Gene–Environment Interaction Between *CAST* Gene and Eye-Rubbing in the Chinese Keratoconus Cohort Study: A Case-Only Study

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Purpose. Keratoconus (KC), characterized by progressive corneal protrusion and thinning, is a complex disease influenced by the combination of genetic and environmental factors. The purpose of this study was to explore potential gene–environment interaction between the calpastatin (*CAST*) gene and eye-rubbing in KC.

Methods. A case-only study including 930 patients (676 patients with eye-rubbing and 254 patients without eye-rubbing) from the Chinese Keratoconus (CKC) cohort study was performed in the present study. Genotyping of single nucleotide polymorphism (SNP) was conducted using the Illumina Infinium Human Asian Screening Array (ASA) Beadchip. The gene–environment interactions between *CAST* gene and eye-rubbing were analyzed using PLINK version 1.90. The interactions between *CAST* genotypes and eye-rubbing were analyzed by logistic regression models. The SNP–SNP–environment interactions were analyzed using generalized multifactor dimensionality reduction (GMDR).

Results. Three SNPs in *CAST* gene, namely, rs26515, rs27991, and rs9314177, reached the significance threshold for interactions (defined as $P < 2.272 \times 10^{-3}$). Notably, the minor alleles of these three SNPs exhibited negative interactions with eye-rubbing in KC. The results of logistic regression models revealed that the minor allele homozygotes and heterozygotes of rs26515, rs27991, and rs9314177 also exhibited negative interactions with eye-rubbing. Furthermore, GMDR analysis revealed the significant SNP–SNP–environment interactions among rs26515, rs27991, rs9314177, and eye-rubbing in KC.

Conclusions. This study identified rs26515, rs27991, and rs9314177 in *CAST* gene existed gene–environment interactions with eye-rubbing in KC, which is highly important for understanding the underlying biological mechanisms of KC and guiding precision prevention and proper management.

Keywords: keratoconus (KC), eye-rubbing, calpastatin (*CAST*) gene, gene–environment interaction, case-only study

Keratoconus (KC) is a corneal disorder characterized by corneal ectasia and protrusion, progressive corneal thinning, and irregular astigmatism.[1] The prevalence of KC is 1.38 of 1000 people worldwide and is reported to be higher in South Asian and Middle-Eastern populations.[2,3] The disease usually occurs in adolescence and progresses until the third to fourth decades, causing variable degrees of vision impairment in early adulthood and legal blindness in severe cases, placing an enormous burden on the economy and society.[4–6]

Currently, the pathogenesis of KC remains unclear. Traditionally, KC is considered a noninflammatory disease due to the lack of typical signs of inflammation.[7] However, an increasing number of studies have discovered high levels of proinflammatory cytokines in the tears of patients with KC, the recruitment of immunoinflammatory cells in keratoconic corneal tissue, and the activation of inflammatory pathways, indicating that inflammation may be involved in the pathological process of KC.[8–10] Several epidemiological studies, as well as our previous studies, have identified a strong association between eye-rubbing and KC, indicating a potential role of eye-rubbing in the pathogenesis of this disease.[11–13] The corneal epithelial microtrauma caused by eye-rubbing can lead to the production of proinflamma-

tory cytokines, ultimately resulting in KC via the induction of ocular surface inflammation.[14] Although eye-rubbing is one of the major risk factors for KC, not all people with eye-rubbing develop the disease, indicating individual differences in the risk of KC induced by eye-rubbing.[13] These differences may be attributed to gene–environment interactions, in which genotype–phenotype associations vary according to the environment (or environment–phenotype associations vary according to the genotype).[15–17] Interestingly, calpastatin, which is encoded by the *CAST* gene, a susceptibility gene of KC, is expressed in the corneal epithelium and influences the process of systemic inflammation.[18] Li et al.[19] first identified the correlation between the *CAST* gene and KC in Caucasian populations and suggested that the differential regulation of the calpain/calpastatin system may be related to the pathogenesis of KC using in silico analysis. The calpain/calpastatin system is composed of two Ca^{2+}-dependent proteases, mu- and m-calpain, and their endogenous specific inhibitor, calpastatin.[20] Previous studies have suggested that an imbalance of calpain and calpastatin could affect the process of inflammation by influencing the immune response of immune cells,[21] the activation of inflammatory mediators,[22] and the induction of cell apoptosis.[23] Overall, both eye-rubbing and the *CAST* gene might be related to the process of inflammation and the occurrence of KC. However, no studies have investigated the interaction between the *CAST* gene and eye-rubbing in KC.

Gene–environment interactions, which can effectively illustrate the missing heritability of complex diseases, have been widely used to explore the pathogenesis of many disorders.[16,24] Both the *CAST* gene and eye-rubbing have been demonstrated to be associated with KC. However, considering the genetic heterogeneity in KC, both factors might account for only a small fraction of the phenotypic variation in the disease. Therefore, a case-only study involving 958 Chinese patients with KC was conducted to explore potential gene–environment interactions between the *CAST* gene and eye-rubbing in KC. This study might provide insight into the pathogenesis of KC that can facilitate precision prevention and proper management of the disease.

Methods

Study Population

A total of 958 patients from the Chinese Keratoconus (CKC) cohort study were recruited from Henan Eye Hospital to explore gene–environment interactions in KC.[25] The diagnosis of KC was based on an asymmetric bowtie pattern with or without skewed axes in corneal topography, as well as a Belin Ambrosio enhanced ectasia total deviation index (BAD) value >2.6, and the presence of clinical characteristics detected by slit lamp examination, such as localized stromal thinning, conical protrusion, Vogt's striae, Fleischer's ring, or anterior stromal scar.[26] Patients with KC who had syndromic diseases (e.g. Down syndrome, Ehlers–Danlos syndrome, and Leber congenital amaurosis) were excluded, as were patients with concomitant corneal dystrophy. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Henan Eye Hospital. All participants and their guardians were informed of the purpose and significance of the study and signed informed consent.

Eye-Rubbing Exposure

The eye-rubbing status of patients with KC was collected by experienced ophthalmologists through face-to-face interviews. Eye-rubbing was defined as a frequency of rubbing the eyes one or more times daily, whereas a frequency of less than once daily was defined as non-eye-rubbing.[11,27] After removing patients who lacked eye-rubbing data, we ultimately included 930 patients in the present study, with 676 patients in the eye-rubbing group and 254 patients in the non-eye-rubbing group.

Genotyping and Quality Control

Total DNA was extracted from EDTA-anticoagulated blood samples using Whole Blood Genomic DNA Extraction Kits according to the manufacturer's recommendations (Concert Bioscience [Xiamen] Co., Ltd.). Genotyping was conducted using the Illumina Infinium Human Asian Screening Array (ASA) Beadchip, which includes 659184 single nucleotide polymorphisms (SNPs). Among these SNPs, 36 were located in the *CAST* gene. PLINK version 1.90 was used to extract the genotyping data and conduct the subsequent analyses. SNPs with a call rate < 95%, minor allele frequency (MAF) < 1%, and Hardy–Weinberg equilibrium (HWE) P value < 0.05 were removed from further analysis. Ultimately, 22 SNPs in the *CAST* gene remained after quality control.

Statistical Analysis

The demographic characteristics of the study population were analyzed using Statistical Product and Service Solutions (SPSS, version 27.0; SPSS Inc., Chicago, IL, USA). Quantitative variables were presented as the mean ± standard deviation and analyzed by the Student's t-test. Qualitative variables were expressed as percentages and analyzed by Pearson's chi-square test. Any 2-tailed P value < 0.05 was considered statistically significant. The interaction was estimated by logistic regression models adjusting for age and sex via PLINK version 1.90, with eye-rubbing status as the dependent variable and SNPs as the independent variable. The Bonferroni correction was used to set a significance threshold (2.272×10^{-3}, 0.05/22). An odds ratio (OR) greater than 1.0 indicates a positive interaction, and a value less than 1.0 indicates a negative interaction for patients with minor alleles compared with those with major alleles. The interactions between the *CAST* SNP genotypes and eye-rubbing were estimated via logistic regression models adjusting for age and sex, with genotypes coded according to dominant and additive models. The SNP–SNP–environment interactions among the *CAST* SNPs and eye-rubbing in KC was analyzed via generalized multifactor dimensionality reduction (GMDR, version 0.7; University of Virginia, Charlottesville, VA, USA). Logistic regression analysis was used to validate the GMDR results. A P value < 0.05 was considered to be of statistical significance.

Results

Demographic Characteristics of the Study Population

The demographic characteristics of the study population are shown in Table 1. A total of 676 patients with KC with eye-rubbing (510 male patients and 166 female patients;

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Table 1. Clinical Characteristics of the Study Population

Parameter	Eye-Rubbing (n = 676)	Non-Eye-Rubbing (n = 254)	P Value
Age at diagnosis, y	20.099 ± 5.705	21.437 ± 6.252	0.003
Sex, M/F	510/166	190/64	0.840

Table 2. Gene–Environment Interactions Between *CAST* SNPs and Eye-Rubbing in Chinese Patients With KC

Variant	Allele 1/2	Minor Allele Frequency		Odds Ratio (95% CI)	P Value*
		Eye-Rubbing	Non-Eye-Rubbing		
rs26515	T/C	0.447	0.534	0.710 (0.576–0.875)	1.340×10^{-3}
rs27991	A/G	0.375	0.457	0.709 (0.574–0.877)	1.148×10^{-3}
rs9314177	G/A	0.463	0.545	0.720 (0.584–0.887)	2.069×10^{-3}
rs75565343	A/C	0.213	0.161	1.360 (1.043–1.774)	0.023
rs26507	C/T	0.426	0.374	1.273 (1.029–1.575)	0.026
rs57889668	T/C	0.421	0.374	1.248 (1.007–1.545)	0.043
rs27524	A/G	0.423	0.382	1.218 (0.984–1.507)	0.070
rs3822683	G/A	0.422	0.383	1.202 (0.971–1.487)	0.092
rs3797815	C/T	0.033	0.022	1.595 (0.807–3.153)	0.179
rs11739478	A/C	0.247	0.279	0.854 (0.675–1.081)	0.189
rs4434401	C/T	0.249	0.280	0.857 (0.677–1.084)	0.197
rs150498302	G/A	0.016	0.022	0.685 (0.324–1.446)	0.320
rs116956641	A/G	0.027	0.032	0.797 (0.437–1.453)	0.459
rs117164783	C/T	0.013	0.018	0.748 (0.330–1.695)	0.487
rs17086593	G/A	0.030	0.024	1.245 (0.645–2.403)	0.514
rs28096	A/G	0.070	0.065	1.080 (0.718–1.625)	0.713
rs13362120	C/T	0.087	0.083	1.054 (0.729–1.523)	0.780
rs11738358	T/C	0.086	0.083	1.044 (0.722–1.510)	0.817
rs117338707	A/G	0.021	0.022	0.923 (0.452–1.889)	0.827
rs11135479	C/T	0.087	0.085	1.030 (0.714–1.484)	0.875
rs1057569	A/G	0.058	0.057	1.028 (0.663–1.594)	0.901
rs2290674	A/G	0.024	0.024	0.965 (0.494–1.885)	0.916

* Adjusted for sex and age.

mean age at diagnosis = 20.099 ± 5.705 years) and 254 patients with KC without eye-rubbing (190 male patients and 64 female patients; mean age at diagnosis = 21.437 ± 6.252 years) were ultimately included in our study. The mean age of patients with KC without eye-rubbing was significantly greater than that of patients with KC with eye-rubbing (P = 0.003). There were no significant differences in sex between the two groups.

Gene–Environment Interactions Between *CAST* SNPs and Eye-Rubbing in Patients With KC

The logistic regression model was used to evaluate the interaction of *CAST* SNPs and eye-rubbing on the risk of KC. As shown in Table 2, there were 3 SNPs in the *CAST* gene, namely, rs26515, rs27991, and rs9314177, that exhibited significant gene–environment interactions with eye-rubbing in KC ($P < 2.272 \times 10^{-3}$). The T allele of rs26515, the A allele of rs27991, and the G allele of rs9314177 showed significant negative interactions with eye-rubbing on the risk of KC (rs26515, OR = 0.710, 95% confidence interval (CI) = 0.576–0.875; rs27991, OR = 0.709, 95% CI = 0.574–0.877; and rs9314177, OR = 0.720, 95% CI = 0.584–0.887).

To further explore the mechanism of the interactions, we also performed interaction analyses between different genotypes and eye-rubbing in KC. As presented in Table 3, there were significant interactions between eye-rubbing and different genotypes of rs26515, rs27991, and rs9314177 (P < 0.05). Compared with major allele homozygotes, minor allele homozygotes and heterozygotes of rs26515, rs27991, and rs9314177 presented negative interactions with eye-rubbing (rs26515 TC and TT versus CC, OR = 0.660, 95% CI = 0.469–0.930; rs27991 AG and AA versus GG, OR = 0.631, 95% CI = 0.460–0.865; and rs9314177 GA and GG versus AA, OR = 0.666, 95% CI = 0.469–0.944). In addition, the joint effects of eye-rubbing and the minor allele homozygotes of the three SNPs were lower than the joint effects of eye-rubbing and the heterozygotes (rs26515 TC versus CC, OR = 0.749, 95% CI = 0.521–1.077; rs26515 TT versus CC, OR = 0.505, 95% CI = 0.333–0.767; rs27991 AG versus GG, OR = 0.677, 95% CI = 0.485–0.944; rs27991 AA versus GG, OR = 0.511, 95% CI = 0.330–0.790; rs9314177 GA versus AA, OR = 0.752, 95% CI = 0.519–1.088; and rs9314177 GG versus AA, OR = 0.519, 95% CI = 0.342–0.790). The results suggested that the risk of KC gradually decreases with the number of minor alleles in individuals with eye-rubbing.

SNP–SNP–Environment Interactions Among rs26515, rs27991, and rs9314177 and Eye-Rubbing in Patients With KC

The rs26515, rs27991, and rs9314177 were shown to interact with eye-rubbing in the occurrence of KC, respectively. To further detect the SNP–SNP–environment interactions among the three SNPs and eye-rubbing in KC, we first identified the significant SNP–SNP interactions model among these three SNPs in patients with KC with eye-

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Table 3. Gene–Environment Interactions Between *CAST* Genotypes and Eye-Rubbing in Chinese Patients With KC

Genotype	Eye-Rubbing Status		OR (95% CI)	*P* Value*
	Eye-Rubbing (*n*, %)	Non-Eye-Rubbing (*n*, %)		
rs26515				
CC	202 (29.926)	55 (21.654)	1.000	
TC and TT	473 (70.074)	199 (78.346)	0.660 (0.469–0.930)	0.017
TC	342 (50.667)	127 (50.000)	0.749 (0.521–1.077)	0.119
TT	131 (19.407)	72 (28.346)	0.505 (0.333–0.767)	0.001
rs27991				
GG	261 (38.724)	72 (28.346)	1.000	
AG and AA	413 (61.276)	182 (71.654)	0.631 (0.460–0.865)	0.004
AG	321 (47.626)	132 (51.969)	0.677 (0.485–0.944)	0.021
AA	92 (13.650)	50 (19.685)	0.511 (0.330–0.790)	0.003
rs9314177				
AA	191 (28.254)	52 (20.472%)	1.000	
GA and GG	485 (71.746)	202 (79.528%)	0.666 (0.469–0.944)	0.022
GA	344 (50.888)	127 (50.000)	0.752 (0.519–1.088)	0.130
GG	141 (20.858)	75 (29.528)	0.519 (0.342–0.790)	0.002

* Adjusted for sex and age.

Table 4. SNP–SNP Interactions Among *CAST* SNPs in Patients With KC with Eye-Rubbing

GMDR Model	Training Accuracy	Testing Accuracy	Sign Test (*P* Value*)	CVC
rs27991	0.553	0.527	5 (0.623)	8/10
rs26515 and rs27991	0.560	0.529	7 (0.172)	7/10
rs26515, rs27991, and rs9314177	0.569	0.543	9 (0.011)	10/10

* Adjusted for sex and age.

rubbing using GMDR analysis, and then analyzed the interactions between different genotype combinations and eye-rubbing in KC using logistic regression analysis. As shown in Table 4, a significant 3-locus model ($P = 0.011$) involving rs26515, rs27991, and rs9314177 was identified, with a cross-validation consistency (CVC) of 10/10 and a testing accuracy of 54.3%. As shown in the Figure, there are 19 genotype combinations composed by genotypes of rs26515, rs27991, and rs9314177. Among the different genotype combinations, 12 combinations are high-risk genotype combinations, and 7 combinations are low-risk genotype combinations. Subsequently, a logistic regression analysis was conducted to validate the results, with eye-rubbing status as the dependent variable and genotype combinations as the independent variable. The results revealed a positive interaction effect between high-risk genotype combinations and eye-rubbing in the occurrence of KC (OR = 1.827, 95% CI = 1.335–2.502; $P < 0.001$).

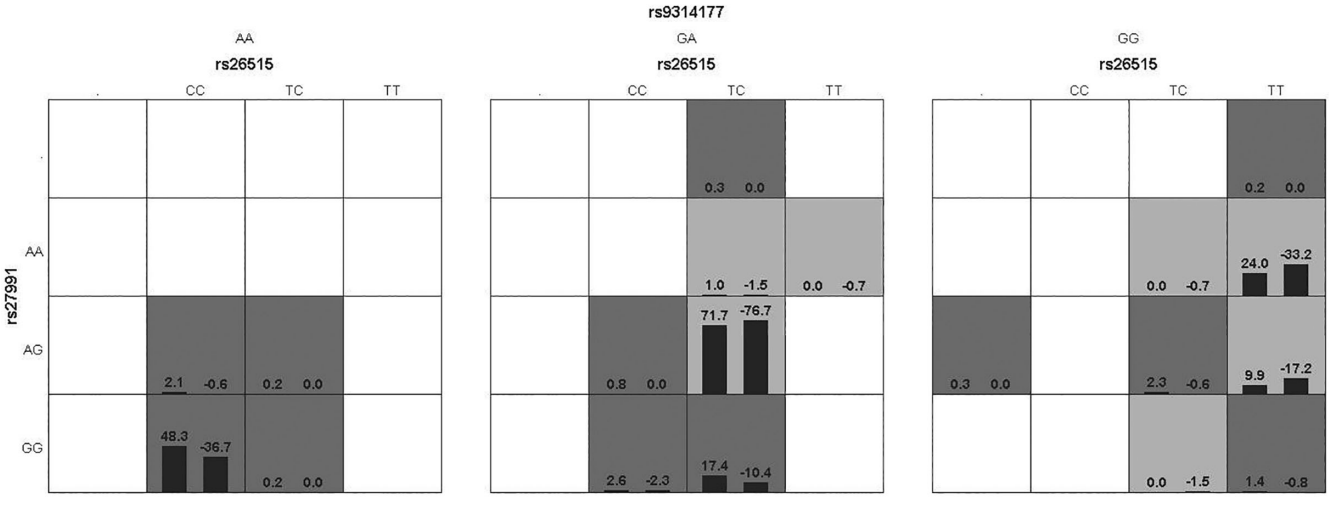

Figure. SNP–SNP interactions of the three-locus model involving rs26515, rs27991, and rs9314177 in patients with KC with eye-rubbing. The *left bars* represent sum scores in patients with KC with eye-rubbing, and the *right bars* represent sum scores in patients with KC without eye-rubbing. The *dark color* cells indicate high-risk genotype combinations, and the *light color* cells indicate low-risk genotype combinations.

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Discussion

This case-only study explored the gene–environment interactions between the *CAST* gene and eye-rubbing in the occurrence of KC. Among the 22 SNPs in the *CAST* gene, the T allele of rs26515, the A allele of rs27991, and the G allele of rs9314177 were found to have negative interactions with eye-rubbing in KC, and the negative interactions tended to increase with the number of minor alleles. In addition, we also identified significant SNP–SNP–environment interactions among rs26515, rs27991, and rs9314177 and eye-rubbing using GMDR.

KC is a complex disease with environmental and genetic factors involved in its pathogenesis.[28] Multiple studies, including ours, have identified numerous genetic loci associated with KC susceptibility.[29–31] However, these variants can explain only a small portion of the phenotypic variation. Recently, several studies have proposed gene–environment interactions in complex diseases, which could account for the missing heritability.[24] Currently, KC is considered a complex disorder with strong heterogeneity, indicating that gene–environment interactions may exist in KC. Case-only studies, first proposed by Piegorsch et al. in 1994, require no controls to explore gene–environment interactions for complex diseases and have been shown to achieve greater testing power and require smaller sample sizes.[32,33] In the present study, a case-only design was first conducted to explore the gene–environment interactions in KC.

To date, many studies have demonstrated that there is a strong correlation between KC and eye-rubbing. Shneor et al.[34] conducted a questionnaire survey of 244 patients with KC and found that 65.6% of the patients rubbed their eyes. A retrospective study of 49 pediatric patients and 167 adult patients with KC conducted by Léoni-Mesplié et al.,[35] reported that 91.84% of children and 70.06% of adults rubbed their eyes. In our previous case-control study of 330 patients with KC and 330 controls, 69.09% of patients had a history of eye-rubbing.[11] These studies provided evidence that eye-rubbing might play an important role in the occurrence of KC. Furthermore, studies have also shown that controlling eye-rubbing is effective in preventing KC. Saad et al.[36] followed a 19-year-old patient with unilateral KC who received no intervention except stopping eye-rubbing for 14 years, and found that both eyes remained in a stable condition, indicating that stopping eye-rubbing may help prevent KC progression. Mazharian et al.[37] conducted a follow-up study including 153 eyes of 77 patients with KC and assessed Pentacam corneal topography at specific timepoints to evaluate the progression of KC after cessation of eye-rubbing, with the increase of maximum keratometry (Kmax) or mean keratometry (Kmean) > 1 diopter (D) or the decrease of thinnest pachymetry (Pachymin) > 5% as the criteria of KC progression. The results suggested that strict cessation of eye-rubbing helps hinder disease progression and maintain a long-term stable state. Dr McGhee[38] provided an overview of KC-related studies and suggested that eye-rubbing may be a "second-hit" factor that can push patients with genetic susceptibility to KC from a subclinical state to a diseased state. Thus, we speculated that interactions may exist between eye-rubbing and genetic variants in KC.

Previous studies have demonstrated that ocular inflammation induced by eye-rubbing might be a possible mechanism resulting in KC.[14] Occasionally, calpastatin, encoded by the *CAST* gene, is correlated with the onset and resolution of systemic inflammation.[21,39,40] Several studies have indicated that altered expression of the calpain/calpastatin system could influence the onset and resolution of systemic inflammation by affecting the apoptosis of neutrophils,[41] the secretion of mature IL-1A,[42] the production of IL-6 and IL-17,[22] and the severity of acute and chronic inflammation.[40] In addition, many studies have indicated that the calpain/calpastatin system participates in the pathological processes of many diseases, such as cancer,[43] Parkinson's disease,[44] sepsis,[45] multiple sclerosis,[46] and retinal neurodegenerative diseases,[47] by mediating the inflammatory response. The association between the *CAST* gene and KC susceptibility was first reported in Caucasian populations by Li et al.,[19] who also reported that the differential regulation of the calpain/calpastatin system may play a role in the pathogenesis of KC. Furthermore, Zhang et al.[48] verified the contribution of the *CAST* gene to KC in the Han Chinese population. Thus, we inferred that a gene–environment interaction may exist between *CAST* gene and eye-rubbing in KC.

In this case-only study, the interaction between the *CAST* gene and eye-rubbing in KC was explored for the first time. The analysis of interactions between the *CAST* SNPs and eye-rubbing revealed that the T allele of rs26515, the A allele of rs27991, and the G allele of rs9314177 have negative interactions with eye-rubbing. This finding indicates that individuals with eye-rubbing who carry minor alleles of rs26515, rs27991, and rs9314177 may have a reduced risk of KC. Furthermore, we performed interaction analyses among the genotypes of the three SNPs and eye-rubbing, with genotypes coded based on dominant models and additive models. The interactions between the *CAST* genotypes and eye-rubbing indicated that the minor allele homozygotes and heterozygotes of rs26515, rs27991, and rs9314177 exhibited negative interactions with eye-rubbing compared with the major allele homozygotes. In addition, the negative interaction increased with the number of minor alleles. These findings of negative gene–environment interactions between eye-rubbing and minor alleles of rs26515, rs27991, and rs9314177 suggested that clinicians can advise patients to avoid eye-rubbing to reduce the risk of KC for noncarriers of minor alleles.

To further investigate the combined effects of rs26515, rs27991, and rs9314177 and eye-rubbing in KC, we investigated the SNP–SNP–environment interactions using GMDR. The GMDR analysis is a widely used tool to assess gene–gene interactions through dimensionality reduction strategies, which can divide multifactor cells in n-dimensional space into high-risk combinations and low-risk combinations according to the threshold.[49,50] We first confirmed the high-risk genotype combinations and low-risk genotype combinations related to eye-rubbing risk in patients with KC using GMDR. The positive interaction between high-risk genotype combinations and eye-rubbing was subsequently validated using logistic regression analysis. These results revealed SNP–SNP–environment interactions among rs26515, rs27991, and rs9314177 and eye-rubbing in KC, and the interactions between high-risk genotype combinations and eye-rubbing might increase the risk of KC. These results suggested that avoiding eye-rubbing might play a role in the prevention of KC in individuals carrying high-risk genotype combinations.

In summary, the present study revealed significant gene–environment interactions between rs26515, rs27991, rs9314177 in the *CAST* gene and eye-rubbing, which might provide a reference for clinicians to guide the precise

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prevention and proper management of KC according to an individual's genetic susceptibility. In addition, the interactions between the *CAST* gene and eye-rubbing may support the correlation between inflammation and KC, suggesting that anti-inflammatory therapies may help manage KC in clinical practice.

Several limitations in this study needed to be addressed. First, a case-only study is not sufficient to determine the main effects of genetic factors and environmental factors, and healthy volunteers for case-control studies should be recruited to determine the main effects of the *CAST* gene and eye-rubbing in the future. Second, as with the clinical characteristics of the CKC study, there were mostly male subjects included in the current study. Additionally, the age-related differences were also identified between patients with and without eye-rubbing. The findings might not be appropriate for other study populations, and further studies in other study populations still need to be explored. Third, this study is from a single center, and selection bias cannot be avoided. Further studies with multicenter analyses are warranted to verify these findings.

Conclusions

In conclusion, the potential gene–environment interactions were identified between three SNPs in the *CAST* gene, namely, rs26515, rs27991, and rs9314177, and eye-rubbing in patients with KC using a case-only study. These findings may help to identify subgroups at higher risk and provide new methods for the prevention and treatment of KC.

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