DPP4 rs16822665 and rs2268694 had Protective Effect on Osteonecrosis of the Femoral Head

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Abstract

Background: It is reported that DPP4 is associated with bone metabolism, osteoporosis and other orthopedic diseases, but the correlation between DPP4 and osteonecrosis of the femoral head (ONFH) is not clear. It was the purpose of this study that was to explore the relationship between DPP4 gene and ONFH.

Methods: We genotyped four single nucleotide polymorphisms (SNPs) from DPP4 gene using the Agena MassARRAY platform. The association between DPP4 variants and ONFH susceptibility was assessed using odds ratio (OR) and 95% confidence intervals (CIs) via logistic regression.

Results: The results showed that the allele C of rs16822665 was related to a lower risk of ONFH (OR = 0.76, 95% CI = 0.63-0.92, p = 0.006). In the case of stratified analysis, we found that rs16822665 could reduce the incidence of ONFH risk in four genetic models (dominant, codominant, log-additive, and recessive models) in drinkers and people age ≤51 years (p < 0.05). In gender stratification analysis, both rs2268694 and rs16822665 were contributed to bring down the risk of disease, which were mainly reflected in the codominant, dominant and log-additive models in female (p < 0.05). The subgroup analysis was conducted based on smokers revealing that rs226894 was vitally correlated with a decreased risk of ONFH in the codominant (C vs. T: OR = 0.51, 95% CI: 0.34-0.76, p = 0.001), dominant (TC-CC vs. TT: OR = 0.53, 95% CI: 0.36-0.77, p = 0.001), and log-additive (OR = 0.65, 95% CI: 0.48-0.88, p = 0.006) models, while it was not found in the non-smokers.

Conclusions: This finding provide evidence that DPP4 variants play a key role in the occurrence of ONFH among the Chinese Han population.

Introduction

Osteonecrosis of the femoral head (ONFH) is a common disease in orthopedics, which is difficult to treat and caused by dislocation of hip joint, improper use of glucocorticoids, long-term drinking and other factors. The insufficient blood supply of bone cells leads to the death of bone cells, and finally leads to the collapse of the surface of femoral head and hip arthritis osteonecrosis of the femoral head an update in year 2000. According to statistics, it usually occurs in people aged 20-40 years. There are 7.5 million patients with femoral head necrosis in China who need timely treatment, and there are 15,0000-20,0000 new cases every year. Femoral head necrosis has a high disability rate, which has a strong impact on the quality of life of patients. In recent years, some progresses have been performed in its treatment, but its specific pathogenesis needs to be further studied.

The human DPP4/CD26 gene, located at 2q24, is a transmembrane glycoprotein with a molecular weight of 110ku and contains 26 exons encoding 766 amino peptides. Its product is a proteolytic enzyme, which has a unique proteolytic effect and belongs to the serine protease family. In addition, scholars found that DPP4/CD26 was involved in physiological processes such as invasion, apoptosis, migration, adhesion, and immune modulation. As a marker of T-cell activation, DPP4/CD26 also plays a key role in immune regulation and activates fibroblast proteins and participate in signal pathways.

It is well known that DPP4 and some of its substrates interact with adipokines, playing a pivotal role in the energy metabolism. In recent years, DPP4 not only plays a vital role in protein regulation in the context of energy metabolism in the literature, but also has a direct and indirect effects on bone metabolism. Many substrates of DPP4 have played role in bone metabolism, and that include incretins, neuropeptides and gastrointestinal peptides. Overall, although some effects are conducive to bone formation, others are intricate and have not been absolutely expounded.

The activity of DPP4 is related to diabetes hyperglycemia, osteoporosis bone loss and general osteoporosis, which indicates that the regulation of DPP4 is not directly involved in pathphysiology of these bone diseases, is also influenced by its development. Experimental studies have clearly confirmed some effects of DPP4 matrix on bone. In addition, some meta-analyses of clinical studies on DPP4 inhibitors and GLP-1-based bone therapy have been published and have indicated that DPP4 inhibitors are relevant to a reduced fracture risk. DPP4 inhibitor can improve vertebral bone mineral density and trabecular structure in vivo, suggesting a harmful effect of DPP4 on bone metabolism. Studies have shown that DPP4 can digest several molecules playing an important role in bone metabolism. It was found that inhibition of DPP4 reduced serum reabsorption markers and trabecular bone volume and increased cortical and bone strength in rats with diabetes. Yeganeh1 et al. showed that DPP4 may be directly involved in bone
resorption. In recent years, studies have displayed that the development of human osteoclasts is hindered when DPP4 signaling is blocked.

So far, DPP4 has been reported to be associated with bone formation, bone loss, and osteoporosis. However, the mechanism of DPP4 gene effect on osteonecrosis remains unclear. In this study, we discussed the association of DPP4/CD26 genetic variations with ONFH susceptibility among Han individuals in China, which will help understand the function of DPP4/CD26 in the development of ONFH and provide new insights into its pathogenesis.

Methods

Study population

In this study, we enrolled 936 subjects, including 468 cases and 468 health controls. All cases were newly diagnosed by the following criteria: 1) diagnosed and selected by anteroposterior and bilateral hip X-ray films and/or magnetic resonance imaging; 2) No other direct trauma, cardiovascular disease, rheumatoid arthritis, ankylosing spondylitis, hip joint disease (such as dysplasia of the hip), diabetes, renal insufficiency, cancer, glucocorticoid, alcohol and family genetic diseases were included. The selection criteria of all healthy people were: 1) they are from the same hospital during the same period; 2) People who are not long-term users of alcohol and steroids; 3) no pain in the buttocks; 4) no lesions were found in the pelvic anteroposterior film and frog leg lateral film.

DPP4 genotyping

Four SNPs (rs2268894, rs16822665, rs6741949, and rs67399148) in DPP4 gene were selected from 1000 Genomes Chinese Han Beijing population, with minor allele frequency (MAF) >5%. 5ml of fasting peripheral venous blood was collected from all subjects, and dispensed in ethylene diamine tetraacetic acid (EDTA)-containing tubes. Genomic DNA was purified with a commercially available DNA extraction kits (GoldMag Co. Ltd, Xi’an, China). Genotype analysis of DPP4 gene polymorphism was performed according to the protocol using Agena MassARRAY platform (Agena Bioscience, San Diego, CA, USA). The genotyping results were managed and analyzed using Agena Bioscience TYPER software (version 4.0).

Statistical analysis

Student’s t-test was used to assess the difference of age, while \( \chi^2 \) test was to compare the distributions of gender between the two groups, assess the frequency of SNP allele between the case and control groups, and figure up the Hardy-Weinberg equilibrium (HWE) of the control group. Odds ratio (OR) and 95% confidence intervals (CIs) were using for evaluating the relationship between DPP4 polymorphisms and ONFH risk by logistic regression analysis. Then, the interaction of SNP-SNP in the risk of ONFH was detected by multifactor dimensionality reduction (MDR).

Results

Basic information of research objects

As rendered in Table 1, there are 468 ONFH patients (272 males and 196 females, mean age: 51.22±14.84 years) and 468 health controls (305 males and 163 females, mean age: 51.10±13.93 years) in this study. Particularly, the mean age was well-matched between the two groups (\( p = 0.299 \)).

Basic information of selected SNPs

Four SNPs (rs2268894, rs16822665, rs6741949, and rs67399148) have been identified and genotyped what we chose in DPP4 gene (Table 2). The genotype distributions of all SNPs were conformed to HWE (\( p>0.05 \)). We found that the allele C of rs16822665 was related to a lower risk of ONFH (OR = 0.76, 95%CI = 0.63-0.92, \( p = 0.006 \)). Simultaneously, no obvious association was found between other three SNPs and ONFH risk.

Association of DPP4 polymorphisms with ONFH susceptibility
As shown in Table 3, we evaluated that the relationship of four SNPs in DPP4 gene with ONFH risk by logistic regression. Our results showed that DPP4 rs2268894 had a decreased effect on ONFH susceptibility in the dominant (OR = 0.76, 95%CI = 0.59-0.98, p = 0.038). We also found that rs16822665 might be a protect factor for ONFH occurrence under the codominant with "CC" genotype (OR = 0.51, 95%CI = 0.32-0.82, p = 0.005), dominant (OR = 0.72, 95%CI = 0.55-0.93, p = 0.014), recessive (OR = 0.59, 95%CI = 0.38-0.92, p = 0.020), and log-additive (OR = 0.74, 95%CI = 0.60-0.90, p = 0.003) models. Nevertheless, no significant correlation was discovered between other two SNPs and ONFH risk.

After stratified analysis (Table 4), we found that rs16822665 could reduce the incidence of ONFH risk in the four genetic models (dominant, codominant, log-additive, and recessive models) in drinkers and patients age ≤ 51 years (p < 0.05). In gender stratification analysis, both rs2268694 and rs16822665 were contribute to bring down the risk of disease, which were mainly reflected in the dominant, codominant, and log-additive models in female (p < 0.05).

The subgroup analysis, which was conducted based on smokers revealed that rs2268894 was vitally related with a decreased risk of ONFH in the codominant (C vs. T: OR = 0.51, 95%CI = 0.34-0.76, p = 0.001), dominant (TC-CC vs. TT: OR = 0.53, 95%CI = 0.36-0.77, p = 0.001), and log-additive (OR = 0.65, 95%CI = 0.48-0.88, p = 0.006) and there was no founding in the non-smokers.

To sum up, we realized that two SNPs (rs2268894 and rs16822665) were not associated with patients at age > 51 years, non-smokers, and non-drinkers, suggesting that age, gender, smoking, and drinking are significant factors that affect the risk of ONFH.

### SNP-SNP interactions

We analyzed the SNP-SNP interactions on the risk of ONFH by using the MDR. Table 5 represents that the best four-locus model including rs2268894, rs16822665, rs6741949, and rs67399148 was the best model to predict ONFH risk (training accuracy: 0.569; testing accuracy: 0.534; CVC = 10/10, p < 0.001).

### Discussion

To the best of our knowledge that genetic studies have offered insights into a great number of diseases, including ONFH. In this study, it was expounded that DPP4 genetic polymorphisms were associated with ONFH risk among Han individuals in China. Allele, genotype of four loci between patients with ONFH and healthy samples were compared, and stratification analysis were marched. We obtained that the allele C of rs16822665 was related to a decreased risk of ONFH in the Chinese Han individuals. In addition, the results showed that rs2268894 and rs13822665 have a protective effect on the risk of ONFH in patients age ≤ 51 years, or females, or smokers, or drinkers. Therefore, all of those date underscore the importance of DPP4 in ONFH development, and those two SNPs may become new biomarker for the early treatment and prevention of ONFH.

Dipeptide peptidase-4 (DPP4), also known as CD26, is an exopeptidase that is widely expressed on diverse types of cell surface. By controlling the activity of its substrate or by physical binding with other proteins, the activity of this transmembrane exopeptidase has a major impact on glucose metabolism, immune regulation, signal transduction, cell migration differentiation. Study have shown that the expression of CD26 mRNA in patients with rheumatoid arthritis is related to disease activity and bone erosion, suggesting that this molecule may play a role in the immunopathology of rheumatoid arthritis and bone erosion. Some researches found that higher plasma DPP4 levels were significantly associated with higher bone turnover and a higher incidence of osteoporotic fracture. These results showed that DPP4 may be related with osteoporotic fracture by mediating bone turnover rate. Carbone et al found that in elderly group, there was no relationship between DPP4 and osteoporosis. These lines of evidence have demonstrated that DPP4 gene played a crucial role in bone-related disease. However, the association between DPP4 gene and osteonecrosis susceptibility has not been reported. Rs2268694 and rs16822665 polymorphisms, located in the DPP4 gene protected susceptibility to ONFH was indicated in our results, but had not been previously reported in other diseases.

Although our study found that the relevant loci of DPP4 gene have a protective effect on ONFH disease, which can offer a new theoretical basis for disease treatment and prevention, several potential limitations are unavoidable in our study. First, all participants were Han ethnicity, so we need more different ethnic populations to confirm our findings. Second, the sample size is too small to confirm our results abundantly. Third, only four polymorphisms in DPP4 gene were studied, more polymorphisms are needed to be investigated.
Conclusions

All in all, we confirmed that DPP4 variants play a key role in the occurrence of ONFH among the Han population in China. This finding may provide new sights for the prevention and diagnosis of ONFH.

Abbreviations

Osteonecrosis of the femoral head: ONFH; odds ratio: OR; confidence intervals: CIs; minor allele frequency: MAF; Hardy-Weinberg Equilibrium: HWE.

Declarations

Ethics approval and consent to participate

The ethical approval of this study is in line with the ethical principles of the Helsinki declaration on human medical research. Our study has been approved by the ethics committee of Hong Hui hospital in Xi’an, China, and all participants have signed informed consent before participating in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author’s contributions

Chang Liu designed this study protocol and supervised the study; Xuan Liu drafted the manuscript and performed the DNA extraction and genotyping; Xiaowei Li performed the data analysis and performed the sample collection and information recording. All authors have read and approved the final manuscript.

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Tables

Table 1

| Variables       | Cases(N=468) | Controls(N=468) | p value |
|-----------------|--------------|-----------------|---------|
| Age, years (mean ± SD) | 51.22±14.84 | 51.10±13.93  | 0.299a  |
| ≤ 51 years      | 249 (49.4%) | 213 (45.5%)  |         |
| > 51 years      | 219 (46.8%) | 255 (54.5%)  |         |
| Gender          |              | 0.027b         |         |
| Male            | 272 (58.1%) | 305 (65.2%)  |         |
| Female          | 196 (41.9%) | 163 (34.8%)  |         |
| Smoking         |              |                |         |
| Yes             | 194 (41.5%) | 252 (53.8%)  |         |
| No              | 274 (58.5%) | 216 (46.2%)  |         |
| Drinking        |              |                |         |
| Yes             | 233 (49.8%) | 241 (51.5%)  |         |
| No              | 235 (50.2%) | 227 (48.5%)  |         |
| Stage           |              |                |         |
| III/IV          | 178 (38.0%) |                  |         |
| I/II            | 66 (14.1%)  |                  |         |
| Missing         | 224 (47.9%) |                  |         |

SD: standard deviation

p^a values were calculated from student’s t-test.

p^b values were calculated from two-sided χ² test.

Table 2

Basic information of SNPs in DPP4 gene
| SNP ID      | Chr: position | Allele | MAF  | O (HET) | E (HET) | HWE  | OR   | (95% CI) | p     |
|------------|---------------|--------|------|---------|---------|------|------|----------|-------|
| rs2268894  | 2: 161999638  | C/T    | 0.298| 0.468   | 0.447   | 0.351| 0.84 | (0.69-1.02)| 0.074 |
| rs16822665 | 2: 162044817  | C/T    | 0.309| 0.496   | 0.466   | 0.197| 0.76 | (0.63-0.92)| 0.006 |
| rs6741949  | 2: 162053713  | C/G    | 0.076| 0.162   | 0.156   | 0.561| 0.88 | (0.63-1.23)| 0.453 |
| rs67399148 | 2: 162056656  | C/T    | 0.046| 0.109   | 0.114   | 0.239| 0.74 | (0.49-1.12)| 0.150 |

SNP: single nucleotide polymorphism; MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium; OR: odds ratio; 95% CI: 95% confidence interval.

*p values were calculated from $\chi^2$ test.*

Table 3

Association of DPP4 polymorphisms with ONFH susceptibility
| Gene | SNP     | Model       | Genotype | Crude analysis |                         | Adjusted analysis |                         |
|------|---------|-------------|----------|----------------|--------------------------|-------------------|--------------------------|
|      |         |             |          | OR (95% CI)    | p                        | OR (95% CI)       | p                        |
| **DPP4** | rs2268894 | **Codominant** | TT       | 1              |                          | 1                  |                          |
|       |         |             | TC       | 0.77 (0.59-1.02) | 0.064                    | 0.76 (0.58-1.00)  | 0.051                    |
|       |         |             | CC       | 0.76 (0.48-1.20) | 0.241                    | 0.75 (0.47-1.18)  | 0.208                    |
|       |         | **Dominant** | TT       | 1              | **0.049**                | 1                  | **0.038**                |
|       |         |             | TC-CC   | 0.77 (0.60-1.00) |                          | 0.76 (0.59-0.98)  |                          |
|       |         | **Recessive** | TT-TG   | 1              |                          | 1.00               |                          |
|       |         |             | CC       | 0.86 (0.56-1.33) |                          | 0.85 (0.55-1.32)  |                          |
|       |         | **Overdominant** | T/T-C/C | 1              | **0.011**                | 1                  | **0.072**                |
|       |         |             | T/C     | 0.81 (0.63-1.05) | 0.071                    | 0.79 (0.60-1.02)  | 0.055                    |
|       |         | **Log-additive** | ——      | 0.83 (0.68-1.02) | 0.071                    | 0.82 (0.67-1.00)  | 0.055                    |
| **DPP4** | rs16822665 | **Codominant** | TT       | 1              |                          | 1                  |                          |
|       |         |             | TC       | 0.79 (0.60-1.04) | 0.088                    | 0.77 (0.58-1.01)  | 0.061                    |
|       |         |             | CC       | 0.52 (0.32-0.82) | **0.005**                | 0.51 (0.32-0.82)  | **0.005**                |
|       |         | **Dominant** | TT       | 1              | **0.021**                | 1                  | **0.014**                |
|       |         |             | TC-CC   | 0.74 (0.57-0.95) |                          | 0.72 (0.55-0.93)  |                          |
|       |         | **Recessive** | TT-TG   | 1              | **0.018**                | 1                  | **0.020**                |
|       |         |             | CC       | 0.59 (0.38-0.91) |                          | 0.59 (0.38-0.92)  |                          |
|       |         | **Overdominant** | T/T-C/C | 1              | 0.390                    | 1                  | 0.200                    |
|       |         |             | T/C     | 0.89 (0.69-1.16) |                          | 0.84 (0.65-1.10)  |                          |
|       |         | **Log-additive** | ——      | 0.75 (0.61-0.91) | **0.004**                | 0.74 (0.60-0.90)  | **0.003**                |
| **DPP4** | rs6741949 | **Codominant** | G/G      | 1              |                          | 1                  |                          |
|       |         |             | C/G     | 0.89 (0.63-1.27) | 0.527                    | 0.89 (0.62-1.27)  | 0.513                    |
|       |         |             | C/C     | 0.49 (0.04-5.43) | 0.562                    | 0.47 (0.04-5.24)  | 0.538                    |
|       |         | **Dominant** | G/G      | 1              | 0.483                    | 1                  | 0.467                    |
|       |         |             | C/G-C/C | 0.88 (0.62-1.25) |                          | 0.88 (0.62-1.25)  |                          |
|       |         | **Recessive** | G/G-C/G | 1              | 0.572                    | 1                  | 0.549                    |
|       |         |             | C/C     | 0.50 (0.05-5.53) |                          | 0.47 (0.043-5.34) |                          |
|       |         | **Overdominant** | G/G-C/G | 1              | 0.540                    | 1                  | 0.560                    |
|       |         |             | C/G     | 0.89 (0.63-1.27) |                          | 0.90 (0.63-1.29)  |                          |
|       |         | **Log-additive** | ——      | 0.88 (0.62-1.23) | 0.443                    | 0.87 (0.62-1.23)  | 0.425                    |
| **DPP4** | rs67399148 | **Codominant** | T/T     | 1              | 0.32                     | 1                  | 0.186                    |
|       |         |             | T/C     | 0.78 (0.51-1.20) | 0.264                    | 0.77 (0.50-1.19)  | 0.246                    |
|       |         |             | C/C     | 0.32 (0.03-3.12) | 0.330                    | 0.34 (0.03-3.26)  | 0.348                    |
|       |         | **Dominant** | T/T     | 1              | 0.197                    | 1                  | 0.186                    |
|       |         |             | T/C-C/C | 0.76 (0.49-1.16) |                          | 0.75 (0.49-1.15)  |                          |
| Model        | Genotype      | OR   | 95% CI          | OR   | 95% CI          |
|-------------|---------------|------|-----------------|------|-----------------|
| Recessive   | T/T-T/C       | 1    | 0.340           | 1    | 0.359           |
|             | C/C           | 0.33 (0.03-3.20) | 0.35 (0.04-3.34) |
| Overdominant| T/T-C/C       | 1    | 0.270           | 1    | 0.200           |
|             | T/C           | 0.79 (0.51-1.21) | 0.75 (0.49-1.17) |
| Log-additive| —             | 0.75 (0.50-1.12) | 0.158 | 0.74 (0.50-1.11) | 0.151 |

SNP: single nucleotide polymorphism; OR: odds ratio; 95% CI: 95% confidence interval. 

*p* values were calculated by logistic regression analysis with adjustments for age, gender, smoke and drink. **Bold** values mean statistically significant (*p*<0.05).

Table 4

Association between *DPP4* polymorphisms and ONFH risk according to the stratification analysis
| SNP ID       | Model       | Genotype | Control | Case | OR (95% CI) | p-value | Control | Case | OR (95% CI) | p-value |
|-------------|-------------|----------|---------|-----|-------------|---------|---------|-----|-------------|---------|
| rs2268894   | Codominant  | T/T      | 84 (39.4%) | 117 (47%) | 1 | 117 (45.9%) | 114 (52%) | 1 | 117 (45.9%) | 114 (52%) | 1 |
|             |             | T/C      | 109 (51.2%) | 109 (43.8%) | 0.68 (0.46-1.01) | 0.058 | 110 (43.1%) | 86 (39.3%) | 0.80 (0.54-1.18) | 0.265 |
|             |             | C/C      | 20 (9.4%) | 23 (9.2%) | 0.84 (0.43-1.65) | 0.622 | 28 (11%) | 19 (8.7%) | 0.69 (0.39-1.32) | 0.271 |
|             | Dominant    | T/T      | 84 (39.4%) | 117 (47%) | 1 | 117 (45.9%) | 114 (52%) | 1 | 10.073 | 0.186 |
|             |             | T/C-C/C  | 129 (60.6%) | 132 (53%) | 0.71 (0.48-1.03) | 0.138 | 138 (54.1%) | 105 (48%) | 0.78 (0.55-1.13) | 0.026 |
|             | Recessive   | T/T-T/C  | 193 (90.6%) | 226 (90.8%) | 1 | 227 (89%) | 200 (91.3%) | 1 | 0.933 | 0.408 |
|             |             | C/C      | 20 (9.4%) | 23 (9.2%) | 1.03 (0.54-1.94) | 0.179 | 28 (11%) | 19 (8.7%) | 0.75 (0.41-1.43) | 0.271 |
|             | Log-additive| — — —     | — — —     | 0.82 (0.61-1.10) | 0.179 | — — —     | 0.82 (0.62-1.09) | 0.169 |
|             |             | Dance    | — — —     | — — —     | 1 | — — —     | — — —     | 1 | 0.82 | 0.169 |

| Gender      | Males       | Females  |
|-------------|-------------|----------|
| rs2268894   | Codominant  |          |
|             | T/T         | 142 (46.6%) | 59 (36.2%) | 1 |
|             | T/C         | 134 (43.9%) | 117 (43%) | 0.94 (0.66-1.32) | 0.710 | 85 (52.1%) | 78 (39.8%) | 0.54 (0.35-0.85) | 0.007 |
|             | C/C         | 29 (9.5%) | 23 (8.5%) | 0.85 (0.47-1.55) | 0.599 | 19 (11.7%) | 19 (9.7%) | 0.59 (0.29-1.21) | 0.153 |
| Dominant            | T/T       | 142 (46.6%) | 132 (48.5%) | 1 | 0.627 | 59 (36.2%) | 99 (50.5%) | 1 | 0.006 |
|---------------------|-----------|-------------|-------------|---|------|-----------|-----------|---|------|
|                     | T/C-C/C   | 163 (53.4%) | 140 (51.5%) | 0.92 (0.66-1.28) | 104 (63.8%) | 97 (49.5%) | 0.55 (0.36-0.85) |
| Recessive           | T/T-T/C   | 276 (90.5%) | 249 (91.5%) | 1 | 0.660 | 144 (88.3%) | 177 (90.3%) | 1 | 0.550 |
|                     | C/C       | 29 (9.5%)   | 23 (8.5%)   | 0.88 (0.50-1.56) | 19 (11.7%) | 19 (9.7%) | 0.81 (0.36-1.60) |
| Log-additive        |           | --          | --          | --          | 0.93 (0.72-1.20) | --          | --          | -- | 0.020 |

rs16822665 Codominant
| Dominant            | T/T       | 127 (41.6%) | 125 (46.3%) | 1 | 0.255 | 52 (31.9%) | 88 (44.9%) | 1 | 0.016 |
|---------------------|-----------|-------------|-------------|---|------|-----------|-----------|---|------|
|                     | T/C       | 139 (45.6%) | 126 (46.7%) | 0.91 (0.65-1.30) | 93 (57.1%) | 92 (46.9%) | 0.57 (0.37-0.90) |
|                     | C/C       | 39 (12.8%)  | 19 (7%)     | 0.50 (0.27-0.90) | 18 (11%) | 16 (8.2%) | 0.53 (0.25-1.13) |
| Log-additive        |           | --          | --          | --          | 0.78 (0.61-1.00) | --          | --          | -- | 0.018 |

Smoking
| Smoking            | Smokers  | Non-smokers |
|--------------------|----------|-------------|
| rs2268894 Codominant | T/T      | 104 (41.3%) | 97 (44.9%) | 0.001 |
|                    | T/C      | 125 (49.6%) | 94 (43.5%) | 1.06 (0.73-1.55) |
|                    | C/C      | 23 (9.1%)   | 25 (11.6%) | 0.87 (0.47-1.59) |
| Log-additive       |          | --          | --          | 0.67 (0.48-0.93) |

| Dominant            | T/T       | 104 (41.3%) | 109 (56.2%) | 1 | 0.001 | 97 (44.9%) | 122 (44.5%) | 1 | 0.914 |
|---------------------|-----------|-------------|-------------|---|------|-----------|-----------|---|------|
|                     | T/C-C/C   | 148 (58.7%) | 85 (43.8%)  | 0.53 (0.36-0.77) | 119 (55.1%) | 152 (55.5%) | 1 | 0.71 (0.47-1.46) |
| Recessive           | T/T-T/C   | 229 (90.9%) | 179 (92.3%) | 1 | 0.643 | 191 (88.4%) | 247 (90.2%) | 1 | 0.558 |
|                     | C/C       | 23 (9.1%)   | 15 (7.7%)   | 0.85 (0.43-1.69) | 25 (11.6%) | 27 (9.8%) | 0.84 (0.47-1.50) |
| Log-additive        |           | --          | --          | --          | 0.65 | --          | --          | 0.97 | 0.848 |
| rs16822665 | Codominant       | T/T      | 96 (38.1%) | 98 (50.5%) | 1 | 83 (38.4%) | 115 (42.3%) | 1 |
|------------|------------------|---------|------------|------------|---|-----------|------------|---|
|            | T/C              | 125 (49.6%) | 81 (41.8%) | 0.62 (0.42-0.94) | 0.021 | 107 (49.5%) | 137 (50.4%) | 0.90 (0.61-1.32) | 0.580 |
|            | C/C              | 31 (12.3%) | 15 (7.7%) | 0.48 (0.24-0.95) | 0.034 | 26 (12%) | 20 (7.3%) | 0.57 (0.29-1.08) | 0.086 |
| Dominant   | T/T              | 96 (38.1%) | 98 (50.5%) | 1 | 0.008 | 83 (38.4%) | 115 (42.3%) | 1 | 0.330 |
|            | T/C-C/C          | 156 (61.9%) | 96 (49.5%) | 0.59 (0.40-0.87) | 0.132 | 133 (61.6%) | 157 (57.7%) | 0.83 (0.57-1.20) | 0.103 |
| Recessive  | T/T-T/C          | 221 (87.7%) | 179 (92.3%) | 1 | 0.132 | 190 (88%) | 252 (92.7%) | 1 | 0.103 |
|            | C/C              | 31 (12.3%) | 15 (7.7%) | 0.61 (0.32-1.16) | 0.007 | 26 (12%) | 20 (7.3%) | 0.60 (0.32-1.11) | 0.133 |
| Log-additive |              |        |        | 0.66 (0.49-0.89) | 0.007 |        | 0.80 (0.61-1.07) | 0.133 |

| Drinking                          | Drinakers | Non-drinkers |
|-----------------------------------|-----------|--------------|
| rs2268894                         |           |              |
| Codominant                        |           |              |
| T/T                               | 101 (41.9%) | 121 (51.9%) | 1 | 100 (44%) | 110 (46.8%) | 1 | 0.73 |
| T/C                               | 121 (50.2%) | 101 (43.4%) | 0.64 (0.43-0.93) | 0.021 | 98 (43.2%) | 94 (40%) | 0.86 (0.58-1.27) | 0.95 |
| C/C                               | 19 (7.9%) | 11 (4.7%) | 0.48 (0.22-1.07) | 0.072 | 29 (12.8%) | 31 (13.2%) | 0.99 (0.56-1.76) | 0.95 |
| Dominant                          |           |              |
| T/T                               | 101 (41.9%) | 121 (51.9%) | 1 | 0.010 | 100 (44%) | 110 (46.8%) | 1 | 0.53 |
| T/C-C/C                          | 140 (58.1%) | 112 (48.1%) | 0.61 (0.42-0.89) | 0.200 | 127 (56%) | 125 (53.2%) | 0.89 (0.61-1.28) | 0.82 |
| Recessive                        |           |              |
| T/T-T/C                          | 222 (92.1%) | 222 (95.3%) | 1 | 0.200 | 198 (87.2%) | 204 (86.8%) | 1 | 0.82 |
| C/C                               | 19 (7.9%) | 11 (4.7%) | 0.60 (0.28-1.31) | 0.009 | 29 (12.8%) | 31 (13.2%) | 1.06 (0.62-1.84) | 0.73 |
| Log-additive                     |           |              |
|                                  |           |              |

| rs16822665 | Codominant       | T/T      | 90 (37.3%) | 110 (47.2%) | 1 | 89 (39.2%) | 103 (44.2%) | 1 |
|------------|------------------|---------|------------|------------|---|-----------|------------|---|
|            | T/C              | 124 (51.5%) | 114 (48.9%) | 0.70 (0.47-1.02) | 0.065 | 108 (47.6%) | 104 (44.6%) | 0.80 (0.54-1.19) | 0.49 |
|            | C/C              | 27 (11.2%) | 9 (3.9%) | 0.28 (0.13-0.63) | 0.002 | 30 (13.2%) | 26 (11.2%) | 0.78 (0.43-1.42) | 0.23 |
| Dominant   | T/T              | 90 (37.3%) | 110 (47.2%) | 1 | 0.013 | 89 (39.2%) | 103 (44.2%) | 1 | 0.23 |

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| Model | Training Bal. Acc. | Testing Bal. Acc. | CVC | OR (95% CI) | p   |
|-------|-------------------|-------------------|-----|-------------|-----|
| rs16822665 | 0.539 | 0.539 | 10/10 | 1.37 (1.06-1.78) | 0.0172 |
| rs2268894,rs16822665 | 0.554 | 0.533 | 8/10 | 1.52 (1.18-1.97) | 0.0014 |
| rs2268894,rs16822665,rs6741949 | 0.561 | 0.531 | 9/10 | 1.65 (1.27-2.15) | <0.001 |
| rs2268894,rs16822665,rs6741949,rs67399148 | 0.569 | 0.534 | 10/10 | 1.73 (1.33-2.23) | <0.001 |

MDR: multifactor dimensionality reduction; Bal. Acc.: balanced accuracy; CVC: cross-validation consistency; OR: odds ratio; 95% CI: 95% confidence interval.

p values were calculated using \( \chi^2 \) tests.

Bold values indicate statistical significance (p < 0.05).