Medicine

Single-Nucleotide Polymorphism of MMP-2 in MMPs/TIMPs Pathway Associated with Alcohol-induced Osteonecrosis of the Femoral Head Risk in Chinese Males

--Manuscript Draft--

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Abstract:	The proportion of alcohol -induced ONFH in all the ONFH patients was 30.7% and males dominated with 70.1% of all ONFH patients in mainland China. MMP-2, which is a member of the matrix metalloproteinases (MMPs) gene family, can promote osteoclast migration, attachment and bone matrix degradation. In this case-control study, we aimed to investigate the association between MMP-2 and the alcohol-induced ONFH in Chinese males. A total of 299 patients with alcohol-induce ONFH and 396 healthy controls were recruited for a case-control association study. Five single nucleotide polymorphisms (SNPs) within the MMP-2 locus were genotyped and examined their correlation with the risk of alcohol-induced ONFH and treatment response using Pearson $\chi 2$ and unconditional logistic regression analysis. We identified three risk alleles for carriers: the allele "T" of rs243849 increased the risk of alcohol-induce ONFH at allele model, log-additive model without adjusted and log-additive model with adjusted for age. Conversely, the genotype "CC" in rs7201 and the genotype "CC" in rs243832 decreased alcohol-induced ONFH risk was revealed by the recessive model. After Bonferroni's multiple adjustment, no significant data existed. Furthermore, the haplotype analysis showed that the "TT" haplotype of MMP-2 more frequent among patients with alcohol-induced ONFH by unconditional logistic regression analysis adjusted for age. There may be an association between MMP-2 and the risk of alcohol-induced ONFH in the Chinese north males. These data can provide a theoretical foundation for other researchers to further study and larger population-based studies are needed to confirm these hypothesis.

Dear editor and reviewers,

Thank you very much for your advice. Your questions and suggestions are very valuable and our manuscript have been revised according your suggestions. We would like to revised it for your consideration. Point by point responses to the reviewer's comments are listed below.

Comments to Author (required)

Reviewer #1:

Question 1: Which 5 SNPs were tested is not clear at introduction and material and methods. It is not clear why the authors choose those 5 polymorphisms.

Response: Thanks for your valuable questions and suggestions. *MMP-2* is a member of the matrix metalloproteinases (MMPs) gene family and the *MMPs/TIMPs* pathway was also found to impact bone metabolism of the human body and several genes in *MMPs/TIMPs* pathway were identified with their polymorphisms related to orthopedic diseases. In this study, five SNPs in *MMP-2* were selected and they were rs1053605, rs243849, rs243847, rs243832 and rs7201. These loci had been found contribute to an individual's disease susceptibility and the association between these five SNPs and alcohol-induced ONFH have not been examined. Thus, the aim of this study was to identified the association between the polymorphisms of these five SNPs with alcohol-induced ONFH in Chinese males. I have supplemented the corresponding information and the reason for the selection of five SNPs at introduction and material and methods.

Question 2: The authors referred only to TT haplotype, which is the frequency of the 5 SNPs haplotype?

Response: In the LD analysis, Red squares display statistically significant associations between a pair of SNPs, as measured by D'; darker shades of red indicate higher D'. From figure 1, two block have a Linkage disequilibrium in five SNPs and D' value of rs243849-rs243847 and rs243832-rs7201 blocks were 1 and 0.99. After haplotype analysis, only "TT" haplotype had a significant association with ONFH risk compared to the wild type "CT".

Reviewer #2:

Question 1: The authors didn't give comprehensive description to the genetic-association research in ONFH. How many genes/variation have been identified in ONFH before? Whether GWAS has been conducted? Any significant association between *MMP-2* and ONFH was identified? The introduction section should provide more logic process for the study and should be more concise while too much non-related contents were attached in the introduction section.

Response: Thanks for your hard working and valuable questions. I have accessed to the relevant reports and supplemented the information. We found that the polymorphisms of angiogenesis- and hypoxia-related genes have been confrimed the association with ONFH, such as *IGFBP3*, *VEGFC* and *ACE* so on. However, GWAS research and *MMP-2* about the ONFH risk are rarely. Some non-related contents in introduction section have been revision.

Question 2: In the current study, there is only one discovery dataset, no further independent validate dataset. It is difficult to eliminate the probability of false-positive finding. Even the author didn't set any positive and negative control, therefore, it is hard to judge whether the result is solid or not. In addition, when there are several SNPs were investigated in same study, multiple test correction should be conducted to control the false positive.

Response: Thanks for your valuable suggestions. In order to eliminate the probability of false-positive finding, Bonferroni's multiple adjustment was applied to the level of significance, which was set at $p<0.0017(0.05/30)$. However, we found no SNPs related to the risk of alcohol-induced ONFH after Bonferroni correction, which is a relative strictly correction approach. There may be an association existed between *MMP-2* and alcohol-induced ONFH, and further study is need to confirmed our hypothesis.

Question 3: In the LD analysis, which samples were used should be provided? Case, control or total samples? Meanwhile, for the LD block analysis, the detail method should be provided rather than only the 'software'.

Response: In the LD analysis, control samples were used to the haplotype

construction. The linkage disequilibrium degree of the Two SNPs is measured by D' value, and D' confidence interval is used to divide haplotype block. The D' value is 0 to 1. The D' value is close to 1, the level of linkage disequilibrium between the loci is stronger. For the LD plot, the color of the box reflects the strength of the linkage disequilibrium, and that darker shades of red indicate higher D' display statistically significant associations between a pair of SNPs. In this study, the D' value of rs243849-rs243847 block and rs243832-rs7201 block were 1 and 0.99 in the figure 1.

Reviewer #3:

Question 1: it appears that there is a significant difference of mean age between the case and control subjects. Meanwhile, there is no comparison of alcohol exposure and other risk factors of ONFH between the case and control subjects. Therefore, I am concerned that the case and control subjects are not well-matched (as shown by age), and may undermine the validity of the subsequent results.

Response: Thanks for your hard working and valuable questions. Many factors influence the risk of alcohol-induced ONFH, such as age and alcohol exposure. In this study, only part of the samples had the alcohol exposure information, thus it was difficult to compare alcohol exposure information in case-control samples. From Table 1, we know that the age in the case and control was not well-matched. To eliminate the influence of the age factor, unconditional logistic regression with adjusted for age was chosen to apply in our data subsequent genotype mode analysis and haplotype analysis.

Question 2: It is problematic to use 0.05 as significance threshold without accounting for multiple comparisons, since the authors performed quite a lot of statistical tests (for 5 SNPs, different models, Table 2-4). I also noticed that most of the p-values claimed as significant by the authors are actually close to 0.05, and am concerned that these findings will no longer be significant after correction for multiple comparison.

Response: Thank you for precious suggestions. I have noticed the problem about the 0.05 as significance threshold. Bonferroni correction was using in our date to

eliminate the probability of false-positive finding, and the level of significance was set at $p<0.0017(0.05/30)$. However, we found no SNPs related to the risk of alcohol-induced ONFH after Bonferroni correction, which is a relative strictly correction approach. It is possible that *MMP-2* has an association with the risk and further study need to confirm our hypothesis.

Question 3: What is the purpose of including AIC and BIC in table 3 and 4? Did the authors attempted to select a best model? Also, there is no details explaining the results on AIC and BIC in the main text.

Response: AIC and BIC in our article were used to selected a suitable model. BIC can select the true model among candidates model when true model is among them, if true model is not among them, AIC is efficient to asymptotically choose the model which minimizes the mean squared error of estimation. The relatively small value corresponding to the model is the appropriate model. In our study, two criterions were used to selected appropriate model.

Question 4: The authors claimed that rs243849 and rs243847 are in strong linkage disequilibrium. However, no r-squared or D' value was given to support the claim. Further, how did the authors determine haplotype phase in haplotype analyses? Data from the 1000 Genome Project East Asians suggested the frequency of rs243847-rs243849 CT haplotype is zero, while the authors showed that such frequency is ~0.4. The authors should explain such discrepancy.

Response: Thank you for your serious work. From the figure 1, rs243849 and rs243847 was in strong linkage disequilibrium and D' value was 1. Present study, we recruited 695 individuals, concluding 299 alcohol-induced ONFH patients and 396 healthy controls. The Sample size was not enough and relatively small. Because our candidate samples only came from Chinese north population, the range was limited. The collection of case samples is relatively difficult, and we need to spend a lot of time and experience to collect these samples. Thus, some randomness was existed in our research and the frequency of rs243847-rs243849 CT haplotype was not well matched for the the 1000 Genome Project East Asians suggestion.

We would like to thank the reviewers for valuable questions and suggestion to us. This is very helpful to our future research. If there are other errors or further requests please contact us and the structure of the manuscript will not changed. We hope that the revised version of the manuscript is acceptable for publication in your journal.

Best wishes for you.

Sincerely,

Heping Zhao

Single-Nucleotide Polymorphism of *MMP-2* in *MMPs/TIMPs* Pathway Associated with Alcohol-induced Osteonecrosis of the Femoral Head Risk in Chinese Males

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Abbreviations:

SNP, single nucleotide polymorphism; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitor of matrix metalloproteinases; ONFH, osteonecrosis of the femoral head; OR, odds ratios; CI, confidence intervals; LD, linkage disequilibrium; AIC, Akaike Information criterion; BIC, Bayesian Information criterion; HWE, Hardy–Weinberg equilibrium.

Abstract

The proportion of alcohol –induced ONFH in all the ONFH patients was 30.7% and males dominated with 70.1% of all ONFH patients in mainland China. *MMP-2*, which is a member of the matrix metalloproteinases (*MMPs*) gene family, can promote osteoclast migration, attachment and bone matrix degradation. In this case-control study, we aimed to investigate the association between *MMP-2* and the alcohol-induced ONFH in Chinese males. A total of 299 patients with alcohol-induce ONFH and 396 healthy controls were recruited for a case-control association study. Five single nucleotide polymorphisms within the *MMP-2* locus were genotyped and examined their correlation with the risk of alcohol-induced ONFH and treatment response using Pearson $\chi 2$ and unconditional logistic regression analysis. We identified three risk alleles for carriers: the allele "T" of rs243849 increased the risk of alcohol-induce ONFH at allele model, log-additive model without adjusted and log-additive model with adjusted for age. Conversely, the genotype "CC" in rs7201 and the genotype "CC" in rs243832 decreased alcohol-induced ONFH risk was revealed by the recessive model. After Bonferroni multiple adjusted, no significant data existed. Furthermore, the haplotype analysis showed that the "TT" haplotype of *MMP-2* more frequent among patients with alcohol-induced ONFH by unconditional logistic regression analysis adjusted for age. There may be an association between *MMP-2* and the risk of alcohol-induced ONFH in the Chinese north males. These data can provide a theoretical foundation for other researchers to further study and larger population-based studies are needed to confirm these hypothesis.

Key words: Single-nucleotide polymorphism (SNP); *MMP-2*; alcohol-induced osteonecrosis of the femoral head (ONFH); Chinese males

Introduction

Osteonecrosis of the femoral head (ONFH) is a kind of intractable and complex orthopedic injury that is characterized by osteocyte apoptosis, bony structure of the femoral head destructed and collapsed, finally leads to femoral head ischemia and death[1-3]. And the following revision is still a outstanding problem in the ONFH treatment procedure[4]. ONFH is divided into two types, traumatic and non-traumatic ONFH.

Alcohol-induced ONFH is a type of non-traumatic ONFH caused by chronic and excessive alcohol consumption, the incidence of this disease is rising in China. And data showed that in mainland China, males dominated with 70.1% of all ONFH patients and the proportion of alcohol–induced ONFH in all the ONFH patients was 30.7%[5]. One previous research had reported that if the alcohol intake was higher than 400ml per week, the risk of ONFH would be a clear increase[6]. Several pathogenic mechanisms associated with alcohol-induced ONFH risk are environmental factors, alcohol intake, dyslipidemia, blood coagulation disorders and ossification dysfunction of mesenchymal stem cells, but the exact pathogenesis of this disease is still controversial and unclear. Genetic researches have offered the potential insight into the occurrence and development of alcohol-induced ONFH. Zhang et al had conducted the association between *ABCB1* polymorphism and ONFH risk[7], and angiogenesis- and hypoxia-related gene polymorphisms have been confrimed the association with ONFH, such as *IGFBP3*, *VEGFC* and *ACE* so on[8]. Recently studies had suggested that *MMP-2* may be a key role in the process of bone metabolism and influence the proliferation/ differentiation of BMSCs in bone and joint diseases[9,10].

MMP-2 is a member of the matrix metalloproteinases (*MMPs*) gene family, that are zinc-dependent enzymes capable of cleaving components of the extracellular matrix and molecules involved in signal transduction. The Matrix metalloproteinases (*MMPs*)/tissue inhibitor of matrix metalloproteinases (TIMPs) pathway was also found to impact bone metabolism of the human body[11] and several genes in *MMPs*/*TIMPs* pathway were identified with their polymorphisms related to orthopedic diseases[12-14]. Some SNPs in *MMP-2* have been found an association with

several diseases, for example, rs1053605 is found associated with stroke outcome[15] but not with abdominal aortic aneurysm[16]; rs243849 polymorphism is significant related to ischemic stroke outcome[15]; rs243847 variant might increased the intracranial aneurysms in Japanese male patients[17]; two single nucleotide polymorphisms (SNPs) (rs243832 and rs7201) that have a strong linkage disequilibrium had an association with endomotriosis[18], furthermore this study found rs7201 risk allele "C" was an independent risk factor for endometriosis in Chinese women[18]. However, insights into whether these five SNPs in *MMP-2* is associated with the alcohol-induced ONFH is still indistinct.Therefore, in this case-control study, our study aimed to investigate whether genetic aspect of *MMP-2* had a protective or risky association with alcohol-induced ONFH in Chinese males.

Materials and Methods

Ethics committee statement

This case-control study strictly obeyed the principles of the Declaration on Helsinki of the World Medical Association and got the permission from the Ethics Committee of Xi'an Hong-Hui Hospital, Xi'an Jiaotong University and Northwest University. All of the participants were informed the case-control study and their consent were obtained.

Research subjects

This case-control study was conducted in Xi'an Hong-Hui Hospital and a total of 695 male pathologically confirmed alcohol-induced ONFH patients were enrolled from January 2016 to September 2014, including 299 patients and 396 healthy controls. ONFH was diagnosed by clinical examination and radiographic analysis. Patients were undergone the checks of anteroposterior and frog view X-rays, and magnetic resonance imaging was needed to further confirm the ONFH if the patients had no X-rays changes. According to Lee HJ, alcohol-induced ONFH patients were the person who consumed more than 400 ml of pure ethanol per week[19]. For many reasons some patients were excluded, such as the individuals who disagreed with this case-control study, consumed the drugs which have an effect on patients liver function and lipid metabolism, did not meet the diagnostic criteria of alcohol-induced ONFH,

needed more steroids to treat and suffered chronic diseases synchronously were excluded. All healthy controls had never been diagnosed with alcohol-induced ONFH and were interviewed by professional interviewers for their age and alcohol exposure.

Genotyping

MMP-2 is a member of the *MMPs* gene family and *MMPs* play a key role in the process of bone metabolism. A number of studies have revealed that some genes in *MMPs/TIMPs* pathway have genetic effects on the risk of ONFH. So, we selected 5 ~~single nucleotide polymorphisms~~ (SNPs) in the *MMP-2* gene based on the minor allele frequencies more than 5% in HapMap Chinese Han population. These five SNPs are rs1053605, rs243849, rs243847, rs243832 and rs7201.

Whole blood of each participant were extracted and placed in the anticoagulant tubes. The extraction kit (GoldMag, China) was used to isolate genomic DNA from whole blood and genomic DNA concentration was measured by spectrometry (DU530 UV/VIS spectrophotometer, Beckman Instruments, Fullerton, CA, USA), then DNA was stored at -20 °C until detection. ~~Using public databases (dbSNP; HAPMAP http://www.ncbi.nlm.nih.gov/SNP/; http://www.Hapmap.org/index.html.en) and past reports, a total of 5 single nucleotide polymorphisms (SNPs) in the *MMP-2* gene were selected on the basis of their location, allele frequencies, and disease relevance.~~ According to the manufacturer agreement, the Sequenom MassARRAY® RS1000 system was used to performed the genotyping, and Sequence MassARRAY Assay Design 4.0 software was used to design Multiplexed SNP Mass extend assay[20]. Data analyses and management were conducted by Sequenom Typer 4.0 Software[21].

Statistical analysis

Microsoft Excel and SPSS 16.0 (SPSS, Chicago, IL, USA) were used to perform statistical analyses. Throughout the document, *p* values were two-sided, furthermore，$p<0.05$ was thought to have a statistical significant. The age difference of case and control was calculated by Welch's t tests and the exact test was used to determine whether the SNPs departure from Hardy–Weinberg equilibrium (HWE). The association between the miner allele and the risk of alcohol-induced ONFH were evaluated by Pearson Chi-squared test. To assess the association between each

genotype and the risk of alcohol-induced ONFH, five models were used, including co-dominant model, dominant model, recessive model, over-dominant model and log-additive model. Finally, the SHEsis software platform and Haploview software package (version 4.2) were used to conduct the patterns of linkage disequilibrium (LD) , haplotype construction and the genetic association at polymorphism loci[22]. Odds ratios (ORs) and 95 % confidence intervals (95%CI) could show whether the frequencies between case and control had a significant difference. Unconditional logistic regression analysis was adjusted for age. In order to eliminate the probability of false-positive finding, Bonferroni's multiple adjustment was applied to the level of significance, which was set at $p<0.0017(0.05/30)$.

Results

In this case-control study, all the participants were males. The age characteristic was presented in Table 1. We selected 695 individuals, concluding 299 alcohol-induced ONFH patients (age: 43.24±13.07) and 396 healthy controls (age: 47.62±10.28). A total of five SNPs were analyzed in present study. Gene, chromosomal position, the minor/major allele frequency, HWE test and Pearson χ^2 test results for all the SNPs were presented in Table 2. ~~Hardy–Weinberg equilibrium~~HWE test was used to check whether the surveyed population approached the genetic equilibrium and achieved random sampling requirements. $P>0.05$ showed that the SNPs meet the Hardy–Weinberg equilibrium and none of the SNPs was excluded. The differences of frequency distributions of alleles between cases and controls were compared by Pearson χ^2 test and found that one significant SNP was associated with the risk of alcohol-induced ONFH in the *MMP-2* gene at a 5% level (rs243849 OR=1.33, 95%CI= 1.02-1.75, $p=0.037$).

Next, we assumed that the minor allele of each SNP was a risk factor and analyzed the association between each variant and alcohol-induced ONFH risk under five genetic models (Co-dominant, Dominant, Recessive, Over-dominant and log-addictive) (Table 3, Table 4). Akaike Information criterion (AIC) and Bayesian Information criterion (BIC) are effectively used to selected the optimal model among candidates model[23]. These two criterion were used to in this study and and the

relatively small value corresponding to the model is the appropriate model. About table 3, the "T" allele of rs243849 was only found under log-addictive model increasing the risk of alcohol-induced ONFH by unconditional logistic regression analysis without adjusted (OR= 1.32, 95%CI=1.01-1.73, *p*= 0.041) (Table 3). Moreover, unconditional logistic regression analysis adjusted for age was used for further analysis between each SNP and alcohol-induced ONFH risk (Table 4). We similarly found rs243849 in the *MMP-2* was associated with increased odds of developing alcohol-induced ONFH in a log-addictive model in the Chinese north male population (OR=1.36, 95%CI=1.03–1.79, *p*=0.027). Conversely, the genotype "CC" in rs243832 and the genotype "CC" in rs7201 decreased alcohol-induced ONFH risk was revealed by the recessive model (rs243832 OR=0.62, 95%CI=0.40-0.98, *p*=0.037; rs7201 OR=0.48, 95%CI=0.24-0.98, *p*=0.035). We did not find any statistically significant association between the alcohol-induced ONFH risk and the loci of rs1053605 and rs243847 under five models. However, after our data was applied by Bonferroni's multiple adjustment, no SNPs in our study was significantly related to alcohol-induced ONFH.

Finally, haplotype analysis results showed two blocks have a linkage disequilibrium, rs243849-rs243847 and rs243832-rs7201, and D' values were 1 and 0.99 respectively (Figure 1). Haplotype "TT" of rs243849 and rs243847 block was found to be associated with an increased risk of alcohol-induced ONFH by Pearson Chi-aquared test ($p<0.05$) (Table 5)， furthermore, under unconditional logistic regression analysis adjusted for age, the "TT" haplotype equally augmented the risk (OR=1.41, 95% CI=1.04-1.90, *p*=0.028). But none of significant haplotype was identified in the rs243832-rs7201 block.

Discussion and Conclusion

The epidemiologic studies were found that SNPs in several genes might associate with patients' susceptibility to orthopedic diseases[24,25], including alcohol-induced ONFH[26]. Investigation of the relationship between different genes and diseases can promote the treatment, prevention and prognosis guidance, and that several genetic markers of alcohol-induced ONFH had already been found[26,27]. In our

study, we identified the association between five SNPs in *MMP-2* gene with the risk of alcohol-induced ONFH in Chinese Han male population from Shanxi or around. Our result showed that *MMP-2* from chromosomes 16 might be associated with the risk of alcohol-induced ONFH. The haplotype "TT"of *MMP-2* was associated with a 1.41-flod increased the risk of alcohol-induced ONFH.

As we all known, alcohol-induced ONFH is a serious and complex injury characterized by the apoptosis of osteocyte and bone metabolism unbalanced, and is caused by the foundation of dynamic equilibrium broken and bone marrow stromal cells prosoplasia [3,28]. The patients could show pain and limitation of hip joint, and then causes bone trabecula breakage and collapse of the subchondral bone, then with the abnormality of lipid metabolism and intravascular coagulation, resulting in necrosis of the femoral head[29,30]. Articles have been reported, the normal repair mechanisms might be disrupted after bone death, and the repair process probably take place as bone remodeling coupled include bone resorption and new bone synthesis [31]. Generally, the Cellular responses in the physiologic bone remodeling cycle involves a series of highly regulated steps, containing "resting," "activation," "resorption," "reversal," and "formation" phases[32]. Both osteoblast and osteoclast are the primarily functional cells involved in the process of bone remodeling. Osteoclast firstly attached to the mineralized bone surface and initiate bone resorption by secreting hydrogen ions and by digesting bone collagen. Afterward osteoblasts migrate to the bone resorption site secret bone matrix, and eventually bone matrix mineralized and new bone formed. The balance of bone remodeling process plays a key role in maintaining the normal bone mass.

MMPs, not only directly involved in bone matrix degradation, but also can promote osteoclast migration and attachment, and it has been proved that osteoblasts can secret matrix metalloproteinases (MMPs) [33,34]. MMPs maintain at a normal level under the regulation of a variety factors, when inflammation occurs, *MMPs* regulate the differentiation of osteoblast and bone resorption of osteoclast to complete the repair of the necrotic bone, moreover *MMP-2* has been suggested make an eventual contribution in bone remodeling[35]. According to Keiichi Inoue, *MMP-2*, which is

located in 16q12.2, plays a crucial role in forming and maintain the osteocytic canalicular network whose formation is a determining factor of bone remodeling and mineralization[36,37]. There is a report suggested that the abnormal expression of *MMP-2* will cause disorder osteolysis[38]. That up regulated *MMP-2* induced the change *MMPs/TIMPs* pathway system can activate osteoclast bone resorption, accelerate osteoblast apoptosis and bone matrix degradation. The balance between bone resorption and bone formation is broken, and the imbalance may be the most important collapse mechanism in ONFH[31]. Therefore, *MMP-2* is a important regulation gene in the alcohol-induced ONFH patients. We have not found any evidence for the role of heredity between the *MMP-2* gene and the risk of alcohol-induced ONFH in Chinese males. In this case-control study, Carriers of the rs243849 "T" allele exhibited a statistically significant increased 1.33-, 1.32-, and 1.36-fold the susceptibility of alcohol-induced ONFH by allele model, log-additive model without adjusted and log-additive model adjusted for age severally, but the patients who had the genotypes "CC" of rs243832 and "CC" of rs7201 was predicted decreased the susceptibility of alcohol-induced ONFH. After Bonferroni's multiple adjustment applied to the date, no significant association existed. However, rs243849 risk allele "T" and rs243847 wild allele "T" constituted the haplotype "TT", compared to wild type "CT", increased the alcohol-induced ONFH risk. Because Bonferroni correction is the most conservative approach, *MMP-2* variant may be a risky factor for the disease.

In conclusion, we identified 5 SNPs in *MMP-2* gene, and found that the variant of *MMP-2* might be a biomarker for alcohol-induced ONFH risk. Though, there were several limitations in our present case-control study, such as limited sample size and lack of corresponding clinical information. Further research is needed to identify our results because of no former report investigated on the association. It is possible that *MMP-2* polymorphisms are related to alcohol-induce ONFH risk and these data can provide a theoretical foundation for other researchers to further study the association in Chinese or other population.

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This work did not have any funding support.

Conflict of interest

The authors declare no conflict of interest.

References

1. Aaron RK. Importance of the early diagnosis of hip pain: new approaches to hip preservation in osteonecrosis. *Medicine and health, Rhode Island.* 1998;81(5):157-161.
2. Zalavras CG, Vartholomatos G, Dokou E, Malizos KN. Genetic background of osteonecrosis: associated with thrombophilic mutations? *Clinical orthopaedics and related research.* 2004(422):251-255.
3. Mont MA, Hungerford DS. Non-traumatic avascular necrosis of the femoral head. *The Journal of bone and joint surgery. American volume.* 1995;77(3):459-474.
4. Scaglione M, Fabbri L, Celli F, Casella F, Guido G. Hip replacement in femoral head osteonecrosis: current concepts. 2015;12:51-54.
5. Cui L, Zhuang Q, Lin J, et al. Multicentric epidemiologic study on six thousand three hundred and ninety five cases of femoral head osteonecrosis in China. *International orthopaedics.* 2016;40(2):267-276.
6. Matsuo K, Hirohata T, Sugioka Y, Ikeda M, Fukuda A. Influence of alcohol intake, cigarette smoking, and occupational status on idiopathic osteonecrosis of the femoral head. *Clinical orthopaedics and related research.* 1988(234):115-123.
7. Zhang Z, Li Y, Liu H, Shi J, Li X, Jiang W. ABCB1 polymorphisms associated with osteonecrosis of the femeral head. *International Journal of Clinical &*

Experimental Pathology. 2015;8(11).

8. Hong JM, Kim TH, Kim HJ, Park EK, Yang EK, Kim SY. Genetic association of angiogenesis- and hypoxia-related gene polymorphisms with osteonecrosis of the femoral head. *Experimental & Molecular Medicine.* 2010;42(5):376-385.
9. Sires UI, Schmid TM, Fliszar CJ, Wang ZQ, Gluck SL, Welgus HG. Complete degradation of type X collagen requires the combined action of interstitial collagenase and osteoclast-derived cathepsin-B. *The Journal of clinical investigation.* 1995;95(5):2089-2095.
10. Vu TH, Shipley JM, Bergers G, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell.* 1998;93(3):411-422.
11. Geoffroy V, Marty-Morieux C, Le Goupil N, et al. In vivo inhibition of osteoblastic metalloproteinases leads to increased trabecular bone mass. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research.* 2004;19(5):811-822.
12. Wang W, Wang L, Xu Z, et al. Effects of estradiol on reduction of osteoarthritis in rabbits through effect on matrix metalloproteinase proteins. *Iranian journal of basic medical sciences.* 2016;19(3):310-315.
13. Uemura Y, Hayashi H, Takahashi T, et al. [MMP-3 as a Biomarker of Disease Activity of Rheumatoid Arthritis]. *Rinsho byori. The Japanese journal of clinical pathology.* 2015;63(12):1357-1364.
14. Ho LJ, Lin LC, Hung LF, et al. Retinoic acid blocks pro-inflammatory cytokine-induced matrix metalloproteinase production by down-regulating JNK-AP-1 signaling in human chondrocytes. *Biochemical pharmacology.* 2005;70(2):200-208.
15. Manso H, Krug T, Sobral J, et al. Variants of the Matrix Metalloproteinase-2 but not the Matrix Metalloproteinase-9 genes significantly influence functional outcome after stroke. *Bmc Medical Genetics.* 2009;11(1):1-9.
16. Smallwood L, Warrington N, Allcock R, et al. Matrix Metalloproteinase-2

Gene Variants and Abdominal Aortic Aneurysm. *European Journal of Vascular & Endovascular Surgery the Official Journal of the European Society for Vascular Surgery.* 2009;38(2):169-171.

17. Low SK, Zembutsu H, Takahashi A, et al. Impact of LIMK1, MMP2 and TNF-|[alpha]| variations for intracranial aneurysm in Japanese population. *Journal of Human Genetics.* 2011;56(3):211-216.
18. Tsai EM, Wang YS, Lin CS, et al. A microRNA-520 mirSNP at the MMP2 gene influences susceptibility to endometriosis in Chinese women. *Journal of Human Genetics.* 2013;58(4):202-209.
19. Lee HJ, Choi SJ, Hong J, et al. Association of a polymorphism in the intron 7 of the SREBF1 gene with osteonecrosis of the femoral head in Koreans. *Annals of human genetics.* 2009;73(1):34-41.
20. Trembizki E, Smith H, Lahra MM, et al. High-throughput informative single nucleotide polymorphism-based typing of Neisseria gonorrhoeae using the Sequenom MassARRAY iPLEX platform. *Journal of Antimicrobial Chemotherapy.* 2014:dkt544.
21. Thomas RK, Baker AC, DeBiasi RM, et al. High-throughput oncogene mutation profiling in human cancer. *Nature genetics.* 2007;39(3):347-351.
22. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21(2):263-265.
23. Vrieze SI. Model selection and psychological theory: a discussion of the differences between the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). *Psychological Methods.* 2012;17(2):228-243.
24. Yin JM, Liu Z, Zhao SC, Guo YJ, Liu ZT. Relationship between the Apolipoprotein AI, B gene polymorphism and the risk of non-traumatic osteonecrosis. *Lipids in health and disease.* 2014;13:149.
25. Kim TH, Baek JI, Hong JM, et al. Significant association of SREBP-2 genetic polymorphisms with avascular necrosis in the Korean population. *BMC medical genetics.* 2008;9:94.
26. Wang Y, Cao Y, Li Y, et al. Genetic association of the ApoB and ApoA1 gene

polymorphisms with the risk for alcohol-induced osteonecrosis of femoral head. *International journal of clinical and experimental pathology.* 2015;8(9):11332-11339.

27. Okazaki S, Nagoya S, Tateda K, et al. Experimental rat model for alcohol-induced osteonecrosis of the femoral head. *International journal of experimental pathology.* 2013;94(5):312-319.
28. Mont MA, Jones LC, Hungerford DS. Nontraumatic osteonecrosis of the femoral head: ten years later. *The Journal of bone and joint surgery. American volume.* 2006;88(5):1117-1132.
29. Wang Y, Li Y, Mao K, Li J, Cui Q, Wang G-J. Alcohol-induced adipogenesis in bone and marrow: a possible mechanism for osteonecrosis. *Clinical orthopaedics and related research.* 2003;410:213-224.
30. JONES JR JP. Intravascular coagulation and osteonecrosis. *Clinical orthopaedics and related research.* 1992;277:41-53.
31. Gou W, Lu Q, Wang X, Wang Y, Peng J, Lu S. Key pathway to prevent the collapse of femoral head in osteonecrosis. *Eur Rev Med Pharmacol Sci.* 2015;19(15):2766-2774.
32. Raisz LG. Physiology and pathophysiology of bone remodeling. *Clinical chemistry.* 1999;45(8):1353-1358.
33. Andersen TL, del Carmen Ovejero M, Kirkegaard T, Lenhard T, Foged NT, Delaissé J-M. A scrutiny of matrix metalloproteinases in osteoclasts: evidence for heterogeneity and for the presence of MMPs synthesized by other cells. *Bone.* 2004;35(5):1107-1119.
34. Tezuka K-I, Tezuka Y, Maejima A, et al. Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. *Journal of Biological Chemistry.* 1994;269(2):1106-1109.
35. Delaissé J-M, Engsig MT, Everts V, et al. Proteinases in bone resorption: obvious and less obvious roles. *Clinica Chimica Acta.* 2000;291(2):223-234.
36. Inoue K, Mikuni-Takagaki Y, Oikawa K, et al. A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism.

Journal of Biological Chemistry. 2006;281(44):33814-33824.

37. Grässel S, Beckmann J, Rath B, Vogel M, Grifka J, Tingart M. Expression profile of matrix metalloproteinase-2 and -9 and their endogenous tissue inhibitors in osteonecrotic femoral heads. *International Journal of Molecular Medicine.* 2010;26(1):127-133.

38. Martignetti JA, Al Aqeel A, Al Sewairi W, et al. Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nature genetics.* 2001;28(3):261-265.

Figure Legend

Figure 1 Haplotype block map for part of the SNPs in *MMP-2* gene. Linkage disequilibrium plots containing five SNPs from 16q12.2. Standard color frame is used to show LD pattern. Two blocks in the figure showed higher LD. That darker shades of red indicate higher D' and display statistically significant associations between a pair of SNPs.

Table1. Characteristics of the male individuals in controls and alcohol-induced ONFH patients

	Group	N	Mean	Std. Deviation	Mean±SD	*P*-value
Age	Case	299	43.24	13.07	43.24±13.07	*P*< 0.001*
	Control	396	47.62	10.28	47.62±10.28	

*: p<0.05 indicates statistical significance. *P* value was calculated by Welch's t test.

Table 2. Basic information summary of candidate SNPs examined in the *MMP2* gene among the cases and controls and odds ratio estimates for alcohol-induced ONFH

Gene	SNP ID	Position	Band	Alleles A/B	MAF		Role	HWE-*P*[a]	OR(95%CI)	*P*[b] value
					Case	Control				
MMP2	rs1053605	55519607	16q12.2	T/C	0.12	0.14	Coding exon	0.84	0.87(0.63-1.19)	0.373
MMP2	rs243849	55523705	16q12.2	T/C	0.21	0.17	Coding exon	1.00	**1.33(1.02-1.75)**	**0.037***
MMP2	rs243847	55523998	16q12.2	C/T	0.38	0.39	Intron	0.11	0.95(0.76-1.18)	0.631
MMP2	rs243832	55539191	16q12.2	C/G	0.36	0.38	Intron (boundary)	0.11	0.92(0.74-1.15)	0.482
MMP2	rs7201	55539614	16q12.2	C/A	0.22	0.25	3' UTR	0.22	0.88(0.68-1.12)	0.298

SNPs: Single nucleotide polymorphisms; A: Miner alleles, B: Major alleles; MAF: Minor allele frequency; OR: Odds ratio. CI: Confidence interval;

HWE: Hardy-Weinberg equilibrium;

a: *p* value were calculated using exact test; b: *p* value were calculated using Chi-square test;

*****: $p<0.05$ indicates statistical significance;

Bonferroni's multiple adjustment was applied to the level of significance, which was set at $p<0.0017(0.05/30)$.

Table 3. Genotypes of rs243849 and the risk of alcohol-induced ONFH without adjusted

Gene	SNP_ID	Model	Genotype	Control(%)	Case(%)	OR (95% CI)	P^a-value	AIC	BIC
MMP-2	rs243849		C/C	275 (69.4%)	190 (63.5%)	1			
		Co-dominant	C/T	110 (27.8%)	92 (30.8%)	1.21 (0.87-1.69)	0.084	950.9	964.6
			T/T	11 (2.8%)	17 (5.7%)	2.24 (1.02-4.88)			
		Dominant	C/C	275 (69.4%)	190 (63.5%)	1	0.1	951.2	960.3
			C/T-T/T	121 (30.6%)	109 (36.5%)	1.30 (0.95-1.79)			
		Recessive	C/C-C/T	385 (97.2%)	282 (94.3%)	1	0.055	950.2	959.3
			T/T	11 (2.8%)	17 (5.7%)	2.11 (0.97-4.57)			
		Over-dominant	C/C-T/T	286 (72.2%)	207 (69.2%)	1	0.39	953.2	962.2
			C/T	110 (27.8%)	92 (30.8%)	1.16 (0.83-1.61)			
		Log-additive	---	---	---	**1.32 (1.01-1.73)**	**0.041***	949.7	958.8

a: *p* value were calculated by unconditional logistic regression without adjusted;

AIC, Akaike's Information criterion; BIC, Bayesian Information criterion;

*****: $p<0.05$ indicates statistical significance;

Bonferroni's multiple adjustment was applied to the level of significance, which was set at $p<0.0017(0.05/30)$.

OA_Table

Table 4. genotypes of dominant tSNPs in *MMP-2* gene and the risk of alcohol-induced ONFH with adjusted for age

Gene	SNP_ID	Model	Genotype	Control (%)	Case (%)	OR (95% CI)	*P*[a]-value	AIC	BIC
MMP-2	rs243849		C/C	275 (69.4%)	190 (63.5%)	1			
		Codominant	C/T	110 (27.8%)	92 (30.8%)	1.28 (0.91-1.80)	0.075	928.7	946.9
			T/T	11 (2.8%)	17 (5.7%)	2.17 (0.98-4.80)			
		Dominant	C/C	275 (69.4%)	190 (63.5%)	1	0.059	928.3	942
			C/T-T/T	121 (30.6%)	109 (36.5%)	1.37 (0.99-1.89)			
		Recessive	C/C-C/T	385 (97.2%)	282 (94.3%)	1	0.077	928.8	942.4
			T/T	11 (2.8%)	17 (5.7%)	2.01 (0.92-4.42)			
		Overdominant	C/C-T/T	286 (72.2%)	207 (69.2%)	1	0.23	930.5	944.1
			C/T	110 (27.8%)	92 (30.8%)	1.23 (0.88-1.72)			
		Log-additive	---	---	---	**1.36 (1.03-1.79)**	**0.027***	927	940.7
MMP-2	rs243832		G/G	159 (40.1%)	116 (38.8%)	1			
		Codominant	G/C	172 (43.4%)	149 (49.8%)	1.14 (0.82-1.58)	0.086	929	947.2
			C/C	65 (16.4%)	34 (11.4%)	0.67 (0.41-1.09)			

		Dominant	G/G	159 (40.1%)	116 (38.8%)	1	0.97	931.9	945.5
			G/C-C/C	237 (59.9%)	183 (61.2%)	1.01 (0.74-1.38)			
		Recessive	G/G-G/C	331 (83.6%)	265 (88.6%)	1	**0.037***	927.5	941.2
			C/C	65 (16.4%)	34 (11.4%)	**0.62 (0.40-0.98)**			
		Overdominant	G/G-C/C	224 (56.6%)	150 (50.2%)	1	0.14	929.7	943.3
			G/C	172 (43.4%)	149 (49.8%)	1.26 (0.93-1.71)			
		Log-additive	---	---	---	0.89 (0.71-1.11)	0.31	930.8	944.5
MMP-2	rs7201	Codominant	A/A	228 (57.7%)	177 (59.2%)	1	0.11	928.6	946.7
			C/A	138 (34.9%)	110 (36.8%)	1.00 (0.72-1.38)			
			C/C	29 (7.3%)	12 (4%)	0.48 (0.24-0.99)			
		Dominant	A/A	228 (57.7%)	177 (59.2%)	1	0.55	930.6	944.3
			C/A-C/C	167 (42.3%)	122 (40.8%)	0.91 (0.67-1.24)			
		Recessive	A/A-C/A	366 (92.7%)	287 (96%)	1	**0.035***	926.6	940.2
			C/C	29 (7.3%)	12 (4%)	**0.48 (0.24-0.98)**			
		Overdominant	A/A-C/C	257 (65.1%)	189 (63.2%)	1	0.69	930.8	944.5
			C/A	138 (34.9%)	110 (36.8%)	1.07 (0.78-1.47)			

Log-additive	---	---	---	0.85 (0.66-1.09)	0.19	929.3	942.9

a: *p* value were calculated by unconditional logistic regression with adjusted for age;

AIC: Akaike's Information criterion; BIC: Bayesian Information criterion;

*****: $p<0.05$ indicates statistical significance.

Bonferroni's multiple adjustment was applied to the level of significance, which was set at $p<0.0017(0.05/30)$.

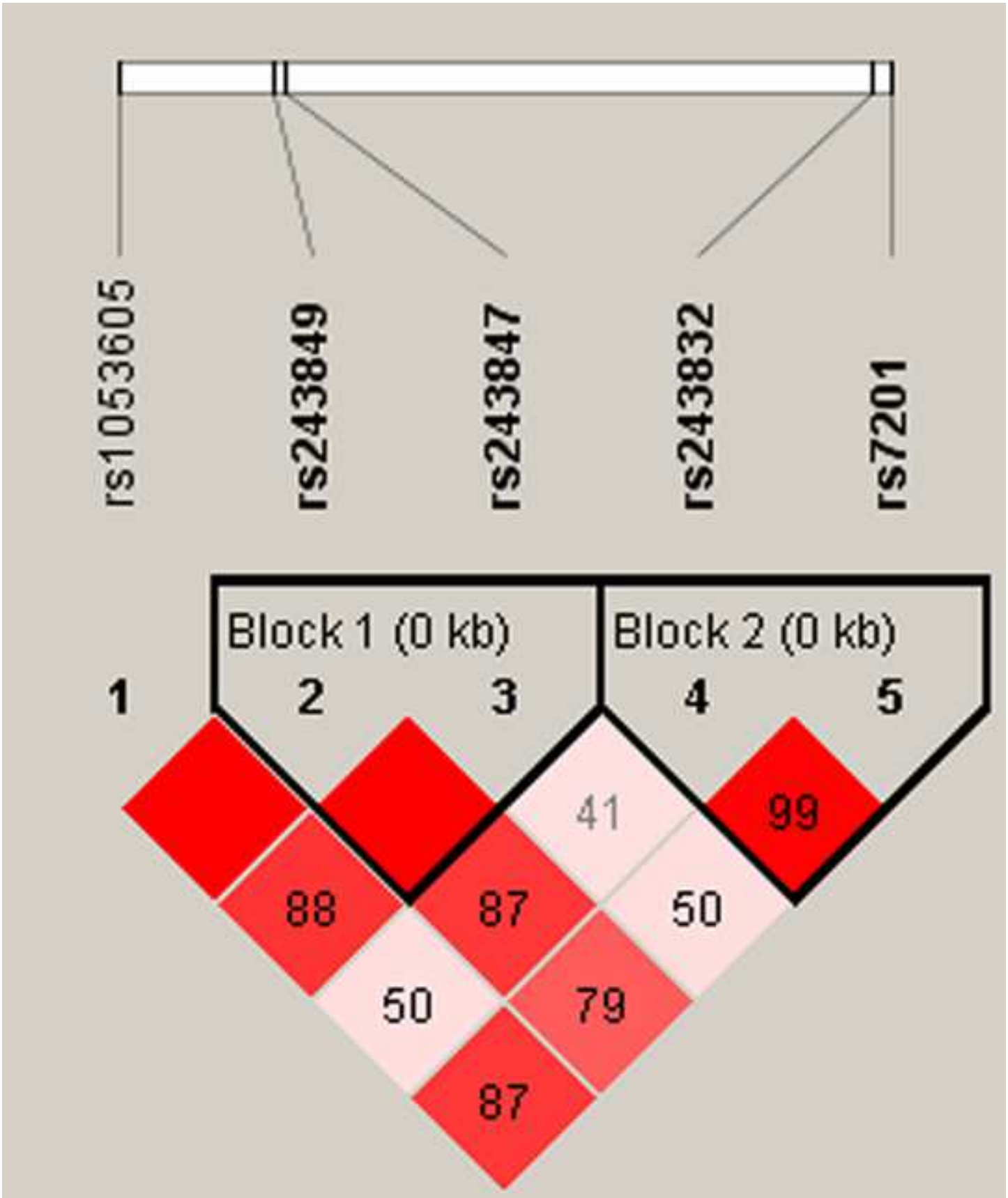
Table 5. *MMP-2* haplotypes frequencies and the association with alcohol-induced ONFH among the cases and controls

SNPs	Haplotype	Freq (case)	Freq (control)	P^a	OR (95% CI)	P^b
	CT	0.408	0.439	--	1.000	--
rs243849|rs243847	CC	0.381	0.394	0.650	1.06 (0.84-1.35)	0.600
	TT	0.211	0.167	0.042*	**1.41 (1.04-1.90)**	**0.028***
	GA	0.637	0.618	--	1.000	--
rs243832|rs7201	CC	0.224	0.248	0.340	0.85 (0.65 - 1.10)	0.210
	CA	0.139	0.135	0.980	0.97 (0.71 - 1.34)	0.870

a: p value were calculated by Pearson Chi-aquared test;

b: p value were calculated by unconditional logistic regression adjusted for age;

*****: $p<0.05$ indicates statistical significance.



STROBE Statement

Checklist of items that should be included in reports of observational studies

Section/Topic	Item No	Recommendation	Reported on Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	P1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	P2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	P3
Objectives	3	State specific objectives, including any prespecified hypotheses	P4
Methods			
Study design	4	Present key elements of study design early in the paper	P4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	P4
Participants	6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants	P4
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case	P4
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	
Data sources/measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	P5
Bias	9	Describe any efforts to address potential sources of bias	P5
Study size	10	Explain how the study size was arrived at	P5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	P5
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	P5
		(*b*) Describe any methods used to examine subgroups and interactions	P5
		(*c*) Explain how missing data were addressed	P5
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	P5
		(*e*) Describe any sensitivity analyses	

Section/Topic	Item No	Recommendation	Reported on Page No
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	P6
		(b) Give reasons for non-participation at each stage	P6
		(c) Consider use of a flow diagram	P6
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	P6
		(b) Indicate number of participants with missing data for each variable of interest	P6
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)	
Outcome data	15*	*Cohort study*—Report numbers of outcome events or summary measures over time	
		Case-control study—Report numbers in each exposure category, or summary measures of exposure	P6
		Cross-sectional study—Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	P7
		(*b*) Report category boundaries when continuous variables were categorized	P7
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	P7
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	P9
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	P9
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	P9
Generalisability	21	Discuss the generalisability (external validity) of the study results	P8
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	P9

**Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.*

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.