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The correlation between the expression of ATF4 and PCT combined with the detection of *RET* mutation and the pathological stage and clinical prognosis of medullary thyroid carcinoma

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ATF4 and PCT combined with the detection of *RET* mutation and the pathological stage

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Abstract

Objective: To explore the correlation between the ATF4 and PCT expressions combined with *RET* mutation and the pathological staging and clinical prognosis of Sporadic MTC (SMTC).

Method: 50 cases (tumor tissue) of SMTC diagnosed by clinicopathology were collected and the patients with nodular goiter were selected as Normal control. The *RET* mutation site was analyzed by detection kit and expressions of PCT and ATF4 in SMTC were analyzed by western blot and IHC. Multiple linear regression was used to analyze the correlation of risk factors (PCT or ATF4 expression, *RET* mutation, tumor differentiation, SMTC stage, lymphatic metastasis) for 5-year recurrence and survival of SMTC.

Results: The ATF4 and PCT expressions were significantly decreased and increased, respectively, with the increase of the SMTC stage. The most frequent mutation of *RET* gene in cancer tissue was M 22458A in exon 16. The ATF4 and PCT expressions as well as *RET* mutation were significantly associated with a 5-year recurrence, while the ATF4 expression was significantly related to better 5-year survival.

Conclusion: ATF4 and PCT expressions combined with *RET* mutation are related to the clinical prognosis of SMTC and can predict SMTC staging.

Keywords: Medullary thyroid carcinoma, ATF4, PCT, *RET* mutation, recurrence, survival

Introduction

Medullary thyroid carcinoma is a common clinical malignant tumor in the endocrine system, which originates from parafollicular cells of the thyroid (also known as C cell) (Daniels 2018). The clinical manifestations of MTC are complex and diverse and it can be divided into Hereditary MTC (HMTc) and SMTC according to genetic characteristics. Among them, SMTC is the most common type, accounting for 70%~80% of total MTC patients (Wells and Franz 2000). Due to the poor prognosis and high degree of malignancy for MTC, early detection and diagnosis are critical for MTC patients. At present, the common methods for diagnosing MTC are fine needle

aspiration cytology and expression analysis by using specific molecular markers, but how to diagnose and treat MTC early is a hot topic (Viola and Elisei 2019). For the treatment of MTC, surgical resection is the only effective treatment. However, this method is only suitable for MTC patients with early-stage, but for MTC patients with postoperative recurrence, metastasis, and advanced stage, it is difficult to eliminate tumor cells only by surgical resection. Therefore, it is extremely important to seek new specific molecular markers or improve the detection methods of existing markers for the early diagnosis, treatment, and prognosis evaluation of TC, which can show a better survival time.

At present, it has been confirmed that the main cause of MTC is the *RET* mutation. *RET* mutation changes the protein conformation of the inner and outer regions of C cells, stimulates autophosphorylation of tyrosine kinases, and induces the excessive proliferation of C cells to cause cancer (Ibáñez 2013). About 95% of HMTc and 70% of SMTC in MTC patients are caused by *RET* germline mutations, and a very small number of HMTc and about 30%~66% of SMTC are related to *RET* somatic mutations (Mohammadi and Hedayati 2017). In SMTC patients, about 50% lesions have *RET* mutations, and most of the mutation points are clustered in exon 10 (608, 611, 618), exon 11 (629, 630, 634, 649), and exon 16 (918, 922), among which M918T mutation is the most common (Marur and Forastiere 2008). In HMTc patients, the mutation sites are mainly distributed in exons 10 and 11, and a small number of codons 804 in exon 14 and codon 883 in exon 15 have mutations (Marur and Forastiere 2008). Different codon mutations affect the activity of RET protein, which in turn determines the malignant degree of MTC. According to the previous reports, MTC patients with mutations in codon M918T are the most common and are distributed in 25-33% of SMTC cases (Mohammadi and Hedayati 2017). Therefore, analysis in the *RET* mutation site has become a breakthrough to realize the early diagnosis and targeted therapy of MTC, which can classify the risk of MTC and take corresponding treatment measures (Mohammadi and Hedayati 2017).

Activating transcription factor 4 (ATF4) is a member of the activated transcription

factor family and plays an important role in regulating cell stress response (Wortel et al. 2017). Recently, many studies have showed that ATF4 expression was significantly up-regulated in primary tumors. For example, ATF4 expression was noticeably increased in colon cancer, liver cancer, breast cancer, and other malignant tumor tissues and was involved in the related processes of regulating tumor occurrence and development (Sharma et al. 2018; Voloshanenko et al. 2018; Zong et al. 2017), suggesting that the up-regulation of ATF4 expression may play a key role in the occurrence and differentiation of many cancers and may become a molecular target for targeted therapies of many cancers and an important indicator of prognosis. In MTC, studies have found that proteasome inhibition leads to the accumulation of ATF4 in cancer cells, and ATF4 induces CHOP expression by binding to the AARE1 element of the CCAAT/CHOP promoter, thereby participating in the cell apoptosis process (Zong et al. 2012). Further studies have shown that the expression of ATF4 is closely related to the survival rate of SMTC patients and that ATF4 can interact with *RET* to affect the apoptosis of SMTC cells (Bagheri-Yarmand et al. 2015; Williams et al. 2021). However, there are few related studies about the relationship between ATF4 and SMTC, more related works need to be carried out.

Procalcitonin (PCT) is a precursor of Calcitonin (CT), a peptide composed of 116 amino acids, but its function is still unclear (Aloisio et al. 2019). At present, serum PCT is a clinically used indicator for the detection of acute severe infections. In recent years, some scholars have suggested using PCT as one of the routine testing items (blood) of MTC. Trimboli et al. reported that PCT was a reliable marker for identifying MTC patients, and its diagnostic sensitivity and specificity in MTC patients were 91% and 96%, respectively (Trimboli et al. 2018). At the same time, studies have confirmed that there is a significant correlation between CT and PCT, and PCT detection can replace CT in the evaluation of MTC (Woliński et al. 2017). Besides, in some tumors with negative CT or low-level CT secretion, the use of detection of PCT combined with CT is a useful strategy for diagnosis and follow-up of patients; PCT seems to be a very promising and reliable serum marker for the diagnosis of MTC, but so far there is not

enough data to propose a specific threshold for normal serum PCT (Karagiannis et al. 2016; Trimboli et al. 2015). Therefore, there are still controversies regarding the level change and possible diagnostic value of PCT in MTC, and the PCT expression in MTC organizations needs further study.

In this study, SMTC patients diagnosed pathologically after surgery were collected and patients with nodular goiter were selected as controls. Direct sequencing of PCR products (two-way sequencing) was used to detect mutations in *RET*, and immunohistochemistry and western blot were used to detect the expression of PCT and ATF4 proteins. Then we analyzed the relationship between *RET* mutation in SMTC tissue and the expression of PCT and ATF4 proteins, and clinicopathological parameters, and preliminarily explored the relationship between the expression of each protein, to provide a theoretical basis for early diagnosis, treatment, and prognosis evaluation of SMTC.

Materials and methods

Patients and samples

A total of 50 patients (including 25 males and 25 females, aged from 30 to 60, with a median age of 49.4 ± 8.8 years, no family history of thyroid cancer, no other malignant tumors, and no chemoradiotherapy)(stage 1:stage 2:stage 3:stage 4 = 24:14:8:4) with SMTC diagnosed clinically and pathologically in Shanghai Xuhui District Central Hospital were collected, and 50 patients (including 25 males and 25 females, aged from 30 to 60, with a median age of 46.5 ± 7.1 years) with nodular goiter during the same period were selected as the normal control (NC) group. This study was approved by the ethics committee of Shanghai Xuhui District Central Hospital. All clinical data and tissue specimens were collected with the informed consent of patients and their families and signed informed consent.

Immunohistochemistry assay

The medullary thyroid carcinoma tissue was prepared into paraffin sections with a thickness of about 4 μm . After dewaxing, hydration, and blocking by endogenous enzymes, they were blocked with goat serum for 2 h and incubated at 37 °C for 30 min.

After washing with PBS, the primary antibody was added and incubated overnight at 4 °C and then washed with PBS 3 times. Next, appropriate biotin-labeled second antibodies including Rabbit anti-human ATF4 polyclonal antibody (1: 100, GTX101943, Gene Tex, CA, USA) and Mouse anti-human PCT monoclonal antibody (1: 100, NB120-14817, Novus, CA, USA) were added respectively and incubated at 37 °C for 60 min. After washing with PBS, then DAB staining solution (Mindray, Shenzhen, China) was added and dyed with hematoxylin for 2 min. After dehydration with gradient alcohol and natural drying, it was sealed with neutral glue, and the protein expression of each tissue was observed under a microscope and was calculated by dividing the integrated optical density by the tissue area.

Western blotting assay

Proteins were extracted from 0.2 g of medullary thyroid carcinoma tissue and separated by SDS-PAGE, and then transferred to a PVDF membrane (Thermo Fisher, Waltham, MA, USA). After the membrane was washed, it was placed in the anti-ATF4 (1: 500, GTX101943, Gene Tex, CA, USA) and anti-PCT (1: 500, NB120-14817, Novus, CA, USA) and anti- β -actin (ab179467, Abcam, 1:500) primary antibody at 4 °C overnight, and then the membrane was rinsed and added to the Goat Anti-Rabbit IgG secondary antibody and incubated at room temperature for 2 h. Then it was put into the mixed color development solution to develop color, and finally, the reaction was stopped and used Image analyzer quantitative system (Tanon 5200, Shanghai, China) for density analysis, and β -actin was used as an internal reference.

DNA extraction

The genomic DNA of medullary thyroid carcinoma tissue in all cases was extracted with E.Z.N.A.TM FFPE DNA Kit (Omega, GA, USA) according to the description of kit instructions.

PCR amplification and DNA sequencing

According to the full sequence of *RET* gene published by NCBI Gene Bank, the corresponding primers were designed using Primer 5.0 and Oligo 6 software to determine the target fragment to be amplified (*RET* ex8 (502 bp): sequence 5'-3' for

Forward TGCTGCCCTGGGTCTGTC and Reverse ACCTTCCCAAGTCCAGAGTGAATC, annealing temperature for 58 °C; *RET* ex10 (389 bp): sequence 5'-3' for Forward CATGGCTTCAGAAAGGCACTG and Reverse CCTTGTGTTGGGACCTCAGATGTG, annealing temperature for 58 °C; *RET* ex11 (430 bp): sequence 5'-3' for Forward CATGCTCGATGGGGTGTTC and Reverse GACCCTCACCAGGATCTTGAA, annealing temperature for 60 °C; *RET* ex13 (336 bp): sequence 5'-3' for Forward CTGCTCTGTGCTGCATTTCA and Reverse GCCCCTCTGATGGAAAGTGAC, annealing temperature for 58 °C; *RET* ex14 (442 bp): sequence 5'-3' for Forward GGAGGCAGAGAGCAAGTGGTT and Reverse CCATATGCACGCACCTTCATCT, annealing temperature for 58 °C; *RET* ex15 (333 bp): sequence 5'-3' for Forward CGACTCGTGCTATTTTTCCTC and Reverse AGGCTGAGCGGAGTTCTAAT, annealing temperature for 60 °C; *RET* ex16 (426 bp): sequence 5'-3' for Forward CTCCAGCCCCCTTCAAAGATGT and Reverse CCATTTGCCTCACGAACACA, annealing temperature for 60 °C). The *RET* mutation site: ex8 (codons 532, 533), ex10 (codons 600, 603, 606, 609, 611, 618 and 620), ex11 (codons 630, 631, 634, 649 and 691), ex13 (codons 768, 769, 777, 778, 781, 790 and 791), ex14 (codons 804, 836, 844, 848 and 852), ex15 (codons 883, 891 and 904) and ex16 (codons 912, 918, 922 and 930). PCR reaction system (50 µL): 10 µL of 5x Prime STAR Buffer, 4 µL of d NTP, 2 µL of Forward Primer, 2 µL of Reverse Primer, 2 µL of Taq DNA polymerase, 3 µL of DNA template, 27 µL of sterile water. PCR conditions consisted of 30 s at 95 °C, 30 s at 58 or 60 °C and 30 s at 72 °C.

The amplified products were obtained to agarose gel electrophoresis and then DNA sequencing (two-way sequencing, each sample mutation detection was sequenced twice with forward and reverse primers). Chromas software was used to compare with the normal sequence of *RET* gene to determine whether there was a mutation.

Statistical analysis

At least three independent measurements were performed and expressed as the mean $\pm$ SD. All data were analyzed using SPSS 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0, followed by the Student's t-test and One-way ANOVA test, the

significant difference was regarded as the p-value less than 0.05, multiple linear regression analysis was performed by SPSS 20.0 software.

Results

ATF4 and PCT expressions in SMTC tissues

As can be seen from the immunohistochemistry assay (Figure 1), ATF4 was located in the nucleus, and its expression order was as follows: NC > SMTC-1 phase > SMTC-2 phase > SMTC-3 phase > SMTC-4 phase. The PCT was located outside the cell nucleus, and its expression order was as follows: SMTC-4 phase > SMTC-3 phase > SMTC-2 phase > SMTC-1 phase > NC. There were significant differences in the expression of partial staging groups. Additionally, western blot assay (Figure 2) showed that the expression of these two proteins was consistent with their corresponding immunohistochemistry expressions. These results indicate that the PCT and ATF4 expressions in SMTC tissue samples in different stages were significantly different.

RET gene mutation analysis of SMTC patients

The *RET* mutation analysis showed that the *RET* mutation rate in SMTC patients was 42% (21/50), among which the mutation rate of M 22458A (Figure 3) in exon 16 was the highest (18%, 9/50), and no *RET* mutation was detected in normal nodular goiter tissue. The prognosis of the SMTC patients was poor. Among them, 12 cases was relapsed and 24 cases was distant metastases within 5 years after surgery.

Multiple linear regression analysis of 5-year recurrence and survival of SMTC patients

As could be seen in Table 1 and Table 2, 5-year recurrence of SMTC patients had a significant correlation with PTC and ATF4 expressions and *RET* mutation (stage 1:stage 2:stage 3:stage 4 = 8:8:4:1) ($P < 0.05$), but there was no significant correlation with tumor differentiation, SMTC stage, and lymphatic metastasis (stage 1:stage 2:stage 3:stage 4 = 9:11:3:1) ($P > 0.05$). Further analysis showed that PTC expression and *RET* mutation (42%) were risk factors for 5-year recurrence of SMTC patients (OR, odd ratio > 1), while ATF4 expression was a protective factor for 5-year recurrence of SMTC patients (OR, odd ratio < 1).

As could be seen in Table 1, Table 3 and Figure 4, better 5-year survival of SMTC patients had a significant correlation with ATF4 expression ($P < 0.05$), but there was no significant correlation with PTC expression, *RET* mutation, tumor differentiation, SMTC stage, lymphatic metastasis ($P > 0.05$). Further analysis showed that ATF4 expression was a protective factor for a 5-year recurrence of SMTC survival ($OR < 1$).

Discussion

RET mutation is the main cause of MTC. The literature has been reported that about 95%~98% of HMTC and 40%~50% of SMTC patients could detect *RET* germline mutation (Romei et al. 2018). Most of the current studies are based on the application value of *RET* mutations to HMTC, and HMTC patients undergo genetic mutation analysis to determine hereditary cases. At the same time, detecting *RET* mutations is also convenient for monitoring other related endocrine abnormalities. In addition, screening tests before the occurrence of HMTC can also help preventive thyroidectomy (Accardo et al. 2017). Among MTC, 70% of SMTC are caused by *RET* germline mutations, and about 30%~66% of SMTC patients are related to *RET* somatic mutations (Mohammadi and Hedayati 2017) and tumor aggressiveness and prognosis (Vuong et al. 2018). The mutation site and type of *RET* are closely related to the disease phenotype, and mutations in different codons result in different penetrance of MTC (Coca-Pelaz et al. 2018). Codon 634 and 918 are the most common mutations in MTC, and they are more aggressive after mutation and are associated with high metastasis rate, high recurrence rate and mortality (Accardo et al. 2017; Romei et al. 2012). The M918T mutation is the most significant in SMTC, but the mutation frequency is different in different populations (Mohammadi and Hedayati 2017). Our current research showed that the *RET* mutation rate in SMTC patients was 42% and the mutation rate of M22458A in exon 16 was the highest (18%), which further confirms that differences in population regions may lead to differences in the mutation sites of *RET*. However, it should be noted that the current conclusions on the evaluation of the prognosis of SMTC by *RET* mutations have not yet reached a unified view. Moreover, detection of *RET* mutation is one of the ways to diagnose MTC, but it is difficult to promote

detection of *RET* mutation in clinical practice due to the low positive rate of pathological screening and expensive factors. Although thyroid cell puncture can confirm the diagnosis, the examination method is invasive and the positive rate is low, which limits its clinical use. Therefore, screening combined with other indicators exhibits great significance for judging the recurrence of MTC and the effect of surgery, and can be used as an auxiliary diagnostic method of MTC, and an important indicator for disease monitoring and observation of efficacy (Papapetrou and Polymeris 2012).

Studies have reported that in the evaluation of PCT as a negative predictive value (NPV) study of MTC markers, serum PCT had a strong NPV, which could be used as a candidate molecular marker for MTC to exclude patients with suspicious thyroid nodules or MTC symptoms (Lim et al. 2016). Therefore, some people suggest that the serum PCT test should be used as a routine test item for MTC. However, there are many factors influencing PCT in serum, such as bacterial infections, endotoxins, and certain cytokines, and false positive or false negative results will inevitably occur during clinical testing (Trimboli et al. 2018). Our results show that the PCT expression in SMTC tissues was significantly correlated with the 5-year recurrence of SMTC patients, which suggested that the high expression of PCT in cancer tissues was related to the occurrence and development of MTC. At the same time, the correlation analysis showed that the PCT expression in SMTC tissues was a risk factor for 5-year recurrence of SMTC, which corroborate the findings of a great deal of the previous work in this field (Papapetrou and Polymeris 2012; Trimboli et al. 2018; Voloshanenko et al. 2018). These reports indicated that the PCT levels in serum or tissue of tumor patients had a significant correlation with recurrence of MTC, and PCT could be used as a biomarker for the diagnosis and prognosis of MTC or to improve the diagnosis and prognostic evaluation of MTC. In addition, our results showed that the PCT expression elevated with the increase of SMTC staging, further indicating that PCT was expected to become a very promising and important marker for the diagnosis of MTC, and PCT also played a role in the development of tumors. However, this study did not analyze the changes in serum PCT levels in SMTC patients and the relationship between tissue PCT

expression and serum PCT changes, which required further research.

The *ATF4* gene is the main transcriptional regulatory element in cells and can be involved in the amino acid synthesis, correct folding of proteins, and degradation of residual proteins, as well as processes such as autophagy and apoptosis (Singleton and Harris 2012). Studies have revealed that *ATF4* may be a candidate molecular marker for MTC. High expression of *ATF4* can be responsible for the poor survival rate of tumor cells and block the activation of downstream signaling pathways of RET. At the same time, *ATF4* can reduce RET expression by promoting the ubiquitination of RET protein. On the contrary, activation of *RET* mutations can prevent tumor cell apoptosis by inhibiting the *ATF4* expression (Bagheri-Yarmand et al. 2015; Bagheri-Yarmand et al. 2017). Our results showed that the *ATF4* expression was localized in the nucleus and was decreased with the rise of the SMTC stage, and the difference in *ATF4* expression between different SMTC stages was statistically significant. Meanwhile, the *ATF4* expression was significantly correlated with 5-year recurrence and better survival of SMTC patients. However, the findings of the current study do not support the previous research that *ATF4* expression was up-regulated in malignant tumor tissues such as laryngeal cancer, ovarian cancer, and liver cancer, and it was highly expressed in patients with larger tumors and lymph node metastasis (Yang et al. 2014; Zong et al. 2017). A possible explanation for this might be that *ATF4* expression is related to the *RET* mutation, thus affecting the *ATF4* activity and inhibit the *ATF4* expression (Bagheri-Yarmand et al. 2017). Bagheri-Yarmand R et al. (Bagheri-Yarmand et al. 2019) found that the decrease or loss of *ATF4* expression in MTC was related to *RET* gene, and low *ATF4* expression was related to the poor prognosis of MTC. At the same time, knocking out *ATF4* in mice could cause up-regulation of *RET* expression and C cell proliferation (C cell proliferation is the process of MTC precancerous lesions). Similarly, knocking out *RET* in MTC cells would activate the *ATF4* expression, and the activated *ATF4* would activate the expression of downstream apoptotic genes to induce tumor cell apoptosis. In addition, low expression of *ATF4* could cause the sensitivity to tyrosinase inhibitor-induced apoptosis. In this study, the correlation analysis revealed

that the ATF4 expression was significantly and negatively correlated with 5-year recurrence and poor survival of SMTC patients. It can be considered that ATF4 and *RET* are jointly involved in the occurrence, development, and metastasis of SMTC. Since the tumor size of MTC and lymph node metastasis are closely related to prognosis, ATF4 may be a protective factor of SMTC and may become an important prognostic indicator of SMTC and a potential molecular target for treatment.

Conclusion

Detection of *RET* mutations is helpful for the differential diagnosis of thyroid benign and malignant tumors and the classification of SMTC risk. PCT and ATF4 can be used as important molecular biological indicators for the diagnosis of SMTC and have guiding value in evaluating prognosis. However, our current research mainly focuses on the protein expression of PCT and ATF4, and their content in the blood has not been analyzed. In addition, the levels of many tumor markers in the blood, especially ctDNA, have not been analyzed. More research on the blood parameters of SMTC patients is needed in the future.

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Figure legends

Figure 1. Immunohistochemical analysis of the PCT and ATF4 expressions in tissue samples of each stage of SMTC. Different letters represent significant differences between groups.

Figure 2. Western blotting analysis of the PCT and ATF4 expressions in tissue samples of each stage of SMTC. Different letters represent significant differences between groups, * represents a significant difference compared with Normal group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3. (a) Sequencing results of the most frequent sites of RET gene mutations; (b) RET gene mutation in medullary thyroid carcinoma.

Figure 4. The survival rate of 5 STMC patients who died within 5 years.

Table legends

Table 1 Distribution of PCT and ATF4 expressions and RET mutations in SMTC development

Patient ID	PCT	ATF 4	RET mutation or not (0,1)	Differentiation (1,2,3)	SMT C stage	Lymphatic metastasis or not	Recurrence or not in 5 years (0,1)	Survival or not in 5 years (0,1)
1	0.49	0.40	1	3	1	0	0	0
2	0.54	0.57	0	3	1	1	0	0
3	0.29	0.58	0	3	1	0	0	0
4	0.46	0.56	1	3	1	0	0	0
5	0.47	0.78	0	3	1	1	0	0
6	0.51	0.75	0	3	1	0	0	0
7	0.56	0.58	0	3	4	1	0	0
8	0.22	0.61	0	3	1	1	0	0
9	0.49	0.59	0	2	1	1	0	0
10	0.32	0.59	0	3	1	0	0	0
11	0.26	0.65	0	3	3	0	0	0
12	0.29	0.41	0	3	1	0	0	0
13	0.27	0.70	1	3	1	0	0	0
14	0.37	0.56	0	3	3	0	0	0
15	0.47	0.62	1	3	1	0	0	0
16	0.58	0.51	0	3	1	0	0	0
17	0.25	0.67	0	2	1	1	0	0
18	0.87	0.42	1	2	1	0	0	0
19	0.55	0.58	0	2	1	0	0	0
20	0.54	0.59	0	2	1	0	0	0
21	0.68	0.67	0	3	1	1	0	0
22	0.47	0.75	1	3	1	0	0	0

23	0.57	0.59	0	1	4	0	0	0
24	0.83	0.70	0	2	1	0	0	0
25	0.71	0.56	0	3	1	0	0	0
26	0.59	0.42	0	2	2	0	0	0
27	0.90	0.25	0	2	2	1	0	0
28	0.82	0.15	0	3	2	1	0	0
29	0.74	0.16	0	2	3	1	0	0
30	0.87	0.33	1	3	3	0	0	0
31	0.60	0.39	1	3	4	0	0	0
32	0.98	0.36	0	3	2	0	0	0
33	0.66	0.33	1	1	2	1	0	0
34	0.94	0.15	1	3	2	1	0	0
35	0.96	0.37	0	3	2	1	0	0
36	0.69	0.28	1	3	2	1	0	0
37	0.58	0.29	1	3	2	1	0	0
38	0.84	0.28	1	3	2	1	0	0
39	0.58	0.31	0	3	1	1	1	0
40	0.75	0.08	1	3	2	0	1	0
41	0.80	0.37	1	2	2	1	1	0
42	0.89	0.23	1	2	2	1	1	0
43	0.87	0.17	0	3	3	0	1	0
44	0.82	0.14	1	2	1	1	1	1
45	0.75	0.35	1	3	3	1	1	0
46	0.89	0.25	1	2	3	1	1	1
47	0.57	0.09	0	2	4	0	1	1
48	0.77	0.24	1	3	1	1	1	1
49	0.51	0.23	0	1	2	1	1	0
50	0.73	0.39	1	2	3	0	1	1

Draft

Table 2 Correlation analysis of risk factors for 5-year recurrence of SMTC

	OR	Wald	95% CI	P
PTC	4.272	4.780	71.731 (1.556~3305.774)	0.028
ATF4	-10.523	9.858	2.689E-5 (3.772E-8~0.019)	0.002
RET	1.347	3.689	3.846 (0.973~15.207)	0.045
Differentiation	-0.824	2.464	0.439 (0.157~1.227)	0.117
SMTC staging	0.546	2.670	1.726 (0.897~3.321)	0.102
Lymphatic metastasis	1.012	2.119	2.750 (0.704~10.737)	0.145

Draft

Table 3 Correlation analysis of risk factors for 5-year survival of SMTC

	OR	Wald	95% CI	P
PTC	3.960	2.055	52.474 (0.233~11786.466)	0.152
ATF4	-8.763	4.575	1.563E-4 (5.094E- 8~0.480)	0.032
RET	1.885	2.643	6.588 (0.678~63.940)	0.104
Differentiation	-0.968	1.992	0.379 (0.099~1.456)	0.158
SMTC staging	0.588	1.723	1.801 (0.748~4.333)	0.189
Lymphatic metastasis	0.639	0.315	1.714 (0.261~11.264)	0.575

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