Abstract: Tumor suppressor p53 directly regulated the abundance of the miR-34b/c. The interaction might contribute to certain cancer. We hypothesized that rs4938723 in the promoter region of pri-miR-34b/c and TP-53 Arg72Pro may be related to the risk of papillary thyroid carcinoma (PTC).

A total of 784 patients with PTC and 1006 healthy controls were recruited to participate in this study. The variants were discriminated using a polymerase chain reaction–restriction fragment length polymorphism method (PCR-RFLP). Additionally, the relative expression levels of miR-34b/c and TP-53 in 44 paired samples were revealed by quantitative reverse transcription polymerase chain reaction (qRT-PCR).

A significantly increased risk of PTC was observed in the miR-34b/c rs4938723 CT, CC, and CT/CC genotypes compared with the TT genotype (CT vs TT: adjusted odds ratio [OR] = 1.51, 95% confidence interval [CI] = 1.23–1.85; CC vs TT: adjusted OR = 1.89, 95% CI = 1.39–2.63; CT/CC vs TT: adjusted OR = 1.59, 95% CI = 1.30–1.92, respectively). Significantly increased PTC susceptibility was also associated with the TP-53 Arg72Pro CC and CG/CC genotypes compared with the GG genotype (CC vs GG: adjusted OR = 2.04, 95% CI = 1.54–2.70; CG/CC vs GG: adjusted OR = 1.35, 95% CI = 1.11–1.67, respectively). Stratification analysis revealed that patients carrying the TP-53 Arg72Pro C allele and CC genotype had a significantly increased risk for developing N1 (C vs G: OR = 1.27, 95% CI = 1.03–1.56; CC vs. GG: OR = 1.62, 95% CI = 1.07–2.46, respectively). Combined analysis showed that the genotypes of rs4938723 CT/CC + TP-53 CG/CC increased the risk of PTC compared with rs4938723 TT + TP-53 GG (OR = 2.25, 95% CI = 1.67–3.03). Additionally, level of miR-34b was significantly upregulated in PTC patients.

These findings indicate that the miR-34b/c rs4938723 and TP-53 Arg72Pro polymorphisms may contribute to the susceptibility of PTC.

INTRODUCTION

Thyroid cancer is the most prevalent type of endocrine cancer with the incidence rates of 4 and 12 per 100,000 in human. 1 Papillary thyroid carcinoma (PTC) represents virtually 80% of all thyroid cancers, which is the fifth leading malignancy in female patients. 2 Although the exact etiology of PTC is not fully clear, numerous pedigree and genomic association studies have indicated that genetic susceptibilities may contribute to PTC. 3–5 MicroRNA (miRNA) are non-coding small RNAs existing extensively in animals, plants, and viruses, at an approximate length of 21 to 23 nt and highly conserved. 6 Mature miRNAs can result in the degradation or translation suppression of mRNA by binding to the 3’ untranslated region of target gene. 7 miRNAs are involved in the regulation of multiple critical biological activities, including cell proliferation, 7 apoptosis, 8 and tumor genesis. 9–10 Altered expression of miR-34 has been found in thyroid tumors, suggesting that miR-34 may have an important role in thyroid carcinogenesis. 11,12 Recently, an rs4938723/C/T polymorphism in the promoter region of pri-miR-34b/c gene has been discovered, which is located in the CpG island. The variation of rs4938723 C to T may affect a predicted GATA-X transcription factor binding and then affect the expression and carcinogenesis. 13–15 It is well known that p53 can regulate the expression of miRNAs, especially the miR-34 family members (i.e., miR-34a, miR-34b, and miR-34c). The 3 mature miRNAs are encoded by 2 different primary miRNAs. miR-34a is encoded by its own transcript, whereas miR-34b and miR-34c shared a common primary transcript (pri-miR-34b/c). 6 The promoter region of miR-34b/c transcripts contains p53-binding sites. 16 Moreover,
the TP-53 gene has an important function in cell cycle control, apoptosis, and maintenance of DNA integrity. The importance of p53 in cell cycle regulation and DNA integrity is such critical that it has been called the “guardian of the genome.”

The TP-53 Arg72Pro SNP results in a change in its protein structure, and this SNP exists only in humans. Given the potential role of the TP-53 Arg72Pro in tumorigenesis, several studies have reported the association between the TP-53 Arg72Pro polymorphism and PTC risk in different populations. However, inconsistent results were obtained. Additionally, there is no report of the polymorphism in Chinese PTC patients. To date, no study has been performed to examine the relationship between the miR-34b/c rs4938723 polymorphism and PTC risk. In this study, we selected the rs4938723 and TP-53 Arg72Pro polymorphisms to investigate the correlation between the 2 polymorphisms and PTC risk in a Chinese population by conducting a case–control study.

**MATERIALS AND METHODS**

**Study Population**

The study population was composed of 784 cases of PTC and 1006 controls. Patients were consecutively recruited from the West China Hospital of Sichuan University between May 2012 and June 2015. The diagnosis of PTC was confirmed by histopathological analysis. Clinical information was obtained from surgical and pathological records, including age, gender, invasion, tumor node metastasis status, clinical stages, and multifocality. Baseline profiles of the study population are summarized in Table 1. The controls were selected from healthy volunteers who visited the hospital for medical examination during the same period. We excluded individuals who had a history of thyroid disease or antecedents of malignancy from the control group. The study was approved by the institutional review board of the hospital, and informed consent was obtained from each participant.

**Genotyping**

Genomic DNA was isolated from EDTA-anticoagulated peripheral blood using a commercial extraction kit (Biobeke, Beijing, China) according to the manufacturer’s instructions. The 2 SNPs were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism assay. For the rs4938723, the primers were as follows: 5′-CTCTGGGAAACCTCTTGGCCATACCCATC-3′ (forward); 5′-TGAGATCAAGCCATACACTAAGA-3′ (reverse). The PCR products were digested with Bcc I (New England BioLabs, Beverly, MA, USA), yielding one band of 147 bp for T allele and two bands of 118 and 29 bp for C allele. For the TP-53 Arg72Pro, the primer sequences, PCR conditions and restriction enzymes used were previously established. To classify the genotypes, digested PCR products were separated on polyacrylamide gels and stained with 1.0 g/L argent nitrate. For quality control, 2 researchers read the gel pictures independently. The samples were reanalyzed and verified by DNA sequencing if conflict results occurred. In addition, about 10% of all samples were randomly selected to be confirmed by DNA sequencing, and the results were 100% consistent.

**Tissue Samples**

Paired tumor and adjacent non-tumor tissue specimens were obtained with informed consent from 44 PTC patients who

| TABLE 1. Demographic and Clinical Characteristics of Study Subjects |
|-------------------------|-------------------------|
|                         | PTC                     | Controls                |
| Age, years              | 44.2 ± 13.1             | 43.1 ± 8.9              |
| Gender, %               |                         |                         |
| Male                    | 191 (24.4)              | 278 (27.6)              |
| Female                  | 593 (75.6)              | 728 (72.4)              |
| T status, %             |                         |                         |
| Tx                      | 48 (6.1)                | 62 (6.2)                |
| T1 and T2               | 247 (31.5)              | 265 (26.4)              |
| T3 and T4               | 489 (62.4)              | 425 (43.5)              |
| N status, %             |                         |                         |
| Nx                      | 3 (0.4)                 | 4 (0.4)                 |
| N0                      | 296 (37.8)              | 335 (33.3)              |
| N1                      | 485 (61.9)              | 488 (49.3)              |
| M status, %             |                         |                         |
| Mx                      | 5 (0.6)                 | 7 (0.7)                 |
| M0                      | 770 (98.2)              | 789 (79.3)              |
| M1                      | 9 (1.1)                 | 7 (0.7)                 |
| Clinical stage, %       |                         |                         |
| I and II                | 496 (65.7)              | 500 (50.0)              |
| III and IV              | 259 (34.3)              | 259 (25.0)              |
| Multifocality, %        |                         |                         |
| No                      | 554 (70.7)              | 583 (58.1)              |
| Yes                     | 230 (29.3)              | 423 (42.0)              |

PTC = papillary thyroid carcinoma.
underwent therapy at the West China Hospital of Sichuan University. All samples, taken during surgery, were immediately frozen in liquid nitrogen for further analysis. All the tumor and non-tumor tissue specimens were diagnosed histopathologically.

RNA Extraction and Quantitative Reverse transcription-PCR Assay

Total RNA was extracted and purified from tissue samples using TRIzol Reagent according to the manufacturer’s protocol (Roche, Indianapolis, IN). Reverse transcription-PCR (RT-PCR) was performed by using commercial kits according to the manufacturer’s instructions (Ribobio, Guangzhou, China; Thermo Scientific, Vilnius, Lithuania). Quantitative real-time PCR was carried out using SYBR green Master Mix (Qiagen, Hilden, Germany) and samples were amplified in a thermocycler as follows: 95 °C for 10 minutes (1 cycle), 95 °C for 10 seconds, 60 °C for 20 seconds, 70 °C for 10 seconds (40 cycles), and melting curve. Data were normalized using comparative threshold cycle method (U6 for miR-34b/c and GAPDH for TP-53). The primers used were as follows: TP-53: CCAGGCCAAGAAGAAACCAC (forward), TGAGTTCCAAGGCCCTCATC (reverse).28 GAPDH: GAAGGTGAAGGTCGGAGTC (forward), GAAGATGGTGATGGGATTTC (reverse). Duplicate Ct values were averaged and miR-34b/c and TP-53 mRNA relative expression levels were determined as $2^{-\Delta\Delta Ct}$.29

Methylation-Specific PCR

A total of 100 genomic DNA samples (including 50 cases and 50 controls) were treated with bisulfite according to the manufacturer’s instructions (Ribobio, Guangzhou, China; Roche, Indianapolis, IN). Reverse transcription-PCR (RT-PCR) was performed by using commercial kits according to Wilcoxon rank sum test. The statistical analyses were performed using the SPSS for windows 13.0 (SPSS Inc, Chicago, IL). Moreover, genotypic association tests in a case–control pattern, assuming codominant, dominant, recessive, and over-dominant genetic models were calculated using SNPstats.32 A P level of <0.05 was regarded as statistically significant.

RESULTS

Totally, 784 patients with PTC and 1006 anonymous controls were enrolled in this study. Demographic information and clinical parameters for all subjects are summarized in Table 1. We have determined the prevalence of the miR-34b/c rs4938723 and TP-53 Arg72Pro polymorphisms in PTC patients and controls in order to evaluate its association with the risk of PTC (Fig. 1). Both polymorphisms followed the Hardy–Weinberg equilibrium in the control group ($P = 0.43$ for rs4938723 and $P = 0.11$ for TP-53 Arg72Pro). The Quanto software (version 1.2.3) was used to calculate the statistical

| TABLE 2. Association Between the miR-34b/c rs4938723 and TP-53 Arg72Pro Polymorphisms and Risk of PTC |
|-----------------------------------------------|
| Genetic Model | Genotypes | Patients n = 784, % | Controls n = 1006, % | OR (95%CI) | P Value$^{\dagger}$ | OR (95%CI) | P Value$^{\dagger}$ |
|----------------|-------------|------------------|------------------|------------|----------------|------------|----------------|
| rs4938723       |             |                  |                  |            |                |            |                |
| Codominant      | TT          | 271 (34.6%)      | 456 (45.3%)      | 1.00       | 1.00           |             |                |
|                 | CT          | 402 (51.3%)      | 451 (44.8%)      | 1.49 (1.22–1.84) | <0.001       | 1.51 (1.23–1.85) | <0.001       |
|                 | CC          | 111 (14.2%)      | 99 (9.8%)        | 1.89 (1.38–2.57) | <0.001       | 1.89 (1.39–2.63) | <0.001       |
| Dominant        | TT          | 271 (34.6%)      | 456 (45.3%)      | 1.00       | 1.00           |             |                |
|                 | CT/CC       | 513 (65.4%)      | 550 (54.7%)      | 1.57 (1.29–1.90) | 0.005       | 1.59 (1.30–1.92) | 0.005       |
| Recessive       | TT/CT       | 673 (85.8%)      | 907 (90.2%)      | 1.00       | 1.00           |             |                |
|                 | CC          | 111 (14.2%)      | 99 (9.8%)        | 1.51 (1.13–2.02) | 0.004       | 1.51 (1.14–2.04) | 0.006       |
| Overdominant    | TT/CC       | 382 (48.7%)      | 555 (55.2%)      | 1.00       | 1.00           |             |                |
|                 | CC          | 402 (51.3%)      | 451 (44.8%)      | 1.29 (1.07–1.56) | 1.30 (1.07–1.56) | 0.006       |
| TP-53 Arg72     |             |                  |                  |            |                |            |                |
| Codominant      | GG          | 217 (27.7%)      | 351 (34.9%)      | 1.00       | 1.00           |             |                |
|                 | CG          | 384 (49.0%)      | 507 (50.4%)      | 1.22 (0.99–1.52) | 0.06       | 1.16 (0.94–1.45) | 0.16       |
|                 | CC          | 183 (23.3%)      | 148 (14.7%)      | 2.00 (1.52–2.63) | <0.001       | 2.04 (1.54–2.70) | <0.001       |
| Dominant        | GG          | 217 (27.7%)      | 351 (34.9%)      | 1.00       | 1.00           |             |                |
|                 | CG/CC       | 567 (72.3%)      | 655 (65.1%)      | 1.40 (1.14–1.72) | 0.001       | 1.35 (1.11–1.67) | <0.001       |
| Recessive       | GG/CG       | 601 (76.7%)      | 858 (85.3%)      | 1.00       | <0.001       |             |                |
|                 | CC          | 183 (23.3%)      | 148 (14.7%)      | 1.76 (1.39–2.24) | 0.001       | 1.85 (1.47–2.38) | <0.001       |
| Overdominant    | GG/CC       | 400 (51.0%)      | 499 (49.6%)      | 1.00       | 0.55           |             |                |
|                 | CG          | 384 (49.0%)      | 507 (50.4%)      | 0.94 (0.78–1.14) | 1.12 (0.93–1.35) | 0.22       |

CI = confidence interval, OR = odds ratio. Boldfaced values indicate a significant difference at the 5% level.

$^{\dagger}$Adjusted for age and gender using the logistic regression model.

$^{\dagger}$P value < 0.001, multiple testing for rs4938723.

$^{\dagger}$P value < 0.001, multiple testing for TP-53 Arg72Pro.
Polymorphism and Clinical Features of PTC Patients

As shown in Table 2, distributions of the miR-34b/c rs4938723 were significantly different between patients and control subjects. Compared with TT genotype, a significantly increased PTC risk was found to be associated with CT (adjusted OR = 1.51, 95% CI = 1.23–1.85, P < 0.001) and CC (adjusted OR = 1.89, 95% CI = 1.39–2.63, P < 0.001) genotype in the codominant model, while CT/CC carriers had a 1.59-fold increased PTC risk (95% CI = 1.30–1.92, P < 0.001) in the dominant model. Compared with TT/CT genotype, CC genotype carriers had a 1.51-fold increased PTC risk (95% CI = 1.14–2.04, P = 0.005) in a recessive model. When compared with TT/CC genotype, CT genotype carriers had a 1.30-fold increased PTC risk (95% CI = 1.07–1.56, P = 0.006).

Significant differences were also observed for genotypes of the TP-53 Arg72Pro polymorphisms. Compared with GG genotype, CC and CG/CC genotype carriers had a 2.04- and 1.35-fold increased PTC risk (95% CI = 1.39–2.63, 1.07–2.46, respectively). Furthermore, compared with GG/CG genotypes, CC genotype carriers had a 1.54-fold increased PTC risk (95% CI = 1.11–1.92, P < 0.001) in the codominant model, while CT/CC carriers had a 1.51-fold increased PTC risk (95% CI = 1.14–2.04, P = 0.005) in a recessive model. When compared with TT/CC genotype, CT genotype carriers had a 1.30-fold increased PTC risk (95% CI = 1.07–1.56, P = 0.006).

Furthermore, we divided the cases by T status, N status, clinical stages, and multifocality (Tables 3 and 4). The patients carrying the TP-53 Arg72Pro C allele and CC genotype had a significantly increased risk for developing N1 when compared with patients carrying the G allele and GG genotype (C vs G: OR = 1.27, 95% CI = 1.03–1.56, P = 0.02; CC vs GG: OR = 1.63, 95% CI = 1.07–2.46, P = 0.02, respectively). Additionally, the frequencies of the TP-53 Arg72Pro C allele and CC genotype in patients with multifocality were lower than other patients (C vs G: OR = 0.78, 95% CI = 0.63–0.97, P = 0.03; CC vs GG: OR = 0.60, 95% CI = 0.38–0.94, P = 0.02, respectively) (Table 4).

We also examined the combined effects of the miR-34b/c rs4938723 and TP-53 Arg72Pro variants on PTC risk. As shown in Table 5, carriers with the combined genotypes of rs4938723CT/CC and TP-53 Arg72Pro CG/CC had a 2.25-fold increased PTC risk (95% CI = 1.67–3.03, P < 0.001) compared with those carrying the rs4938723TT and TP-53 Arg72ProGG genotypes.

We analyzed the differential expression of miR-34b/c and TP-53 mRNA in 44 paired PTC tissues and adjacent non-tumor tissues. Level of miR-34b was significantly upregulated in PTC patients (P = 0.02). However, there was no significant difference of the miR-34c and TP-53 mRNA levels between PTC tissues and adjacent non-tumor tissues (P > 0.05) (Fig. 2). Furthermore, no significant association between miR-34b/c rs4938723 polymorphism and miR-34b/c methylation level has been found (data not shown).

### DISCUSSION

Growing evidence has shown that miR-34b/c is downregulated in a series of cancers, such as colorectal cancer,

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**Table 3. Association Between the miR-34b/c rs4938723 Polymorphism and Clinical Features of PTC Patients**

| Clinical Features | Genotype Frequency | N, % | N, % | OR (95% CI) | P |
|-------------------|--------------------|------|------|-------------|---|
| **T status**      |                    |      |      |             |   |
| TT                | 88 (35.6)          | 169 (34.6) |      | 1.00        |   |
| CT                | 124 (50.2)         | 249 (50.9) | 1.05 (0.75–1.46) | 0.80 |
| CC                | 35 (14.2)          | 71 (14.5)  | 1.06 (0.65–1.71) | 0.82 |
| T                 | 300 (60.7)         | 587 (60.0) |      |            |   |
| C                 | 194 (39.3)         | 391 (40.0) | 1.03 (0.83–1.29) | 0.79 |
| **N status**      |                    |      |      |             |   |
| TT                | 91 (30.7)          | 178 (36.7) |      | 1.00        |   |
| CT                | 159 (53.7)         | 242 (49.9) | 0.78 (0.56–1.07) | 0.13 |
| CC                | 46 (15.5)          | 65 (13.4)  | 0.72 (0.46–1.14) | 0.16 |
| T                 | 341 (57.6)         | 598 (61.6) |      |            |   |
| C                 | 251 (42.4)         | 372 (38.4) | 0.85 (0.69–1.04) | 0.11 |
| **Clinical stages** |                  |      |      |             |   |
| TT                | 164 (33.1)         | 99 (38.2)  |      | 1.00        |   |
| CT                | 264 (53.2)         | 120 (46.3) | 0.75 (0.54–1.05) | 0.09 |
| CC                | 68 (13.7)          | 40 (15.4)   | 0.97 (0.61–1.55) | 0.91 |
| T                 | 592 (59.4)         | 318 (61.3) |      |            |   |
| C                 | 400 (40.6)         | 200 (38.7) | 0.93 (0.75–1.16) | 0.52 |
| **Multifocality** |                    |      |      |             |   |
| No                | 191 (34.5)         | 80 (34.8)   |      | 1.00        |   |
| Yes               | 291 (52.5)         | 111 (48.3) | 0.91 (0.65–1.28) | 0.59 |
| **Boldfaced values indicate a significant difference at the 5% level.**

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**Table 4. Association Between the TP-53 Arg72Pro Polymorphism and Clinical Features of PTC Patients**

| Clinical Features | Genotype Frequency | N, % | N, % | OR (95% CI) | P |
|-------------------|--------------------|------|------|-------------|---|
| **T status**      |                    |      |      |             |   |
| TT                | 72 (29.7)          | 132 (27.0) |      | 1.00        |   |
| CG                | 118 (47.8)         | 244 (49.9) | 1.13 (0.79–1.62) | 0.51 |
| CC                | 57 (23.1)          | 113 (23.1) | 1.08 (0.70–1.66) | 0.72 |
| G                 | 262 (52.2)         | 508 (51.9) |      | 1.00        |   |
| C                 | 232 (47.8)         | 470 (48.1) | 1.05 (0.84–1.30) | 0.69 |
| **N status**      |                    |      |      |             |   |
| N0                | 91 (30.7)          | 126 (26.0) |      | 1.00        |   |
| N1                | 149 (50.3)         | 233 (48.0) | 1.13 (0.81–1.59) | 0.48 |
| **Clinical stages** |                  |      |      |             |   |
| I and II          | 435 (39.3)         | 261 (44.1) | 1.27 (1.03–1.56) | 0.02 |
| III and IV        | 139 (28.0)         | 69 (26.0)   |      | 1.00        |   |
| **Multifocality** |                    |      |      |             |   |
| No                | 268 (48.4)         | 116 (50.4) | 0.87 (0.61–1.25) | 0.45 |
| Yes               | 141 (25.5)         | 42 (18.3)   | 0.60 (0.38–0.94) | 0.02 |

**Boldfaced values indicate a significant difference at the 5% level.**

Growing evidence has shown that miR-34b/c is downregulated in a series of cancers, such as colorectal cancer,
TABLE 5. The Combined Genotypes Frequencies of the miR-34b/c rs4938723 and TP-53 Arg72Pro Between Patients With PTC and Controls

| Patients N = 784, % | Controls N = 1006, % | OR (95% CI) | P     |
|---------------------|----------------------|-------------|-------|
| rs4938723TT + TP-53GG | 80 (10.2)            | 182 (18.1)  | 1.00  |
| rs4938723CT/CC + TP-53CG/CC | 376 (48.0) | 381 (37.9)  | **2.25 (1.67–3.03)** | **<0.001** |
| rs4938723TT + TP-53CG/CC | 191 (24.4)            | 274 (27.2)  | 1.00  |
| rs4938723CT/CC + TP-53GG | 137 (17.5)            | 169 (16.8)  | 1.16 (0.87–1.56) | 0.31  |

Boldfaced values indicate a significant difference at the 5% level.
factors may influence the results. Another is that the molecular mechanism is not involved in the study, which should be characterized in the future. Finally, the PCR-restriction fragment length polymorphism method may be subject to false positive results. To minimize the false positive rate, we have taken quality control.

In summary, we evaluated the impact of the genetic variability of the miR-34b/c rs4938723 and TP-53 Arg72Pro on PTC risk, and for the first time, we found that the 2 polymorphisms were associated with a significantly increased risk of PTC in the Chinese population. Further studies in different populations are warranted to confirm these findings.

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