A Functional Insertion/Deletion Polymorphism in the Promoter Region of the *NFKB1* Gene Increases the Risk of Papillary Thyroid Carcinoma

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This study aimed to assess whether an insertion/deletion polymorphic variation rs28362491 in the *NFKB1* promoter region was related to the risk of papillary thyroid carcinoma (PTC). Genomic DNA was extracted from the peripheral venous blood of 352 patients with PTC and 459 controls. The *NFKB1* rs28362491 polymorphism was genotyped by using a polymerase chain reaction assay. We found that the frequency of the heterozygous genotype ATTG1/ATTG2 was significantly higher in the cases compared to the controls (odds ratios [OR]=1.44, 95% confidence intervals [CI]=1.05–1.96, $p=0.02$). Moreover, the frequency of ATTG1/ATTG2+ATTG1/ATTG1 genotypes was significantly elevated in the cases compared to the controls (OR=1.38, 95% CI=1.03–1.85, $p=0.03$). These findings suggest that the –94 insertion/deletion ATTG polymorphism in the *NFKB1* promoter might be associated with an increased risk of PTC.

Introduction

PAPILLARY THYROID CARCINOMA (PTC) is the most common malignant thyroid neoplasm, comprising up to 80% of all thyroid carcinomas (Gimm, 2001). PTC is associated with an excellent prognosis and the overall survival is more than 90% (Caron and Clark, 2006). However, during the past several decades, the incidence of thyroid carcinoma has steadily increased in the Americas, Europe, Asia, and Oceania (Kilfoy *et al.*, 2009). Although the exact etiology of PTC is unknown, it is known that the majority of PTCs have genetic alterations, such as mutations in *RAS* and *BRAF*, *RET/PTC* rearrangements, and loss of *TP53* and *PTEN* (Giordano *et al.*, 2005).

The NF- κ B family of transcription factors regulates the expression of a wide spectrum of genes involved in cell survival, immune and inflammatory response, and oncogenesis (Ghosh *et al.*, 1998; Baldwin, 2001; Tak and Firestein, 2001; Yang *et al.*, 2005). In mammals, the NF- κ B family is composed of five members: NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB, and c-Rel (Vallabhapurapu and Karin, 2009). The members can form up to 15 NF- κ B complexes in the form of homo- and heterodimers with each other, which mediate transcriptional activities. Among the

dimeric complexes of NF- κ B, the most abundant form is p50/p65, which is present in almost all cell types. In most normal cells, NF- κ B complexes are inactivated by its inhibitor I κ B (Aggarwal, 2004). In 1997, Visconti *et al.* (1997) were the first to report that activation of NF- κ B is critical in thyroid carcinoma. Since then, more evidence has emerged to reveal the oncogenic and antiapoptotic role of NF- κ B in thyroid carcinoma (Pacifico *et al.*, 2004; Starenki *et al.*, 2004; Vasudevan *et al.*, 2004; Pacifico and Leonardi, 2010). Meanwhile, several signaling pathways have been shown to induce neoplastic transformation of thyroid cells through activation of the NF- κ B signaling pathway (Kato *et al.*, 2006; Palona *et al.*, 2006; Guigon *et al.*, 2009).

Recently, a number of studies have reported that an insertion/deletion polymorphic variation rs28362491 in the *NFKB1* promoter region might be associated with the risk of different malignancies, such as ovarian cancer (Fan *et al.*, 2011), gastric cancer (Lo *et al.*, 2009), nasopharyngeal carcinoma (Zhou *et al.*, 2009), and oral squamous cell carcinoma (Lin *et al.*, 2006), suggesting its possible role in carcinogenesis. The *NFKB1* gene, located at chromosome 4q24, encodes p50 and p105. rs28362491 (–94 insertion/deletion ATTG) is located between two putative key promoter

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regulatory elements in the *NFKB1* gene, which seems to be the first potential functional *NFKB1* genetic variation (Karban *et al.*, 2004). The presence of a 4-bp deletion (ATTG1 allele) results in the loss of binding to nuclear proteins, thereafter, leading to a reduced promoter activity and influencing the p50-mediated transcriptional activity.

To date, no association has been identified between the *NFKB1*-94 insertion/deletion ATTG polymorphism and PTC risk. In our study, we evaluated the *NFKB1* polymorphism in association with the risk of PTC through a hospital-based case-control study.

Materials and Methods

Study subjects

In the case-control study, a total of 352 PTC patients and 459 cancer-free controls were recruited from June 2012 to May 2014 at the West China Hospital of Sichuan University. This study was approved by the Ethics Committee of Sichuan University and informed consent was obtained from all participating subjects involved in this study. The cases were newly diagnosed PTCs, confirmed by histopathological analysis, without prior history of other malignancies or previous chemotherapy or radiotherapy. All controls were healthy subjects who were seeking healthcare at the outpatient department of the same hospital over the same period and were frequency matched to the cases for sex and age. Approximately 2% of controls were excluded because they were diagnosed with Hashimoto thyroiditis or nodular goiter. Demographic data and clinical information of PTC patients were collected from surgical and pathological records, including age, gender, the number of foci, and tumor-node-metastasis (TNM) status. The disease stage was determined based on the American Joint Committee on Cancer TNM classification. The case group was composed of 72 males and 280 females with a mean age (standard deviation) of 44.0 (13.7) years. The control group consisted of 112 males and 347 females with a mean age (standard deviation) of 42.8 (11.6) years.

DNA extraction and genotyping

Peripheral venous blood samples were collected from all individuals into ethylenediaminetetraacetic acid (EDTA)-anticoagulant vacutainers and stored at -20°C for DNA extraction. Genomic DNA was extracted from 200 μL peripheral blood samples using a commercial DNA extraction kit (Bioteke Corporation, Beijing, China) according to the manufacturer's protocol. The *NFKB1*-94 insertion/deletion ATTG polymorphism was genotyped employing a polymerase chain reaction (PCR)-polyacrylamide gel electrophoresis (PAGE) method. The PCR fragment containing the polymorphism was amplified with the primer pairs (F, 5'-tgacgcgcatgactctatca-3' and R, 5'-ggctctggcttctagcag-3'). PCR was performed as previously described (Zhou *et al.*, 2009). PCR products were visualized on 6% PAGE gels stained with argenti nitrate, where allele ATTG1 had a 154-bp band and allele ATTG2 had a 158-bp band. For quality control, two investigators independently read all gel pictures, and 5% of samples were reanalyzed in duplicate and confirmed by DNA sequencing.

Statistical analyses

All data analyses were performed using SPSS 19.0 statistical software (IBM, Armonk, NY). Allelic and genotype

frequencies of the *NFKB1*-94 insertion/deletion ATTG polymorphism were obtained by direct counting. The Hardy-Weinberg equilibrium was analyzed by the chi-square test. The effects of allelic and genotype distributions on PTC were determined by deriving the odds ratios (ORs) and the respective 95% confidence intervals (CIs). A two-sided p -value of <0.05 was considered statistically significant.

Results

Characteristics of PTC patients and controls

The clinical and pathological characteristics of 352 cases and 459 controls are listed in Table 1. Among the cases, 72 (20.5%) were male and 280 (79.5%) were female, while among the controls, 112 (24.4%) were male and 347 (75.6%) were female. The cases and controls were matched with respect to age and gender ($p > 0.05$). All 352 patients had PTC. There were 96 (27.3%) patients in disease stage T1, 9 (2.6%) patients in T2, 209 (59.3%) patients in T3, 13 (3.7%) patients in T4, and 24 (6.8%) patients in Tx.

Association between the *NFKB1* polymorphism and risk of PTC

The allelic and genotype frequencies of the *NFKB1*-94 insertion/deletion ATTG polymorphism as well as their associations with risk of PTC are presented in Table 2. We successfully identified three genotypes of the *NFKB1*-94 insertion/deletion ATTG polymorphism (ATTG1/ATTG1, ATTG1/ATTG2, and ATTG2/ATTG2). No deviation from the Hardy-Weinberg equilibrium was observed in genotype distributions of the *NFKB1* polymorphism, both in the PTC patients and the controls. The frequency of the heterozygous genotype ATTG1/ATTG2 was significantly higher in the cases compared to the controls (OR = 1.44, 95% CI = 1.05–1.96, $p = 0.02$). The frequency of ATTG1/ATTG2 + ATTG1/ATTG1 genotypes was significantly elevated in the cases compared to the controls (OR = 1.38, 95% CI = 1.03–1.85, $p = 0.03$). No significant difference was observed between the cases and the controls in the ATTG1/ATTG1 versus ATTG2/ATTG2 comparison. Moreover, when we stratified the cases

TABLE 1. DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF STUDY SUBJECTS

Variables	Patients (n = 352)	Controls (n = 459)
Age (mean $\pm$ SD)	44.0 $\pm$ 13.7	42.8 $\pm$ 11.6
Gender (%)		
Male	72 (20.5)	112 (24.4)
Female	280 (79.5)	347 (75.6)
T status (%)		
T1 + T2	105 (29.8)	
T3 + T4	223 (63.4)	
Tx	24 (6.8)	
N status (%)		
N0	131 (37.2)	
N1	221 (62.8)	
Focus (%)		
Single	260 (73.9)	
Multiple	92 (26.1)	

SD, standard deviation.

TABLE 2. THE GENOTYPE AND ALLELE DISTRIBUTIONS OF THE *NFKB1* POLYMORPHISM IN PTC PATIENTS AND CONTROLS

Variables	Patients (n=352)	Controls (n=459)	OR (95% CI)	P-value
<i>NFKB1</i> -94 genotypes (%)				
ATTG1/ATTG1	60 (17.0)	79 (17.2)	1.23 (0.81–1.85)	0.34
ATTG1/ATTG2	186 (52.8)	209 (45.5)	1.44 (1.05–1.96)	0.02
ATTG2/ATTG2	106 (30.1)	171 (37.3)	Ref.	Ref.
ATTG1/ATTG2 + ATTG1/ATTG1	246 (69.9)	288 (62.7)	1.38 (1.03–1.85)	0.03
<i>NFKB1</i> -94 alleles (%)				
ATTG1	306 (43.5)	367 (40.0)	1.15 (0.95–1.41)	0.16
ATTG2	398 (56.5)	551 (60.0)		

Bold values indicate significant differences at the 5% level.

CI, confidence intervals; OR, odds ratios; PTC, papillary thyroid carcinoma; Ref., reference.

by age, gender, the number of foci, and TNM status, no significant association was observed.

Discussion

In general, PTC is a consequence of genetic and environmental factors (Ron *et al.*, 1995; Dardano *et al.*, 2009; Gudmundsson *et al.*, 2009, 2012; He *et al.*, 2009; Jazdzewski *et al.*, 2009; Jendrzewski *et al.*, 2012; Jones *et al.*, 2012). PTCs (5%–10%) are known to be familial and likely have several genetic alterations (Kouniavsky and Zeiger, 2010). Recent studies have identified multiple genetic susceptible loci for the development of thyroid carcinoma. Until now, at least four genome-wide association studies have provided several lines of evidence on genetic predisposition to PTC among multiple ethnic populations (Gudmundsson *et al.*, 2009, 2012; Takahashi *et al.*, 2010; Kohler *et al.*, 2013). More evidence about gene polymorphism associated with the risk of PTC has been reported (Dardano *et al.*, 2009; He *et al.*, 2009; Jendrzewski *et al.*, 2012; Jones *et al.*, 2012). In thyroid carcinoma, previous studies demonstrated that NF- κ B plays a crucial role in maintaining the transformed phenotype and conferring resistance to apoptosis (Pacifico and Leonardi, 2010). At present, it is accepted that activation of NF- κ B both in cell lines and primary tumors is more than a consequence of the neoplastic transformation, but rather a factor that leads to neoplasia. Multiple signaling pathways leading to the neoplastic transformation of thyroid cells require NF- κ B activation (Kato *et al.*, 2006; Palona *et al.*, 2006; Guigon *et al.*, 2009), and an elevated NF- κ B activity might at least partly render chemo- or radioresistance to thyroid cancer cells; so, NF- κ B can be a promising therapeutic target in thyroid carcinoma. Inhibition of NF- κ B by stable expression of a repressor form of I κ B α in the undifferentiated thyroid cancer cell line enhanced the sensitivity to drug-induced apoptosis and the block of tumor growth (Pacifico *et al.*, 2004). Hence, a number of NF- κ B inhibitors have been designed to suppress the NF- κ B activity as an anticancer agent by targeting either NF- κ B or its upstream I κ B. Dehydroxymethyl epoxymycin, a derivative of the antibiotic epoxymycin C, which inhibits nuclear translocation of NF- κ B, showed its inhibitory effect on thyroid cancer cells both *in vitro* and *in vivo* (Starenki *et al.*, 2004). Other drugs, such as curcumin (Aggarwal *et al.*, 2005) and genistein (Li *et al.*, 2005), are I κ B kinase inhibitors.

The *NFKB1*-94 insertion/deletion ATTG polymorphism has been implicated in a variety of neoplastic diseases

through case-control studies, suggesting its association with increased susceptibility for cancers, including ovarian (Fan *et al.*, 2011), nasopharyngeal (Zhou *et al.*, 2009), oral (Lin *et al.*, 2006), gastric (Lo *et al.*, 2009), bladder (Tang *et al.*, 2010), and other cancers (Bu *et al.*, 2007; Zhang *et al.*, 2009; Zhou *et al.*, 2010; Song *et al.*, 2011; Cai *et al.*, 2013; Cheng *et al.*, 2013; Huang *et al.*, 2013). Although these studies have found some hints about the association between the –94 insertion/deletion ATTG polymorphism and cancer risk, the results were inconsistent (Riemann *et al.*, 2006; Lewander *et al.*, 2007). Two meta-analyses have been carried out to assess the effect of –94 insertion/deletion polymorphism on cancer risk and made similar conclusions (Duan *et al.*, 2014; Xu *et al.*, 2014). Both showed that the –94 insertion/deletion ATTG polymorphism is a risk factor for cancer in the Asian population, but not in the Caucasian population. They also observed that it was associated with the risk of oral squamous cell carcinoma and ovarian cancer, but no associations were found in bladder and lung cancers. These results suggest the *NFKB1*-94 insertion/deletion ATTG polymorphism might be ethnic and cancer subtype specific.

Our study attempted to assess the association between the –94 insertion/deletion ATTG polymorphism in the promoter region of *NFKB1* and PTC. In the study, we found that PTC patients with the heterozygous ATTG1/ATTG2 genotype had a significantly higher susceptibility to PTC than those with the homozygous ATTG2/ATTG2 genotype ($p=0.02$). Moreover, individuals with the ATTG1 allele were at a 1.38-fold risk to develop PTC compared to individuals homozygous for the ATTG2 allele ($p=0.03$). However, when stratified by age, gender, the number of foci, and TNM status, no association between primary PTC and the –94 insertion/deletion ATTG polymorphism was observed. The difference of PTC risk may be attributable to the –94 insertion/deletion ATTG polymorphism in the *NFKB1* promoter region.

Most studies have reported that the *NFKB1* ATTG2 allele increases cancer risk in different types of cancers, such as ovarian cancer (Fan *et al.*, 2011), oral squamous cell carcinoma (Lin *et al.*, 2006), and others. The probable mechanism for this observation might be the activation of p50, which regulates cellular events such as apoptosis and cell death independent of the NF- κ B complex (Yu *et al.*, 2009). p50, which does not contain a C-terminal transactivation domain in the other members of the NF- κ B family, may repress proinflammatory gene transcription in the form of homodimers (Elsharkawy *et al.*, 2010; Shih *et al.*, 2011). Riemann *et al.* (2007) and Li

et al. (2013) have reported that bladder cancer with the ATTG2/ATTG2 genotype had higher expression of p50 (*NFKB1*) than those with the -94 del ATTG1 allele. Similar findings were also observed in colorectal cancer (Song *et al.*, 2011; Mohd Suzairi *et al.*, 2013). A previous study showed that the ATTG2 allele in the promoter sequence leads to an approximately twofold higher transcriptional activity compared to that of the ATTG1 allele, thus resulting in a higher level of p50 in the cytoplasm (Karban *et al.*, 2004), which may easily form p50/p65 heterodimers to mediate the inflammatory activity. However, Li *et al.* (2013) indicated that bladder cancer risk was significantly increased in the ATTG1/ATTG1 compared to the ATTG2/ATTG2 genotype. One plausible explanation for the association is that the ATTG1/ATTG1 genotype, which decreases p50 synthesis, may lead to a reduction of p50 homodimers and hence a weakened anti-inflammatory response (Karban *et al.*, 2004). The diversity of results may be attributed to different types of cancers, genetic background, environmental factors, and others.

However, our study bears several limitations. First, our study is a hospital-based case-control study. Selection bias cannot be excluded as subjects pertaining to a specific genotype might be enrolled. Second, the relatively small sample size of our study might compromise the statistical power. Overall, our study could be an initial step in investigating the association between the -94 insertion/deletion ATTG polymorphism of the *NFKB1* promoter and PTC risk.

To the best of our knowledge, this is the first study to assess the risk of PTC associated with the -94 insertion/deletion ATTG polymorphism in the *NFKB1* promoter. In conclusion, we report that ATTG1/ATTG2 and ATTG1/ATTG1 genotypes are associated with an increased risk of PTC in a Chinese population.

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Author Disclosure Statement

No competing financial interests exist.

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