 may participate in the onset and progression of PTC. The degree of differentiation, TNM stage and lymph node metastasis are crucial indices for evaluating the malignancy of PTC. In terms of the above clinicopathological characteristics, Atg5 showed significantly different low expressions, implying that low Atg expression was related to the biological behaviours of PTC. The 3-year survival rate was significantly lower in patients with low Atg5 expression, indicating

Table 4: Factors affecting prognosis of PTC patients

	β	*S.E.*	*Wald*	*OR*	*95%CI*	*P*
TNM stage	0.432	0.186	6.584	1.548	1.056~4.356	0.032
Lymph node metastasis	0.325	0.128	4.862	4.155	1.025~8.862	0.015
EGFR	1.354	0.642	9.548	5.648	1.893~12.624	0.008
Atg5	-0.871	0.368	10.265	2.436	1.364~9.545	<0.001
p62	0.657	0.254	10.574	3.542	1.632~6.752	0.012

CI: Confidential interval; EGFR: epidermal growth factor receptor; OR: odds ratio; PTC: papillary thyroid carcinoma; S.E.: standard error; TNM: tumor-node-metastasis

that such expression level reflected the prognosis of PTC patients to some extent. Additionally, the analysis results of the Cox's proportional hazards regression model revealed that low Atg5 expression was associated with the prognosis of PTC patients.

As an autophagy substrate protein, p62 has multiple domains and is capable of binding ubiquitin-modified proteins and transporting them to autophagic vacuoles for degradation (Nawas et al. 2016). In the cytoplasm, p62 is continuously degraded during the autophagy of normal cells, and the inactivation of autophagy leads to p62 protein accumulation in the cytoplasm. Thus, p62 is a marker protein for cell autophagic activity (Moscat et al. 2016). In addition, the accumulation of p62 affects the expressions of NF-êB signalling pathway and downstream genes, thus exacerbating pro-tumour inflammation (Duran et al. 2016). One of the mechanisms of autophagy in impeding tumour formation is lowering the level of p62 in cells (Ye et al. 2016). P62 is able to separate Beclin1 (the first tumour suppressor gene regulating autophagy found in mammals) and Bcl-2, releasing Beclin1 and thus promoting autophagic activity (Li et al. 2018). In this study, the expression rate of p62 in PTC and metastatic lymph node tissues was higher than that in normal paracancerous tissues and normal lymph node tissues, suggesting that p62 may be involved in the development and progression of PTC. Besides, the p62 expression rate in PTC tissues was elevated with increasing TNM stage and degree of differentiation, and also raised in patients with lymph node metastasis, implying that high expression of p62 can indirectly reflect the malignant biological behaviours of PTC. Moreover, p62 was negatively correlated with Atg5 in PTC and metastatic lymph node tissues, which may be attributed to the mutual expression of them influenced by changes in the internal environment of tumour tissues. Finally, the 3-year survival rate of patients with high p62 expression was significantly lower than that of patients with low p62 expression, and high p62 expression was an independent risk factor for prognosis.

EGFR tyrosine kinase is capable of phosphorylating Beclin1 to inhibit autophagic activity, and facilitating cell survival through its kinase-independent activity (Qin et al. 2018). EGFR is highly expressed in many solid tumours (Ndoye et al. 2017). Likewise, in this study, PTC and metastatic lymph node tissues had higher expression rates of EGFR than that of normal tissues, indicating that EGFR was closely associated with the onset and progression of PTC. Besides, EGFR had no correlations with the clinical pathological parameters of PTC patients. Moreover, EGFR was not associated with Atg proteins Atg5 and p62, possibly because the regulation of autophagy was correlated with the level of EGFR phosphorylation, and the total EGFR protein level cannot reflect the degree of phosphorylation and had no significant relationship with autophagy. Furthermore, patients with high EGFR expression had a lower 3-year survival rate, and EGFR was an independent predictor for the prognosis of PTC patients.

CONCLUSION

In summary, EGFR and p62 have high expressions in PTC and metastatic lymph node tissues, whereas Atg5 has low expression. In addition, Atg5 and p62 have correlations with the biological behaviours of PTC.

RECOMMENDATIONS

EGFR, p62 and Atg5 can be used as independent predictors for the prognosis of PTC patients. Atg5 and p62 are potential indices for diagnosing PTC.

ABBREVIATIONS

DAB: diaminobenzidine
EGFR: epidermal growth factor receptor
PTC: papillary thyroid carcinoma
TNM: tumour-node-metastasis

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DECLARATION OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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