Effect of TIMP2/TIMP3 genes on the risk of osteosarcoma in Zhejiang population

Zhongwei Wu, MDa, Huali Chen, MDb, Liwei Pan, MDc, Weiyang Yu, MDa, Chao Lou, MDa, Jian Chen, MDa, Dengwei He, PHDa,b,c

Abstract
Osteosarcoma is a malignant tumor that develops from a mesenchymal cell line and is caused by gene–environment interactions. This study aimed to explore whether TIMP2/TIMP3 polymorphisms influenced the osteosarcoma risk.

The expression of the TIMP2 and TIMP3 genes in osteosarcoma histiocytes was analyzed by immunohistochemistry. In this case-control study, which includes samples from 499 patients and 500 healthy controls, 10 single-nucleotide polymorphisms (SNPs) in TIMP2 and TIMP3 were selected. Furthermore, we used the Agena MassARRAY platform for genotyping. The statistical analysis was performed using χ² test/Fisher exact test, and logistic regression analysis.

The immunohistochemistry results showed that the expression of TIMP2 is obvious higher in osteosarcoma histiocytes than in the normal histiocytes. The association study indicated that the allele of rs2277698 and rs4789936 were protective SNPs reducing the risk of osteosarcoma (odds ratios > 1, P < .05) by the χ² test. In the genetic model, logistic regression analyses revealed that the rs2277698 and rs4789936 were associated with decreasing the risk of osteosarcoma under the codominant model, dominant model, and log-additive model. Stratification analysis revealed that 2 SNPs (rs2277698 and rs4789936) were significantly associated with a reduced risk of osteosarcoma in allele and genetic model after stratification by gender or age (P < .05). In addition, the haplotype “Trs2277698Crs2009169Crs7342880” of TIMP2 was associated with decreasing the osteosarcoma risk. The “Ars9609634Trs11547635” of TIMP3 was associated with reducing the osteosarcoma risk. This finding shed new light on the high expression of TIMP2 polymorphisms may contribute to decreasing the osteosarcoma risk in Zhejiang populations.

Abbreviations: 95% CI = 95% confidence interval, HWE = Hardy–Weinberg equilibrium, LD = linkage disequilibrium, MMPs = matrix metalloproteinases, OR = odds ratio, TIMPs = the tissue inhibitors of metalloproteinases.

Keywords: genetic polymorphism, osteosarcoma, TIMP2, TIMP3, Zhejiang populations

1. Introduction
Osteosarcoma, one of the most common primary bone tumors, is highly aggressive and easily metastasizes which mainly occurs in teenagers and young adults.[1] It develops from the mesenchymal cell line.[2] The tumor grows rapidly and its prognosis is generally poor, accompanied by high mortality. Annual morbidity rate of osteosarcoma is about 0.3 to 0.5 per 10 million people across the world, and it presents a bimodal age distribution with peaks at 15 to 19 years old and 70 years old.[3] The estimated 5-year survival rate of patients with distal metastasis is less than 30%, which makes osteosarcoma a severe threat to young patients.[4,5] It is known to all that osteosarcoma is complex and multifactorial disease, and the carcinogenesis of those malignant bone tumors is still uncertain.[6]

At present, a lot of research has been reported that there are gene–environment interactions in the carcinogenesis of malignant bone tumors.[7,8] However, under the same risk factors, the onset of different individuals is different, which suggests that individual genetic background may play an essential role in determining the development of osteosarcoma.[9,10] And this genetic background differences in the population mainly manifested as the single-nucleotide polymorphism (SNP). Therefore, the genetic susceptibility factors play a vital role in the development of osteosarcoma. Previously, genetic linkage analysis and candidate gene association studies in osteosarcoma have implicated several loci and candidate genes, for example, several study showed that the X-ray repair cross-complementing group-1 (XRCC1)[10] excision repair cross-complementation (ERCC)[10,11] 5,10-methylenetetrahydrofolate reductase (MTHFR),[12] insulin-like growth factor 1 (IGF-1)[13] the apurinic/apyrimidinic endonuclease (APE1)[14] and tumor suppressor gene TP53.[15] The tissue inhibitors of metalloproteinases (TIMPs) including TIMP2 and TIMP3 are the key physiological inhibitors of matrix
metalloproteinases (MMPs) and along with MMPs, TIMPs play a vital role in the basement membrane that represent the barriers to any malignant tumor invasion and progression.[16] Many studies have reported TIMP2 and TIMP3 may be risk factors developing complex diseases,[17] including colorectal cancer,[15] urinary bladder cancer,[16] coronary artery disease and myocardial infarction,[11] and lumbar disc degeneration.[10] However, few studies investigated the association of the TIMP2 and TIMP3 genes susceptibility to the osteosarcoma. Therefore, we performed a case-control study to analyze the association between the TIMP2 and TIMP3 genes and the risk of osteosarcoma from the teenagers in Zhejiang Province.

2. Materials and methods

2.1. Subject recruitment and ethics committee statement
We performed a case-control study to determine the association between TIMP2/TIMP3 polymorphisms and osteosarcoma risk. A total of 499 osteosarcoma cases, and 500 controls were recruited from The Central Hospital of Lishui City between January 2016 and January 2019. Detailed recruitment and exclusion criteria were used. All the osteosarcoma cases were newly diagnosed and histologically confirmed. Patients who had any previous history of other cancers and who had undergone radiotherapy or chemotherapy before surgery were excluded. Control subjects were randomly selected from the medical examination center at the same hospital during the similar period.

All participants were informed both in writing and verbally of the procedures and purpose of the study, and they signed informed consent documents. The use of human tissue and the protocol in this study were strictly conformed to the principles expressed in the Declaration of Helsinki, and this study was carried out with approval from the ethics committee of The Central Hospital of Lishui City. All the subsequent research analyses were carried out in accordance with the approved guidelines and regulations.

2.2. Immunohistochemical (IHC) evaluation
The expression of TIMP2 in the osteosarcoma tissue was also detected using immunohistochemistry. Specimens obtained from surgical resection were fixed in 10% formalin prior to being processed in paraffin. Immunohistochemical staining was performed using an EnVision TM HRP-polymer anti-mouse IHC Kit (K8002, Dako BioTECH, Shenzhen, China) according to the manufacturer’s guidelines. The sections were stained within 5 days of cutting using an Autostainer Link48 (Dako, California, USA) in strict accordance with the manufacturer’s instructions. The primary antibodies specific for TIMP2 (mouse TIMP2 (sc-21,735; Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:50) were obtained from Sigma-Aldrich (St. Louis, MO). Finally, we observed the images of the scanned tissue slices through Aperio ImageScope (Version 11.1.2.752).

2.3. SNP selection and genotyping
A GoldMag–Mini Purification Kit (GoldMag Co Ltd, Xi’an City, China) was used to extract genomic DNA from whole-blood samples. DNA samples were stored at –20°C prior to analysis. At the same time, the concentrations and purity of the DNA were measured by using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) at a wavelength of A260 and A280 nm.

Ten tag SNPs in TIMP2 and TIMP3 were selected for our study. These SNPs had minor allele frequencies greater than 5% according to the 1000 Genomes Project (http://www.internatio nalgenome.org/). The primers were designed online (https:// agenacx.com/online-tools/). Agena MassARRAY Assay Design 4.0 software was used to design a multiplexed SNP MassEXTEND assay, and SNP genotyping was performed using the Agena MassARRAY RS1000 with manufacturer protocols. Agena Typer 4.0 software was used to perform data management and analyses.

2.4. Statistical analysis
Data analysis was performed using Microsoft Excel (Redmond, WA) and SPSS 19.0 statistical package (SPSS, Chicago, IL). Each SNP frequency in the control subjects was assessed for departure from Hardy–Weinberg Equilibrium (HWE) using an exact test. We calculated genotype frequencies of cases and controls using a χ² test. Odds ratios (ORs) and 95% confidence intervals (CIs) were determined using unconditional logistic regression with adjustment for age and sex. Five genetic models (codominant, dominant, recessive, and additive) were performed using PLINK software (http://zzz.bwh.harvard.edu/plink/anal.shtml), to characterize the potential association of TIMP2/TIMP3 polymorphisms and osteosarcoma risk. Finally, we used Haploviz software package (version 4.2) to evaluate pairwise linkage disequilibrium (LD), haplotype construction, and genetic association of the polymorphic loci. All P values were 2-sided, and P < .05 was indicated statistical significance.

3. Result

3.1. The expression of TIMP2/TIMP3 in the primary osteosarcoma histiocytes
As shown in Figure 1, we observed the morphological observation of normal histiocytes and osteosarcoma histiocytes by hematoxylin–eosin staining showed that there are obvious differences in morphology between osteosarcoma histiocytes and normal histiocytes under the electron microscope (×20), and the size and shape of osteosarcoma histiocytes are inconsistent, and the volume of nucleus increased (Fig. 1, A and B). Representative photomicrographs of staining intensity of TIMP2 and TIMP3 expressions in osteosarcoma histiocytes and normal histiocytes are shown in Figure 1C to F. Compared with Figure 1C, TIMP2 expression was obviously enhanced in osteosarcoma histiocytes (Fig. 1D). However, there was no significant difference in the expression of TIMP3 between osteosarcoma histiocytes (Fig. 1E) and normal histiocytes (Fig. 1F).

3.2. Characteristics of the participants
This study included 999 subjects, including 499 patients (321 males and 178 females) and 500 healthy subjects (297 males and 203 females). The mean ages of teenagers were 15.12 ± 4.26 years for patients and 15.61 ± 5.73 years for controls. The mean ages of old peoples were 66.34 ± 3.76 years for patients and 67.08 ± 5.32 years for controls. The cases and controls were matched by age and sex, and there were no significant differences in the
Table 1

The characteristic of case and control.

| Variable                  | Case   | %   | Control | %   | P  |
|---------------------------|--------|-----|---------|-----|----|
| Total                     | 499    |     | 500     |     |    |
| Gender                    |        |     |         |     |    |
| Male                      | 321    | 64.3| 297     | 59.4| >.05*
| Female                    | 178    | 35.7| 203     | 40.6|    |
| Teenagers Age (yr, SD)    | 15.12±4.26| | 15.61±5.73| | >.05† |
| Age<24                    | 386    | 77.3| 221     | 44.2|    |
| Old people Age (yr, SD)   | 66.34±3.76| | 67.08±5.32| | >.05† |
| Age>56                    | 112    | 22.7| 279     | 55.8|    |
| Clinical stages           |        |     |         |     |    |
| Stage II                  | 194    | 38.9|         |     |    |
| Stage III                 | 122    | 24.4|         |     |    |
| Stage IV                  | 183    | 36.7|         |     |    |

* P values were calculated from 2-sided χ² tests.
† P values were calculated by Student t tests.
distributions of age and sex between osteosarcoma patients and healthy controls ($P > .05$) (Table 1).

### 3.3. Associations between TIMP2 and TIMP3 SNPs and osteosarcoma risk

Ten SNPs in TIMP2 and TIMP3 were analyzed in this study. Allele frequencies and basic information for all SNPs are shown in Table 2. All SNPs were in HWE in the controls ($P > .05$). We used the $\chi^2$ test to assess the risk of gene polymorphisms in the allele model, the frequency of the “T” allele of rs2277698 was significantly lower in cases than in controls (32.7% vs 33.3%), which suggested that “T” allele of rs2277698 was associated with decreasing the risk of osteosarcoma ($OR = 0.62, 95\% CI: 0.25–0.97, P = .015$). The frequency of the “T” allele of rs715572 was significantly lower in cases than in controls (22.1% vs 28.3%), which suggested that “T” allele of rs715572 was a risk allele reducing the development of osteosarcoma ($OR = 0.32, 95\% CI: 0.17–0.25, P = .004$).

Furthermore, we assumed that the minor allele of each SNP as a risk factor compared with the wild-type allele. Four genetic models (codominant, dominant, recessive, and additive) were applied to analyze the associations between the SNPs and osteosarcoma risk using a logistic regression test. Our analyses showed that the rs2277698 in the TIMP2 (consisted of rs2277698, rs2009169, and rs7342880) was associated with decreasing the osteosarcoma risk ($OR = 0.66, 95\% CI: 0.48–0.96, P = .031$). The “AT” haplotype in the TIMP3 (consisted of rs9609643 and rs11547635) was associated with decreasing the osteosarcoma risk ($OR = 0.64, 95\% CI: 0.43–0.91, P = .046$).

### 3.4. LD and haplotype association analysis

Linkage disequilibrium and haplotype analyses of the SNPs in the case and control samples were further studied. Linkage disequilibrium structure is shown in Figure 2. We observed that the SNPs rs2277698, rs2009169, and rs7342880 in the TIMP1 had very strong linkage disequilibrium, it forms one LD block. One block was detected in studied TIMP2 SNPs (rs9609643 and rs11547635) by haplotype analyses.

The haplotypes of the different blocks of each gene were calculated as shown in Table 4. The most frequent haplotype was used as reference, haplotype analysis of genes TIMP2 and TIMP3 detected significant association with the risk of osteosarcoma. The result showed that the “TCC” haplotype in the TIMP2 (consisted of rs2277698, rs2009169, and rs7342880) was associated with decreasing the osteosarcoma risk ($OR = 0.65, 95\% CI: 0.48–0.97, P = .015$). The “TT” haplotype in the TIMP3 (consisted of rs9609643 and rs11547635) was associated with decreasing the osteosarcoma risk ($OR = 0.64, 95\% CI: 0.43–0.91, P = .046$).

### 3.5. Stratification analysis

As shown in Table 5, we implemented a stratification analysis by gender and age to evaluate sex and age-specific associations between SNP alleles and osteosarcoma risk. In the allele model, we found that rs2277698 (TIMP2) significantly reduced the risk of osteosarcoma in males ($OR = 0.57, 95\% confidence interval [95\% CI]: 0.25–0.97, P = .004$). The frequency of the “T” allele of rs715572 was significantly lower in cases than in controls (22.1% vs 28.3%), which suggested that “T” allele of rs715572 was a risk allele reducing the development of osteosarcoma ($OR = 0.32, 95\% CI: 0.17–0.25, P = .004$).

Furthermore, we assumed that the minor allele of each SNP as a risk factor compared with the wild-type allele. Four genetic models (codominant, dominant, recessive, and additive) were applied to analyze the associations between the SNPs and osteosarcoma risk using a logistic regression test. Our analyses showed that the rs2277698 in the TIMP2 (consisted of rs2277698, rs2009169, and rs7342880) was associated with decreasing the osteosarcoma risk ($OR = 0.66, 95\% CI: 0.48–0.96, P = .031$). The “AT” haplotype in the TIMP3 (consisted of rs9609643 and rs11547635) was associated with decreasing the osteosarcoma risk ($OR = 0.64, 95\% CI: 0.43–0.91, P = .046$).

### Table 2

| SNPs       | Locus     | Gene(s) | Alleles A/B | Case   | Control | HWE-p | OR (95% CI) | $P^*$-values |
|------------|-----------|---------|-------------|--------|---------|--------|-------------|--------------|
| rs2277698  | 17q25.3   | TIMP2   | T/C         | 0.327  | 0.333  | 0.25   | 0.19 (0.16–0.23) | .015         |
| rs2009169  | 17q25.3   | TIMP2   | A/C         | 0.218  | 0.271  | 0.086  | 0.48 (0.17–1.89) | .216         |
| rs7342880  | 17q25.3   | TIMP2   | C/T         | 0.212  | 0.251  | 0.277  | 0.33 (0.24–1.70) | .614         |
| rs11654470 | 17q25.3   | TIMP2   | C/T         | 0.116  | 0.131  | 0.134  | 0.46 (0.28–0.92) | .142         |
| rs2003240  | 17q25.3   | TIMP2   | T/C         | 0.221  | 0.283  | 1.000  | 0.32 (0.17–0.88) | .0014        |
| rs4789396  | 17q25.3   | TIMP2   | T/C         | 0.102  | 0.119  | 0.579  | 1.02 (0.81–1.28) | .864         |
| rs1238603  | 17q25.3   | TIMP2   | A/G         | 0.302  | 0.319  | 0.777  | 0.96 (0.79–1.18) | .721         |
| rs8609643  | 17q25.3   | TIMP2   | T/G         | 0.058  | 0.058  | 0.226  | 0.62 (0.31–1.84) | .135         |
| rs11547635 | 17q25.3   | TIMP2   | T/C         | 0.151  | 0.129  | 0.861  | 1.04 (0.85–2.81) | .323         |

MAF = minor allele frequency. HWE = Hardy–Weinberg equilibrium. $P^*$-values were calculated using 2-sided $\chi^2$ test. $P < .05$ indicates statistical significance.
| SNPs     | Models     | Genotype | Control | Case   | OR (95% CI) | P value | AIC   | BIC   |
|----------|------------|----------|---------|--------|-------------|---------|-------|-------|
| rs2277698 | Codominant | C/C      | 217     | 183    | 0.75 (0.66–1.63) | .012    | 519.8 | 540.9 |
|          |            | C/T      | 237     | 258    | 0.64 (0.43–0.83)  |         |       |       |
|          |            | T/T      | 46      | 59     |             |         |       |       |
|          | Dominant   | C/C      | 217     | 183    | 0.56 (0.21–0.92)  | .004    | 517.8 | 534.7 |
|          |            | C/T-T/T  | 283     | 317    |             |         |       |       |
|          | Recessive  | C/C-C/T  | 454     | 441    | 0.84 (0.43–2.43)  | .960    | 517.9 | 534.7 |
|          |            | T/T      | 46      | 59     |             |         |       |       |
|          | Log-additive | –      |        |        | 0.38 (0.29–0.89)  | .039    | 517.8 | 534.7 |
| rs2009196 | Codominant | C/C      | 300     | 398    | 0.75 (0.66–1.63) |         |       |       |
|          |            | C/T      | 178     | 106    | 1.19 (0.65–2.18)  |         |       |       |
|          |            | T/T      | 29      | 3      |             |         |       |       |
|          | Dominant   | C/C      | 300     | 398    | 0.56 (0.21–0.92)  |         |       |       |
|          |            | C/T-C/T  | 283     | 317    |             |         |       |       |
|          | Recessive  | C/C-C/T  | 454     | 441    | 1.43 (0.87–2.37)  |         |       |       |
|          |            | T/T      | 46      | 59     |             |         |       |       |
|          | Log-additive | –      |        |        | 1.08 (0.80–1.46)  | .160    | 517.6 | 534.4 |
| rs7342880 | Codominant | C/C      | 116     | 266    | 0.75 (0.45–1.26)  |         |       |       |
|          |            | C/A      | 268     | 196    |             |         |       |       |
|          |            | A/A      | 123     | 37     |             |         |       |       |
|          | Dominant   | C/C      | 116     | 266    | 0.88 (0.54–1.42)  |         |       |       |
|          |            | C/A-A/A  | 391     | 232    |             |         |       |       |
|          | Recessive  | C/C-C/A  | 384     | 461    | 1.05 (0.67–1.63)  |         |       |       |
|          |            | A/A      | 123     | 37     |             |         |       |       |
|          | Log-additive | –      |        |        | 1.08 (0.80–1.46)  | .160    | 517.6 | 534.4 |
| rs11654470 | Codominant | T/T      | 209     | 124    | 0.96 (0.61–1.51)  |         |       |       |
|          |            | T/C      | 239     | 257    | 1.02 (0.48–2.14)  |         |       |       |
|          |            | C/C      | 59      | 118    |             |         |       |       |
|          | Dominant   | T/T      | 209     | 124    | 0.97 (0.63–1.50)  |         |       |       |
|          |            | T/C-C/C  | 298     | 375    |             |         |       |       |
|          | Recessive  | T/T-T/C  | 448     | 381    |             |         |       |       |
|          |            | C/C      | 59      | 124    |             |         |       |       |
|          | Log-additive | –      |        |        | 1.03 (0.71–1.37)  | .296    | 518.8 | 535.6 |
| rs2003241 | Codominant | T/T      | 327     | 203    | 0.75 (0.45–1.26)  |         |       |       |
|          |            | T/C      | 154     | 248    | 0.97 (0.36–2.59)  |         |       |       |
|          |            | C/C      | 18      | 48     |             |         |       |       |
|          | Dominant   | T/T      | 327     | 203    | 1.02 (0.48–2.14)  |         |       |       |
|          |            | T/C-C/C  | 172     | 296    |             |         |       |       |
|          | Recessive  | T/T-T/C  | 481     | 451    |             |         |       |       |
|          |            | C/C      | 16      | 48     |             |         |       |       |
|          | Log-additive | –      |        |        | 0.99 (0.71–1.37)  | .296    | 518.8 | 535.6 |
| rs4789036 | Codominant | C/C      | 260     | 209    | 0.65 (0.42–1.96)  | .034    | 515.7 | 536.7 |
|          |            | C/T      | 197     | 236    |             |         |       |       |
|          |            | T/T      | 43      | 55     |             |         |       |       |
|          | Dominant   | C/C      | 260     | 209    | 0.62 (0.25–0.91)  |         |       |       |
|          |            | C/T-T/T  | 240     | 301    |             |         |       |       |
|          | Recessive  | C/C-C/T  | 457     | 445    |             |         |       |       |
|          |            | T/T      | 43      | 55     |             |         |       |       |
|          | Log-additive | –      |        |        | 0.72 (0.51–0.95)  | .023    | 514.1 | 530.9 |
| rs715572  | Codominant | G/G      | 227     | 316    | 1.18 (0.75–1.87)  |         |       |       |
|          |            | A/A      | 248     | 172    | 0.88 (0.43–1.80)  |         |       |       |
|          | Dominant   | G/G      | 227     | 316    | 1.11 (0.72–1.71)  |         |       |       |
|          |            | G/A-A/A  | 273     | 191    |             |         |       |       |
|          | Recessive  | G/G-G/A  | 475     | 488    |             |         |       |       |
|          |            | A/A      | 25      | 19     |             |         |       |       |
|          | Log-additive | –      |        |        | 1.01 (0.74–1.39)  | .193    | 517.6 | 534.4 |
| rs8136803 | Codominant | G/G      | 205     | 179    | 0.59 (0.29–1.19)  | .031    | 519.3 | 540.3 |
|          |            | G/T      | 231     | 237    |             |         |       |       |
|          |            | T/T      | 63      | 83     |             |         |       |       |
|          | Dominant   | G/G      | 205     | 179    | 0.59 (0.29–1.19)  |         |       |       |
|          |            | G/T-T/T  | 294     | 320    |             |         |       |       |
|          | Recessive  | G/G-G/T  | 436     | 416    |             |         |       |       |
|          |            | T/T      | 63      | 83     |             |         |       |       |

(continued)
Table 3 (continued).

| SNPs      | Models       | Genotype | Control | Case | OR (95% CI) | P value | AIC   | BIC   |
|-----------|--------------|----------|---------|------|-------------|---------|-------|-------|
| rs9609643 | Log-additive | –        | –       | –    | 0.59 (0.29–1.18) | .113    | 517.3 | 534.2 |
|           | Codominant   | G/G      | 241     | 248  | 1           | .279    | 521   | 542   |
|           |              | G/A      | 203     | 191  | 0.95 (0.57–1.57) |       |       |       |
|           |              | A/A      | 56      | 60   | 1.86 (0.28–12.48) |       |       |       |
|           | Dominant     | G/G      | 241     | 251  | 1           | .394    | 519.4 | 536.3 |
|           |              | G/A-A/A  | 259     | 151  | 0.98 (0.60–1.61) |       |       |       |
|           | Recessive    | G/G-G/A  | 444     | 439  | 1           | .251    | 519   | 535.8 |
|           |              | A/A      | 56      | 60   | 1.88 (0.28–12.58) |       |       |       |
| rs11547635| Log-additive | –        | –       | –    | 1.02 (0.65–1.61) | .193    | 519.4 | 536.2 |
|           | Codominant   | C/C      | 278     | 218  | 1           | .188    | 517.7 | 538.8 |
|           |              | T/C      | 164     | 231  | 1.06 (0.68–1.67) |       |       |       |
|           |              | T/T      | 58      | 50   | 1.22 (0.56–2.66) |       |       |       |
|           | Dominant     | C/C      | 278     | 218  | 1           | .171    | 515.9 | 532.7 |
|           |              | T/C-T/T  | 222     | 281  | 1.09 (0.71–1.67) |       |       |       |
|           | Recessive    | C/C-T/C  | 442     | 449  | 1           | .166    | 515.8 | 532.6 |
|           |              | T/T      | 58      | 50   | 1.18 (0.56–2.49) |       |       |       |
|           | Log-additive | –        | –       | –    | 1.09 (0.78–1.52) | .162    | 515.8 | 532.6 |

AIC = Akaike’s Information criterion, BIC = Bayesian Information criterion, CI = confidence interval, OR = odds ratio.
P values were calculated from Wald test adjusted for age and sex.
P < .05 indicates statistical significance.

Table 4

Haplotype analysis results in this study.

| Chromosome | Gene  | SNPs                      | Haplotype | OR (95% CI) | P values |
|------------|-------|---------------------------|-----------|-------------|----------|
| chr17      | TIMP2 | rs2277689(rs2009169)rs7342880 | CGC       | 1           | –        |
|            |       |                           | TCC       | 0.66 (0.48–0.96) | .031    |
|            |       |                           | CCA       | 0.90 (0.59–1.38) | .620    |
|            |       |                           | CCC       | 0.76 (0.43–1.34) | .350    |
|            |       |                           | GC        | 1           | .631    |
|            |       |                           | AT        | 0.64 (0.43–0.91) | .046    |
|            |       |                           | GT        | 0.89 (0.59–1.36) | .189    |
| chr22      | TIMP3 | rs9609643(rs11547635)     | GC        | 1           | .631    |
|            |       |                           | AT        | 0.64 (0.43–0.91) | .046    |
|            |       |                           | GT        | 0.89 (0.59–1.36) | .189    |

CI = confidence interval, OR = odds ratio, SNP = single-nucleotide polymorphism.
P indicates adjusted by gender and age.
P < .05 indicates statistical significance.

Figure 2. Haplotype block map for the TIMP2 and TIMP3 SNPs genotype in this study. SNP = single-nucleotide polymorphism.
### Table 5

The association between sex and age stratification and osteosarcoma risk in allele and genotype models.

| SNPs   | Alleles | Male OR (95% CI) | Male p | Female OR (95% CI) | Female p | Age < 24 OR (95% CI) | Age < 24 p | Age ≥ 56 OR (95% CI) | Age ≥ 56 p |
|--------|---------|------------------|--------|-------------------|----------|----------------------|------------|----------------------|-----------|
| rs2277698 | C/C    | 1.034            | 1      | 0.041             | 1        | 0.057                | 1          | 0.037                | 1         |
|         | C/T    | 0.79 (0.53–1.28) | 1      | 0.95 (0.77–1.14)  | 1        | 0.80 (0.74–1.50)     | 1          | 0.92 (0.88–1.90)     | 1         |
|         | T/T    | 0.57 (0.25–0.92) | 1      | 0.6 (0.33–0.95)   | 1        | 0.43 (0.26–0.91)     | 1          | 0.51 (0.24–0.76)     | 1         |
|         | C      | 1.029            | 1      | 0.616             | 1        | 0.28                 | 1          | 0.47                 |           |
|         | T      | 0.35 (0.26–0.77) | 1      | 1.49 (0.30–1.92)  | 1        | 0.32                 | 1          | 0.43 (0.23–0.81)     | 1         |
| rs2009196 | C/C    | 1.123            | 1      | 0.085             | 1        | 0.21                 | 1          | 0.56                 |           |
|         | C/G    | 0.54 (0.38–1.78) | 1      | 0.83 (0.67–1.13)  | 1        | 0.71                 | 1          | 0.77 (0.59–1.01)     | 1         |
|         | G/G    | 0.68 (0.21–2.57) | 1      | 1.15 (0.84–1.97)  | 1        | 0.62                 | 1          | 1.03 (0.65–1.64)     | 1         |
| rs7342880 | C/C    | 1.186            | 1      | 0.206             | 1        | 0.59                 | 1          | 0.89                 |           |
|         | C/G    | 0.52 (0.82–2.83) | 1      | 1.17 (0.67–2.40)  | 1        | 1.12                 | 1          | 0.98                 |           |
|         | G/G    | 1.359            | 1      | 0.73 (0.54–1.99)  | 1        | 0.54                 | 1          | 1.16                 |           |
| rs11654470 | T/T    | 1.039            | 1      | 0.087             | 1        | 0.07                 | 1          | 0.90                 |           |
|         | T/C    | 1.01 (0.70–1.56) | 1      | 0.97 (0.79–1.19)  | 1        | 0.98                 | 1          | 0.90                 |           |
|         | C/C    | 0.89 (0.56–2.31) | 1      | 1.21 (0.90–2.00)  | 1        | 0.75                 | 1          | 1.01                 |           |
| rs2003241 | T/T    | 1.342            | 1      | 0.542             | 1        | 0.19                 | 1          | 0.21                 |           |
|         | T/C    | 1.26 (0.89–2.04) | 1      | 1.19 (0.86–1.61)  | 1        | 1.07                 | 1          | 1.02                 |           |
|         | C/C    | 0.77 (0.71–2.16) | 1      | 1.24 (0.59–2.07)  | 1        | 1.20                 | 1          | 0.98                 |           |
|         | T      | 1.176            | 1      | 1.149             | 1        | 1.15                 | 1          | 0.96                 |           |
| rs4789936 | C/C    | 1.15 (0.94–1.84) | 1      | 1.02 (0.64–1.86)  | 1        | 0.79                 | 1          | 0.96                 |           |
|         | C/T    | 1.05 (0.78–1.43) | 1      | 0.97 (0.66–1.43)  | 1        | 0.98                 | 1          | 0.90                 |           |
|         | C/C    | 0.89 (0.56–2.31) | 1      | 1.21 (0.90–2.00)  | 1        | 0.75                 | 1          | 1.01                 |           |
| rs9509643 | T/T    | 1.342            | 1      | 0.542             | 1        | 0.19                 | 1          | 0.21                 |           |
|         | C/C    | 1.039            | 1      | 0.087             | 1        | 0.08                 | 1          | 0.92                 |           |
|         | C/T    | 1.01 (0.70–1.56) | 1      | 0.97 (0.79–1.19)  | 1        | 0.98                 | 1          | 0.90                 |           |
|         | T/T    | 0.89 (0.56–2.31) | 1      | 1.21 (0.90–2.00)  | 1        | 0.75                 | 1          | 1.01                 |           |
| rs11547635 | C/C    | 1.12 (0.86–1.91) | 1      | 1.25 (0.84–1.93)  | 1        | 0.77                 | 1          | 1.01                 |           |
|         | C/T    | 1.12 (0.86–1.91) | 1      | 1.25 (0.84–1.93)  | 1        | 0.77                 | 1          | 1.01                 |           |
|         | T/T    | 1.12 (0.86–1.91) | 1      | 1.25 (0.84–1.93)  | 1        | 0.77                 | 1          | 1.01                 |           |
|         | C      | 1.12 (0.86–1.91) | 1      | 1.25 (0.84–1.93)  | 1        | 0.77                 | 1          | 1.01                 |           |
|         | T      | 1.12 (0.86–1.91) | 1      | 1.25 (0.84–1.93)  | 1        | 0.77                 | 1          | 1.01                 |           |

95% CI = 95% confidence interval, OR = odds ratio.
P = values were calculated from Wald test adjusted for age.
P* = values were calculated from Wald test adjusted for gender.
P<.05 indicates statistical significance.

P = .026, females (log-additive model: OR = 0.65, 95% CI = 0.36–0.89, P = .042), the population under 24 years of age (dominant model: OR = 0.66, 95% CI = 0.47–0.93, P = .031; log-additive model: OR = 0.72, 95% CI = 0.55–0.94, P = .029), and over 56 years of age (dominant model: OR = 0.62, 95% CI = 0.35–0.81, P = .036). Also, rs4789936 has a protective effect in reducing the risk of osteosarcoma in males (dominant model: OR = 0.58, 95% CI = 0.36–0.91, P = .029 for the “C/T-T/T”
genotype; log-additive model: OR = 0.56, 95% CI = 0.33–0.94, P = .041), the population under 24 years of age (dominant model: OR = 0.67, 95% CI = 0.34–0.96, P = .011; log-additive model: OR = 0.66, 95% CI = 0.32–0.97, P = .042), and over 56 years of age (log-additive model: OR = 0.61, 95% CI = 0.49–0.88, P = .019).
4. Discussion
Genetic studies have provided insight into many diseases, including osteosarcoma. In the present case–control study, we investigated the associations between 10 SNPs in TIMP2 and TIMP3 genes and osteosarcoma risk in Zhejiang population. Our results show that the rs2277698 and rs4789936 in the TIMP2 were associated with decreasing the risk of osteosarcoma. These results suggested that the polymorphisms of TIMP2 gene may contribute to be a protective role reducing the osteosarcoma risk. In addition, we first used IHC to detect the expression of the TIMP2 and TIMP3 gene in normal histiocytes and osteosarcoma histiocytes. We found that the expression level of TIMP2 in osteosarcoma histiocytes was significantly higher than the normal histiocytes. We predicted that this gene may be a risky gene for osteosarcoma.

The TIMP2 is located on the long arm of chromosome 17 at position 25.3 (17q25.3). However, in addition to the MMP inhibitory activities, TIMPs play essential roles in many physiological processes including modulation of cell proliferation, migration, and invasion and synaptic plasticity. TIMPs influence tumor progression and metastasis through the inhibition of MMPs and through direct modulation of angiogenesis and apoptosis. Many studies have shown that TIMP2, as a disease susceptibility gene, can affect the development of cancers and other diseases. For examples, Mikolajczyk-Stecyna et al. reported that TIMP2 was associated with increasing the risk of abdominal aortic aneurysm in the Polish population. Banday and Sameer demonstrated that there was a strong and highly significant association between the TIMP2-418G/C promoter SNPs and the risk of developing CRC in ethnic Kashmiri population. An et al. showed the TIMP2 G>C (rs8179090) and G>A (rs2277698) alleles were strongly associated with primary ovarian insufficiency (POI), which suggested that the minor TIMP2 alleles may increase POI risk in Korean women. This study identified that the rs2277698 and rs4789936 in the TIMP2 were associated with decreasing the risk of osteosarcoma in Zhejiang populations, and found the expression level of TIMP2 in osteosarcoma histiocytes was significantly higher than the normal histiocytes.

Tissue inhibitor of metalloproteinase 3, a member of the TIMP family, is located on the long arm of chromosome 22 at position 12.3 (22q12.3), which functions as the antagonist of MMPs to guard homeostasis and affect physiological tissue remodeling and developmental processes by regulating cell growth, invasion, migration, apoptosis, and angiogenesis. Furthermore, genetic variation in TIMP3 has been linked with susceptibility to cardiovascular disorders and cancers. Peerea et al. found that the rs9862 variant of the TIMP3 gene was associated with severity of lumbar disc degeneration and modic changes. Srivastava et al. reported that TIMP3 gene was associated with reducing the risk of prostate cancer in North Indian cohort. Banday and Sameer demonstrated that the TIMP3-1296TC promoter SNPs was associated with decreased risk of colorectal cancer in ethnic Kashmiri population. However, few previous studies have reported associations between TIMP3 gene polymorphism and osteosarcoma risk. Moreover, there was no significant difference in the expression level of TIMP3 between normal tissue and osteosarcoma tissue.

Our study aimed to report the association between the polymorphisms of TIMP2 and TIMP3 and the osteosarcoma risk in the Zhejiang teenagers, which may provide new data to facilitate earlier diagnosis and promote early prevention, and shed light on the new candidate genes and new ideas for the study of subsequent occurrence mechanism of osteosarcoma. However, some potential limitations of our current study should be considered when deciphering the results. Our study only is a preliminary basic research, further functional studies and larger population-based prospective studies are required to understand the genetic factors underlying osteosarcoma in the subsequent research.

5. Conclusion
The results indicate that the expression level of TIMP2 in osteosarcoma histiocytes was significantly higher than the normal histiocytes. The polymorphisms of TIMP2 (rs2277698 and rs4789936) were significantly associated with decreasing the osteosarcoma risk.

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Author contributions
Conceptualization: Zhongwei Wu, Huali Chen.
Data curation: Liwei Pan, Jian Chen.
Formal analysis: Jian Chen.
Investigation: Weiyang Yu.
Methodology: Weiyang Yu.
Project administration: Dengwei He.
Resources: Weiyang Yu.
Supervision: Chao Lou, Dengwei He.
Writing – original draft: Zhongwei Wu, Huali Chen.
Writing – review & editing: Zhongwei Wu, Huali Chen.

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