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ORIGINAL ARTICLE

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Lack of association between *LTF* gene polymorphisms and different caries status in primary dentition

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Abstract

Objective: Dental caries is related to cariogenic bacteria, salivary components, oral hygiene and host susceptibility. Lactoferrin is an important antimicrobial glycoprotein in saliva; however, the role of the *LTF* gene in caries susceptibility is unclear. We investigated the association between *LTF* polymorphisms and the severity of caries.

Design: Our study included 910 healthy paediatric subjects (aged 24–48 months) categorised into three groups: 403 with no caries or white-spot lesions; 230 with moderate caries (8 ≤ dmft ≤ 12); and 277 with severe caries (13 ≤ dmft ≤ 20). Information regarding the subjects' oral habits was gathered using questionnaires. The *LTF* rs1126477 and rs1126478 polymorphism alleles were genotyped by Sanger sequencing.

Results: The three groups showed no significant differences in *LTF* polymorphisms alleles, genotypes or haplotypes distribution. Multifactor dimensionality reduction analysis showed that the interactions between breastfeeding for a duration >24 months, night feeding >24 months and high frequency of sweet food intake increased the risk of caries ($p = 0.0014$); however, we detected no interaction effect between the *LTF* polymorphisms and oral habits on caries susceptibility.

Conclusions: The *LTF* rs1126477 and rs1126478 polymorphisms showed no association with the different levels of caries risk in our Chinese paediatric cohort.

KEYWORDS
caries, genetics, oral habits

1 | INTRODUCTION

Dental caries remains a significant issue in paediatric health and quality of life in modern society. Cavities begin with demineralisation and organic degradation of the tooth structure, further develop into pulp and periapical inflammation, and eventually cause toothache and premature tooth loss (Hunter, 1988). Untreated early childhood caries (ECC) can lead to low self-esteem, malocclusion, impaired masticatory function and developmental problems (Anil & Anand, 2017).

According to the 4th Chinese National Oral Epidemiological Survey, released in September 2017, the caries prevalence rate in 5-year-old was 70.9%, up by 5.8% over the corresponding period 10 years earlier. In Beijing, the mean number of decayed, missing and filled teeth (dmft) in 36- to 42-month-old children was 2.17 ± 3.31, with 59.77% of the carious teeth occurring in 30% of the surveyed children (Li, Miao, & Zhang, 2012). The prevalence rate of caries varies among areas by age group, ethnicity, family income and socioeconomic status (Congiu, Campus, & Lugliè, 2014). Secchi, Maciver, Zeidel, and Zwieniecki (2009) reported a caries prevalence of 3.0% in a population of 787 1- to 3-year-old children attending paediatric clinics in Boston, USA. However, the caries prevalence rates in less developed countries were comparatively higher. In India, the

prevalence rate was 19.2% among 2- to 6-year-old children (Tyagi, 2008), while in Brazil, severe early childhood caries (S-ECC) had a prevalence rate of 37.0% among 4-year-old children (Feldens, Giugliani, Vigo, & Vítolo, 2010).

Caries formation is influenced by cariogenic bacteria, carbohydrate consumption, salivary components, oral hygiene, fluoride and host susceptibility (Vieira, 2012). Although it is a multifactorial disease, genetic factors are gaining more attentions due to their effects on individual susceptibility. In heritability analyses conducted among 732 households, Wang at el. found that caries phenotypes were heritable in primary dentition, with genes accounting for 54%–70% of the variation in caries scores ($p < 0.01$), and the heritability was higher than that in permanent dentition (35%–55%, all $p < 0.01$; Wang et al., 2010).

Previous researches reported an association of host gene polymorphisms with caries susceptibility (Kulkarni et al., 2013; Patir et al., 2008; Tannure et al., 2012; Wang et al., 2010); these polymorphisms can serve as risk factors or confer protection, even when oral habits are included as covariates in the analyses (Abbasoğlu et al., 2015). Researches for candidate genes usually encompass four main types of genes, that is, genes involved in enamel development, saliva composition, the immune response and taste perception (Werneck, Mira, & Trevilatto, 2010). Saliva contains a wide variety of innate and acquired defence factors, such as lysozyme, peroxidase, immunoglobulins and lactoferrin (LTF). LTF, an iron-binding glycoprotein in mammalian secretions (such as saliva, milk and tears), shows broad-spectrum antimicrobial activity, participates in inflammation and regulates the immune response (Fine, 2015). The cationic and hydrophobic *N*-terminus of LTF exerts a direct antimicrobial function by destroying the outer membrane of bacteria (Naidu, Svensson, Kishore, & Naidu, 1993) and works indirectly by sequestering the iron required for bacterial survival. Beyond its bacteriostatic and bactericidal effects, LTF can decrease the lipopolysaccharide-activated innate immune response. LTF regulates the adaptive immune system by promoting the maturation of T-cell precursors into T-helper cells and the differentiation of immature B cells into efficient antigen-presenting cells (Actor, Hwang, & Kruzel, 2009). LTF acts as a front-line mediator in the immune system, playing an important role in physiologic homoeostasis, which is in turn related to disease development. (Kruzel, Zimecki, & Actor, 2017). LTF is involved in many types of inflammatory, autoimmune and tumour diseases such as endotoxaemia, tuberculosis, inflammatory bowel disease, multiple sclerosis and nasopharyngeal cancer. In addition, LTF is involved in the most common oral infectious disease, namely caries.

In our study, the tag single nucleotide polymorphisms (SNPs) were selected according to the following points: (a) minor allele frequency (MAF) exceeding 5%; (b) SNPs with functional effects on protein coding, splicing and transcriptional regulation, which were possibly directly associated with disease (Lee & Shatkay, 2008); and (c) SNPs which were previously studied in oral diseases including caries (Wu et al., 2009; Zupin et al., 2017). Fine at el. reported that LTF accounted for 60%–80% of all anti-*Streptococcus mutans* activity in the saliva and *LTF* rs1126478 can influence caries development (Fine et al., 2013). In addition, the AA genotype of rs1126478 displays bioactivities against other acid-producing microbes (Velliyagounder et al., 2003) and regulates the formation of dental plaque biofilm, thus influencing caries status (Azevedo et al., 2010). However, previous studies showed inconsistent among different populations (Volckova et al., 2014; Wang, Qin, & Xia, 2017). *LTF* rs1126477 was proposed as a risk factor for periodontitis and might contribute to the immune response against periodontitis bacteria (Zupin et al., 2017). The tag SNPs cause missense substitution in the functional region of LTF protein; however, it is unknown whether these SNPs have an effect on caries susceptibility.

In this case–control study, we investigated the allele and genotype distributions of two nonsynonymous *LTF* SNPs (rs1126477 and rs1126478) in children with no caries, those with moderate caries ($8 \le$ dmft ≤ 12) and those with severe caries ($13 \le$ dmft ≤ 20). The relationship between these polymorphisms and caries severity was assessed.

2 | MATERIALS AND METHODS

2.1 | Participants

This study included 910 unrelated paediatric subjects (age range: 24–48 months) with the integrity of the primary dentition. All participants were Han Chinese living in Beijing, China, and were free from systemic diseases. We excluded children whose mothers had experienced pregnancy complications, such as hypertensive disorder complicating pregnancy, gestational diabetes mellitus, acute fatty liver of pregnancy or pregnancy-associated cardiomyopathy; those born at an early gestational age (<37 weeks) or with a low birthweight (<2,499 g); and those showing enamel hypoplasia, enamel hypomineralisation, dentin hypoplasia, dental fluorosis or discoloured or stained lesions.

The guardians of all participants signed informed consent forms prior to enrolment. The Ethics Committee of Peking University School and Hospital of Stomatology approved the study design, protocol and informed consent procedure (PKUSSIRB-201628050).

The participants were divided into three groups according to caries status:

The caries-free group (dmft = 0, with no white-spot lesions) comprised 403 children recruited from 16 kindergartens in the Haidian District of Beijing from August 2015 to September 2016. The patients with caries were outpatients of the Paediatric Department of Peking University Hospital of Stomatology. The moderate caries group consisted of 230 children with a dmft score of 8–12, and the severe caries group contained 277 children with a dmft score of 13–20.

2.2 | Questionnaire

Information regarding oral habits was acquired using questionnaires completed by the guardians of the children. The questionnaire included

questions on the duration of breastfeeding without teeth brushing (≤24 or >24 months), the duration of night feeding without teeth brushing (in months), the frequency of sweet food intake (1–2 times per week, 1–2 times per day, or ≥ 3 times per day) and age (in months) at which teeth brushing was first performed by guardians. Young children lack the capacity to remove food debris and dental plaque, and children who brush their teeth under the supervision of parents have been shown to have a lower risk of caries (Miao, Wang, Zou, & Lin, 2015). All of these oral habits can affect the initiation and development of caries (Congiu et al., 2014).

2.3 | Dental examination

For the oral examinations at the hospital, each participant was seated in a paediatric dental chair in an independent dental consulting room. During the oral examinations at the kindergartens, each participant was seated in a chair with their head resting on the examiner's knees; all examinations were performed in the classroom. During the examinations, the examiner was seated behind the participant's head. Examination for caries was conducted using an artificial light, mouth mirror and tip probe. The subjects were required to fast for 1 hr before the examination. Food debris was gently removed before recording the caries status.

Caries was diagnosed, using the modified World Health Organisation (WHO, 1997) caries diagnostic criteria without radiographs. Caries is recorded when a lesion had an unmistakable cavity, undermined enamel or a decayed/softened floor or wall; a filled tooth is defined as a tooth with permanent restorations or sealant, and no sign of decay (WHO, 1997). In our study, we recorded the number of decayed and filled teeth. Children with suspicious and white-spot lesions were excluded from the caries-free group.

A single paediatric dentistry student performed the caries examinations; prior to the study, the student and an experienced paediatric dentist (reference examiner) examined 20 children to ensure consistency. The κ value for interexaminer agreement was 0.82 and that for intra-examiner agreement was 0.86.

2.4 | SNP selection

Two nonsynonymous exon SNPs (rs1126477 and rs1126478) of *LTF* were chosen as tag SNPs. The potential functions of the tag SNPs were predicted using the *F*-snp online software (https://compbio.cs.queensu.ca/F-SNP/). The global minor allele frequencies (GMAFs) of rs1126477 and rs1126478 in NCBI were A = 0.47 and G = 0.37, respectively.

2.5 | DNA collection and genotyping

The children were required to fast for 1 hr before the collection of exfoliated cells from the oral mucosa. The examiner wiped the buccal mucosa using sterile swabs. Genomic DNA was extracted using the TIANamp Swab DNA Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions, and the isolated DNA was stored at −20°C until use.

The forward (5′-TGTGGAGAATGGCTGGACAT-3′) and reverse (3′-CCATTCAGCTTGGTCCCAAC-5′) primers were designed by Primer 3 online software (https://bioinfo.ut.ee/primer3-0.4.0/). Genes were amplified using 2 × Taq polymerase chain reaction (PCR) Master Mix (Tiangen Biotech). PCR was performed in a Mastercycler Gradient thermal cycler (Eppendorf, Hamburg, Germany) as follows: denaturation at 94°C for 3 min, 29 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, with a 5-min extension step at 72°C. The products were sequenced using an ABI 3730XL Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) to generate genotyping data.

2.6 | Power calculation

The prevalence rate of dental caries in 5-year-old children in Beijing is 58.1% (Wang, Miao, Zou, & Wang, 2015). Assuming a type I error rate of 0.05, with an expected odds ratio >1.6 (showing a medium to strong association), we calculated that a sample size exceeding 230 children in each group would provide sufficient statistical power ($\beta > 0.79$). The statistical power was calculated using Quanto software (https://biostats.usc.edu/Quanto.html).

2.7 | Statistical analyses

Hardy–Weinberg equilibrium of the population was tested using PLINK 1.07 software (https://pngu.mgh.harvard.edu/~purcell/plink/). The chi-squared test was used to analyse the duration of night feeding, frequency of sweet food intake and age at first teeth brushing using SPSS software (ver. 20.0; SPSS Inc., Chicago, IL, USA). The allele and genotype distribution of the *LTF* SNPs, and linkage disequilibrium (LD) and haplotype data were analysed using SHEsis online software (https://analysis.bio-x.cn/myAnalysis.php). The interactions between SNPs and oral habits were assessed using multifactor dimensionality reduction (MDR) software (https://www.multifactordimensionalityreduction.org/). A p-value < 0.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | General information

This study included 910 children (age range: 24–48 months): 403 were caries-free and 507 had dental caries. The dmft score in the caries group ranged from 8 to 20, with a mean of 13.26 ± 3.00. The average ages of the participants were 42.82 ± 3.92 months in the caries-free group, 41.94 ± 6.67 months in the moderate caries group and 42.58 ± 5.62 months in the severe caries group. The proportion of males was 48.9% in the caries-free group, 55.2% in the moderate caries group and 49.8% in the severe caries group. The groups showed no significant differences in terms of age ($p = 0.29$) or sex ($p = 0.13$), but did show significant differences in terms of duration of breastfeeding without teeth brushing ($p < 0.01$), duration of night feeding without teeth brushing

TABLE 1 Personal and oral habit information of the participants

Information	Caries-free group (*N* = 403)	Moderate caries group (*N* = 230)	Severe caries group (*N* = 277)	*F*/χ2	*p*-Value
Mean age ± *SD* (month)	42.82 ± 3.92	41.94 ± 6.67	42.58 ± 5.62	2.07	0.13
Sex distribution					
Male, *N* (%)	197 (48.9)	127 (55.2)	138 (49.8)	2.49	0.29
Female, *N* (%)	206 (51.1)	103 (44.8)	139 (50.2)		
Duration of breastfeeding					
≤24 months, *N* (%)	265 (65.8)	101 (43.9)	117 (42.2)	46.83	*<0.01*
>24 months, *N* (%)	138 (34.2)	129 (56.1)	160 (57.8)		
Duration of night feeding (month)	16.69 ± 10.05	19.49 ± 9.49	20.04 ± 9.27	11.72	*<0.01*
Frequency of sweet food intake					
1–2 times per week, *N* (%)	226 (56.1)	72 (31.3)	69 (24.9)	95.30	*<0.01*
1–2 times per day, *N* (%)	152 (37.7)	111 (48.3)	138 (49.8)		
≥3 times per day, *N* (%)	25 (6.2)	47 (20.4)	70 (25.3)		
Age at first teeth brushing (month)	20.89 ± 10.15	22.00 ± 10.08	23.97 ± 10.56	7.42	*0.01*

p-values are presented in italics when the differences are significant (p < 0.05)

($p < 0.01$), sweet food intake frequency ($p < 0.01$) and age at which teeth brushing was done for the first time by guardians ($p = 0.01$). Participant characteristics and oral habit information are provided in Table 1.

3.2 | Genetic analyses

The two SNPs were in Hardy–Weinberg equilibrium in the caries-free and caries groups ($p > 0.05$). The allele and genotype frequencies of rs1126477 and rs1126478 are displayed in Tables 2–4. The caries-free and moderate caries groups (Table 2), caries-free and severe caries groups (Table 3), and moderate and severe caries groups (Table 4) were compared in terms of genetic profile. The allele frequencies of rs1126477 and rs1126478 showed no significant differences among the caries-free, moderate caries and severe caries groups ($p > 0.05$). In addition, the genotype distributions showed no significant differences among the caries-free, moderate caries and severe caries groups ($p > 0.05$). The LD analysis, done to determine the difference between observed and expected haplotype frequencies, revealed the nonrandom association of alleles at rs1126477 and rs1126478 loci in the study population. The extent of LD was measured by D' and r^2. When D' or r^2 was equal to 1, there was complete linkage disequilibrium between loci. Our results showed that rs1126477 and rs1126478 were in LD ($D' = 0.99$, $r^2 = 0.36$). Haplotype analysis of the caries-free and caries groups showed no significant differences in the distributions of the A-G, G-A or G-G haplotypes between the two groups ($p > 0.05$; Table 5).

TABLE 2 Gene analysis of caries-free and moderate caries groups

LTF polymorphism	Caries-free group (*N* = 403)	Moderate caries group (*N* = 230)	χ2	*p*-Value
rs1126477, *N* (freq)				
Allele G	463 (0.57)	264 (0.57)	<0.01	0.99
Allele A	343 (0.43)	196 (0.43)		
Genotype GG	138 (0.34)	76 (0.33)	0.32	0.85
Genotype AG	187 (0.46)	112 (0.49)		
Genotype AA	78 (0.19)	42 (0.18)		
rs1126478, *N* (freq)				
Allele A	270 (0.34)	151 (0.33)	0.06	0.81
Allele G	536 (0.66)	309 (0.67)		
Genotype AA	51 (0.13)	28 (0.12)	0.06	0.97
Genotype AG	168 (0.42)	95 (0.41)		
Genotype GG	184 (0.46)	107 (0.47)		

TABLE 3 Gene analysis of moderate and severe caries groups

LTF polymorphism	Moderate caries group (*N* = 230)	Severe caries group (*N* = 277)	$\chi 2$	*p*-Value
rs1126477, *N* (freq)				
Allele G	264 (0.57)	328 (0.59)	0.34	0.56
Allele A	196 (0.43)	226 (0.41)		
Genotype GG	76 (0.33)	99 (0.47)	0.41	0.82
Genotype AG	112 (0.49)	130 (0.49)		
Genotype AA	42 (0.18)	48 (0.17)		
rs1126478, *N* (freq)				
Allele A	151 (0.33)	199 (0.36)	1.06	0.30
Allele G	309 (0.67)	355 (0.64)		
Genotype AA	28 (0.12)	35 (0.13)	1.76	0.41
Genotype AG	95 (0.41)	129 (0.46)		
Genotype GG	107 (0.47)	113 (0.41)		

TABLE 4 Gene analysis of the caries-free and severe caries groups

LTF polymorphism	Caries-free group (*N* = 403)	Severe caries group (*N* = 277)	$\chi 2$	*p*-Value
rs1126477, *N* (freq)				
Allele G	463 (0.57)	328 (0.59)	0.42	0.52
Allele A	343 (0.43)	226 (0.41)		
Genotype GG	138 (0.34)	99 (0.47)	0.48	0.79
Genotype AG	187 (0.46)	130 (0.49)		
Genotype AA	78 (0.19)	48 (0.17)		
rs1126478, *N* (freq)				
Allele A	270 (0.34)	199 (0.36)	0.85	0.36
Allele G	536 (0.66)	355 (0.64)		
Genotype AA	51 (0.13)	35 (0.13)	1.79	0.41
Genotype AG	168 (0.42)	129 (0.46)		
Genotype GG	184 (0.46)	113 (0.41)		

TABLE 5 Haplotype analysis of the participants

Haplotype	Caries-free group (*N* = 403)	Caries group (*N* = 507)	$\chi 2$	*p*-Value	Odds ratio [95% CI]
A-A	2.57 (<0.01)	0.00 (0.00)	–	–	–
A-G	340.43 (0.42)	422.00 (0.416)	0.11	0.75	0.97 [0.80–1.17]
G-A	267.43 (0.33)	350.00 (0.345)	0.30	0.58	1.06 [0.87–1.28]
G-G	195.57 (0.24)	242.00 (0.239)	0.06	0.81	0.97 [0.79–1.21]

3.3 | Multifactor dimensionality reduction analysis

To facilitate our statistical analyses, the data on duration of night feeding (≤12, 13–24 and >24 months) and age at first teeth brushing (≤12, 13–24 and >24 months) were converted to numerical values. Multifactor dimensionality reduction analysis comparing the caries-free and caries groups using the optimal third-order model showed that interactions between breastfeeding of >24 months duration, night feeding of >24 months duration and high frequency of sweet food intake increased the risk of caries (OR = 4.10, 95% CI = [1.69–9.94], $p = 0.0014$; Figure 1). No significant interaction effect between the oral habits and genetic factors on caries susceptibility was found.

4 | DISCUSSION

Our previous report on the genotypic distribution of *LTF* rs1126478 in caries and caries-free groups (*N* = 1,005) suggested

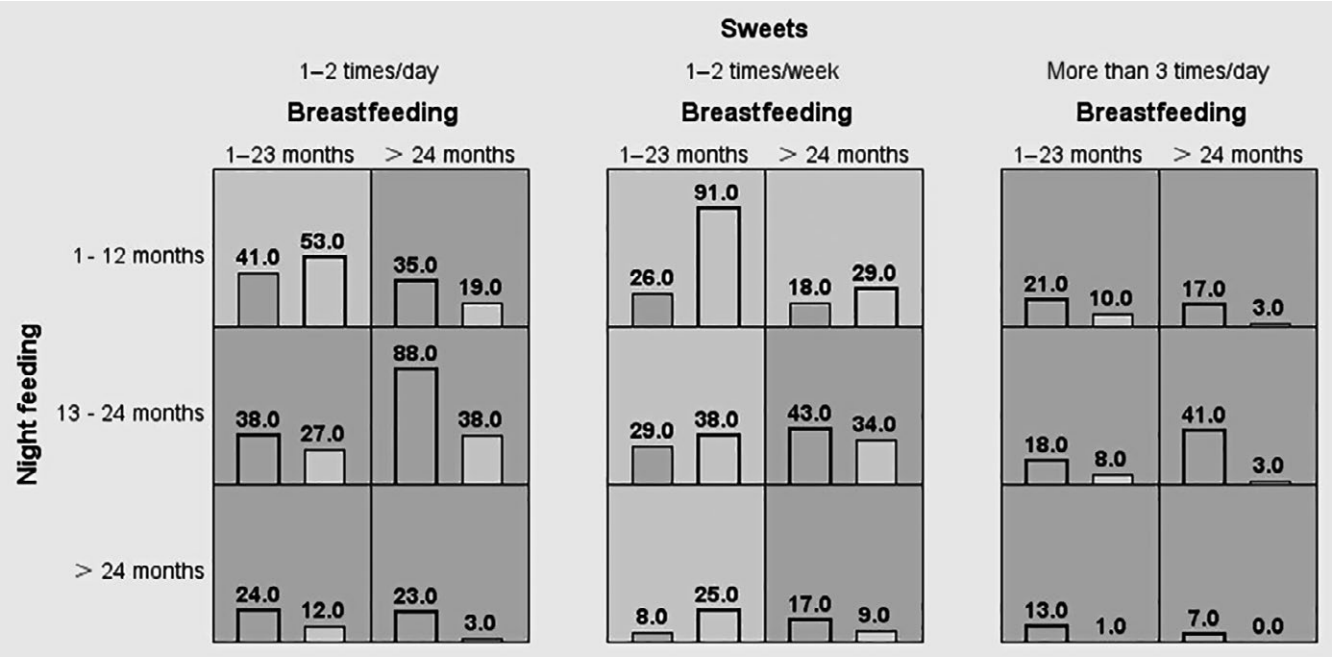

FIGURE 1 Multifactor dimensionality reduction (MDR) analysis of the participants. The MDR model was composed by the combination of risk cells, and light grey cells are low risk, while dark grey cells are high risk. The number of caries patient is shown in the histogram on the left in each cell, while caries-free children are shown by the histogram on the right

no positive association between this SNP and caries (Wang et al., 2017). To further investigate the impact of the *LTF* gene polymorphisms on different caries severity, we chose participants aged 24–48 months, and with complete primary dentition, from among our former study population, and grouped the patients according to whether they had moderate (8 ≤ dmft ≤ 12) or severe caries (13 ≤ dmft ≤ 20). Previous epidemiological studies indicated a polarisation distribution of caries among the paediatric population, with a large majority of carious teeth occurring in a small proportion of children (Li et al., 2012). Such patients have higher dmft scores than the average and thus constitute a key population with respect to the requirement for caries identification and treatment. Although prevention methods including dietary guidance, oral hygiene and fluoride application are effective in reducing caries prevalence worldwide, disease control among highly susceptible individuals remains a challenge, partly due to the role of caries-related genes (Doetzer et al., 2015). Therefore, to explore the impact of the *LTF* SNPs on different levels of caries status, we focused on younger children (age range: 24–48 months) with poor oral health (dmft range: 8–20, i.e., higher than that of the general population). Furthermore, genetic factors showed differential effects on caries severity. Karayasheva et al. analysed the effect of *MMP3* rs679620 in students who were caries-free (DMFT = 0), had low caries status (DMFT ≤ 5) or had high caries status (DMFT > 5). The results showed differences in genotype frequencies between the low and high caries groups. The GG genotype had a stronger association with high vs low caries severity ($p = 0.008$). (Karayasheva, Glushkova, Boteva, Mitev, & Kadiyska, 2016). We also hypothesised that the *LTF* SNPs would show differences among the caries-free, moderate caries and severe caries groups. However, no associations were found between different caries status and the *LTF* rs1126477 or rs1126478.

Rs1126477 and rs1126478 variations in *LTF* exon 2 lead to a shift from alanine to threonine at amino acid position 29, and from arginine to lysine at position 47. These two nonsynonymous SNPs are associated with amino acid changes in the *N*-terminal region of LTF, which is a key region for antimicrobial function. LTF was investigated as a potential biomarker of periodontal disease in previous studies. Huynh et al. (2015) found higher level of LTF in the gingival crevicular fluid of periodontitis patients. Yadav et al. (2014) also reported a higher LTF level in the periodontitis patients than in the healthy controls, and LTF quantification could reduce after periodontal therapy. Gene polymorphisms influence the concentration and activity of LTF, Zupin et al. (2017) observed an association between rs1126477 and risk of chronic periodontitis, among 439 North-Eastern Italian subjects, possibly because of the influence of these loci on the antibacterial activity of LTF. Jordan indicated that the frequency of *LTF* rs1126477 G allele was higher in patients with aggressive periodontitis, suggesting that this polymorphism was associated with aggressive periodontitis in an African American, but not a Caucasian population (Jordan et al., 2005). Zhou et al. (2012) suggested that the *LTF* "A-G-G-T" haplotype (rs1126477, rs1126478, rs2073495 and rs9110) was a protective factor for nasopharyngeal carcinoma and further influenced the expression levels of JNK2 (Zhou, Liu, Yang, Li, & Xu, 2016). Cao, Zhou, Li, and Yi (2011) showed that the frequency of rs1126477 A allele was higher in 700 ovarian cancer patients than that in the healthy group of 700 cases ($p < 0.01$), suggested

that rs1126477 is associated with ovarian carcinoma physiological processes in the Chinese population. An in vitro functional study of the association between *LTF* variants and antimicrobial activity revealed that the lysine residue (AA genotype) at rs1126478 had stronger anti-*Streptococcus mutans* ability than the arginine residue (GG genotype) in saliva ($p = 0.001$, RR = 3.6); it also influenced other acid-producing microbes. Furthermore, the *LTF* rs1126478 AA genotype acted as a protective factor against smooth and proximal surfaces caries (Fine et al., 2013). Genetic polymorphism analysis in 110 12-year-old Caucasian students from Curitiba showed an association of the *LTF* rs1126478 A allele with a high salivary flow rate (>0.5 ml/min; $p = 0.06$, OR = 2.48) and a low DMFT score (DMFT ≤ 2, OR = 0.16, $p = 0.01$; Azevedo et al., 2010). Fine suggested that rs1126478 had opposite effects on proximal caries and localised aggressive periodontitis, where the K29K genotype reduced caries susceptibility but increased periodontitis risk, likely due to the influence of the lysine variant on biofilm composition and disease outcome (Fine, 2015). Doetzer et al. (2015) identified *LTF* as a functional candidate gene for caries, as the rs6441989 A allele occurred significantly less frequently in the high-level caries group (DMFT ≥ 2, $n = 253$) than in the low caries group (DMFT ≤ 1), showing a protective effect against caries. Even considering the role of the environment, Abbasoğlu et al. (2015) suggested that the CT genotype of *LTF* rs4547741 is a protective factor for ECC. Despite these positive findings, our study failed to show an association of *LTF* variants with caries severity in children. Brancher et al. (2011) genotyped the SNPs in the promoter region of the *LTF* gene in 50 students with no caries or an extreme caries phenotype (DMFT = 0 and DMFT ≥ 4, respectively), but failed to identified polymorphisms associated with caries status. Analysis of the *LTF* genotype distribution among 637 Czech children, aged 11–13 years with and without caries, revealed no significant association between allele frequency, genotype distribution and caries (Volckova et al., 2014), which was consistent with our results. We classified our participants into no, moderate and severe caries groups to assess the role of *LTF* SNPs in caries severity; however, the distribution of alleles, genotypes and haplotypes was similar among the three groups. Multifactor dimensionality reduction analysis showed that dietary factors affect caries status without interacting with *LTF* gene polymorphisms. A meta-analysis that included five case–control studies involving 720 patients with caries and 412 caries-free controls showed no significant difference in *LTF* rs1126478 polymorphism prevalence between the caries and caries-free groups, indicating that the rs1126478 polymorphism is not associated with caries susceptibility (Zhou et al., 2016). As the relatively large sample size provided adequate statistical power, we suggest that rs1126477 and rs1126478 are not associated with the severity of caries in primary dentition of Chinese Han children. Yu et al. (2013) found that *LTF* rs10865941 may be associated with caries in a Chinese Han population, but the distributions of the rs10865941, rs1126477 and rs9110 genotypes showed no significant differences between case (DMFT ≥ 3) and control (DMFT ≤ 2) groups in a Chinese Yugur population. As gene polymorphisms vary by geographical region and ethnicity, genetic heterogeneity may be one reason for such inconsistencies. Random patient selection and a sufficient sample size are required in case–control studies to minimise possible error and bias, sometimes it is really difficult for gene association designs. In addition, consideration of the caries phenotype may be critical to such analyses, as genetic factors may have a differential impact on low and high caries status patients (Volckova et al., 2014). Furthermore, greater attention to the host factors in susceptible individuals with extreme caries phenotype may be needed for effective prevention and treatment. But definitions of low and high caries status show variability; moreover, the DMFT/dmft score represents only the number of decayed and filled teeth, and the disease grade (i.e., severity) and location (i.e., anterior or posterior teeth) can be easily overlooked. A recent study showed that the impact of genes associated with caries risk may differ between pit and fissure vs smooth surface caries (Shaffer et al., 2012). On the other hand, the caries inheritance pattern may differ between primary and permanent teeth (Megan et al., 2018; Wang et al., 2010). In contrast to previous studies, we chose young children as our subjects, which may explain the inconsistent results. Future studies with accurate classification of caries are needed to excavate more information.

Caries is a complex disease, and all of the various genetic and environmental factors may exert a small effect on individual susceptibility. However, the interactions between them likely play vital roles in the progression of caries. We used MDR analysis to explore such interactions. MDR analysis is modelled by disease susceptibility, converts multiple factors into a combination to reduce data dimensionality and uses cross-validation and permutation tests to evaluate the ability of combinations of factors to predict disease. This nonparametric method can reduce the type I and II errors associated with logistic linear models, thus constituting a reliable means of studying the aetiology of polygenic disorders (Tang, Li, Chen, & Hu, 2007). Our MDR analysis revealed that, in our population, dietary habits played a larger part in caries occurrence than *LTF* SNPs. Sugar consumption is recognised as a risk factor for caries. According to the WHO classification, free sugar includes artificial monosaccharides and disaccharides additives, as well as the natural sugars present in honey and pure fruit juices. Excessive consumption of free sugars is linked to health problems, such as caries, overweight, diabetes, cardiopathy and neoplasms. In a longitudinal study, Chaffee, Feldens, and Vítolo (2014) found that frequent breastfeeding, particularly beyond 24 months, also increased caries risk, possibly due to the cariogenic characteristics of human milk and supplementation with other sweet foods. Our results showed breastfeeding of >24 months duration without teeth brushing, night feeding of >24 months duration without teeth brushing and high frequency of sweet food intake increased the caries risk fourfold, indicating that feeding habits are associated with oral health in children.

5 | CONCLUSIONS

Our study included 910 Han Chinese participants (age range: 24–48 months): 403 had no caries, 230 had moderate caries and

277 had severe caries. We found no association between different levels of caries risk and the two *LTF* SNPs (rs1126477 and rs1126478). MDR analysis showed no interaction effect between the *LTF* SNPs and oral habits on caries susceptibility. Ad libitum feeding is one of the major risk factors for caries. To further investigate whether *LTF* SNPs influence caries status, multicentre, large-sample phenetic classification studies are required to improve our understanding of the genetic components of caries progression.

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AUTHOR CONTRIBUTIONS

Mengchen Wang conducted the experimental process and wrote the manuscript. Man Qin designed the study and reviesed it.

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