

Department of Stomatology, Yuyao People's Hospital of Zhejiang Province, Yuyao, Zhejiang, China

Association between *DEFB1* polymorphisms and periodontitis: a meta-analysis

SHICHUN ZHONG, CAIJUN WANG*, RENHUI GAO, SHUANG SHU, CHENGJUN SHU

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*Corresponding author: Caijun Wang, Department of Stomatology, Yuyao People's Hospital of Zhejiang Province, NO.800 East Road, Yuyao, Zhejiang 315400, China
caijunwang06@126.com

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Previous studies showed that *DEFB1* gene polymorphisms may impact the development and progression of periodontitis; nevertheless, inconsistent conclusions were described. This study meta-analytically explored the association between periodontitis the *DEFB1* gene polymorphisms and periodontitis. We searched PubMed, Embase, Springer and Cochrane Library for the relevant case-control studies of periodontitis up to February 13th, 2019. Two reviewers selected studies according to the predefined inclusion and exclusion criteria. Newcastle-Ottawa Scale (NOS) was used to assess the quality of studies, and the combined effect size was calculated using R 3.12 software. A total of 9 studies involving 4113 patients and 2373 controls were included. Meta-analysis of *DEFB1*-G1654A gene polymorphisms showed that there were significant differences in model A vs. G (OR = 3.7876, 95%CI = 2.9051-4.9382, $P < 0.001$), AA vs. GG (OR = 4.6743, 95%CI = 3.0900-7.0710, $P < 0.001$), AA vs. GG + AG (OR = 3.5131, 95%CI = 2.4496-5.0384, $P < 0.001$), AA + AG vs. GG (OR = 4.3087, 95%CI = 2.8827-6.4402, $P < 0.001$) and AG vs. GG (OR = 3.0639, 95%CI = 1.6804-5.5863, $P = 0.003$). However, no significant differences were found between *DEFB1* rs11362, rs1799946 and rs1800972 and periodontitis. Sensitivity analysis implied that our results were robust and no publication bias was noticed. Our meta-analysis showed that the *DEFB1*-G1654A polymorphism may be a genetic susceptibility factor for periodontitis.

1. Introduction

Periodontitis is a destructive periodontal disease, which is characterized by microbially associated and host-mediated inflammation (Slots 2017; Tonetti et al. 2018). Currently, two principal forms of periodontitis have been recognized, chronic periodontitis (CP) and aggressive periodontitis (Papapanou and Susin 2017). It mainly manifests in the formation of periodontal pockets, loss of attachment, alveolar bone resorption, and ultimately the loss of teeth. This disease may start in childhood or adolescence (Botero et al. 2015), but frequently debuts in early adulthood (Baelum and Lopez 2013). It has been well acknowledged that bacteria on plaque biofilm are the initiating factors of periodontitis (Ji et al. 2015; Larsen and Fiehn 2017). However, the inflammatory and immune responses of the host caused by bacteria are the decisive factors of periodontal tissue destruction (Hajishengallis 2015). Periodontitis is a multifactorial disease associated with environmental and genetic factors (da Silva et al. 2017). Changes in genetic factors are the basis of host response to disease (Chu et al. 2010; Stabholz et al. 2010). The gene polymorphisms of inflammatory factors may interfere with gene expression of important immune defense mediators, thus regulating the immune response of individuals (Laine et al. 2010).

Human β -defensin 1 (hBD-1), constitutively presented in the oral mucosa, is a small (36 amino acid) peptide that plays critical roles in the host innate defense (Weinberg et al. 1998). It has been reported that hBD-1 contributes to a broad spectrum antimicrobial activity including against viruses, fungi, gram negative and positive bacteria (Weinberg et al. 1998). *DEFB1* is one of the different single nucleotide polymorphisms (SNPs) within the hBD-1 encoding gene. It affects peptide expression of hBD-1 at 5' untranslated region (UTR) of *DEFB1* at g.-52G>A (rs1799946), g.-44C>G (rs1800972) and g.-20G>A (rs11362) (Prado-Montes de Oca 2010). Recently, an increasing number of studies have been focused on the association between *DEFB1* and periodontitis, however, some results are inconsistent. For example, previous studies suggested that *DEFB1*

is associated with chronic and aggressive periodontitis among European Caucasian patients and Japanese patients (Ikuta et al. 2015; Schaefer et al. 2010), but not in North American whites (Ozturk et al. 2010; Wohlfahrt et al. 2006). In addition, these studies are almost limited in sample size of individuals, and there are also differences in population ethnicity.

To address the above problems, in the present study, we systematically searched and assessed the case-control studies exploring the association between the common promoter *DEFB1*-G1654, rs11362, rs1799946 and rs1800972 polymorphism and the susceptibility of periodontitis. We aimed at providing more improved

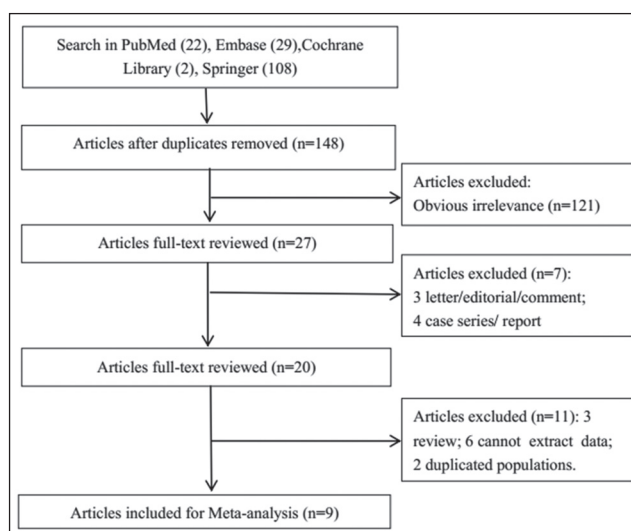


Fig. 1: Literature search and study selection

Table 1: Characteristics of the included literature

Author	Public Year	Location	Genotyping Method	diagnostic criteria	Gene	Score*	Group	Characters		
								N	M/F	Age(years)
Cimoes R	2014	United Kingdom	RT-PCR	A loss of attachment of ≥ 5 mm with one or more of those sites having a pocket of ≥ 4 mm.	rs11800972	6	Periodontitis	44	37/82	45.5(18-82)
Ikuta T	2013	Japan	PCR-Invader	American Academy of Periodontology in 1999.	rs1799946, rs1800972 rs11362	9	Periodontitis	62	22/40	48.6 \pm 13.9
Loo WT	2012	Hong Kong	PCR-HincII restriction enzyme	American Academy of Periodontology in 1999.	G1654A	7	Periodontitis	44	17/26	41.9 \pm 15.6
Ozturk A	2010	USA	DNA Self-Collection kits	Probing depth, clinical attachment loss, and bleeding on probing index.	rs11362, rs1800972, rs179946	5	Periodontitis	108	69/39	42.9 \pm 9.7
Rayanne SAD-MD	2018	Brazil	RT-PCR	American Academy of Periodontology in 1999.	rs11362, rs1800972, rs179946	9	CP	93	NA	NA
Schaefer AS	2009	Netherlands	Applied Biosystems BigDye	NA	rs11362, rs1800972, rs179946	8	Healthy	203	NA	NA
Tian Y	2013	China	PCR-RFLP	American Academy of Periodontology in 1999.	G1654A	7	CP	1337	54 \pm 8	118/4
Wohlfahrt JC	2006	USA	PCR	Eight or more teeth having ≥ 5 mm of proximal CAL.	rs11362	8	Healthy	69	37 \pm 10	70/462
Zupin L	2017	Italy	EZI DNA	Periodontal probing, an X-ray examination.	rs11362, rs1800972, rs179946	8	Periodontitis	137	61/76	53.3 \pm 10.5
							Healthy	82	55/27	56.0 \pm 10.9
							Periodontitis	439	357/448	51.52 \pm 16.19
							Healthy	155		

CP: chronic periodontitis; *: NOS Score (The Newcastle-Ottawa Scale); M/F: Male/Female; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; RT-PCR: real-time polymerase chain reaction; CAL: Clinical attachment loss.

Table 2: Genotype distribution

Author	Public Year	SNP	Wild/mutant	Case				Control				HWE in control	
				N	WH	HT	MH	N	WH	HT	MH	χ^2	P
Cimoes R	2014	rs1800972	C/G	44	22	15	7	34	18	12	4	0.742	0.3890
		rs1799946	G/A	62	20	33	9	43	16	20	7	0.032	0.8588
Ikuta T	2013	rs1800972	C/G	62	52	8	2	43	29	14	0	1.626	0.2023
		rs11362	G/A	62	16	33	13	43	14	20	9	0.140	0.7085
Loo WT	2012	G1654A	G/A	44	9	5	30	108	58	12	38	71.119	<0.0001
		rs11362	G/A	84	32	37	15	203	76	91	36	0.915	0.3387
Ozturk A	2010	rs1800972	C/G	93	60	29	4	203	137	60	6	0.035	0.8524
		rs1799946	G/A	91	36	38	17	203	80	87	36	2.046	0.1526
Rayanne SADMD	2018	rs1799946	G/A	95	31	49	15	69	22	28	19	2.423	0.1196
		rs1800972	C/G	116	86	25	5	95	65	25	5	1.348	0.2457
Schaefer AS	2009	rs11362	G/A	116	18	58	40	69	38	25	6	0.397	0.5286
		rs11362	G/A	1133	354	561	218	1617	513	820	284	1.981	0.1593
Tian Y	2013	rs1800972	C/G	1360	835	472	53	2826	1653	1027	146	0.697	0.4038
		rs1799946	G/A	1359	587	613	159	2834	1222	1302	310	1.777	0.1825
Wohlfahrt JC	2006	G1654A	G/A	122	27	16	79	532	291	54	187	133.612	<0.0001
		rs11362	G/A	137	45	63	29	82	38	32	12	1.414	0.2343
Zupin L	2017	rs11362	G/A	439	169	194	76	155	41	80	34	0.183	0.6687
		rs1800972	C/G	439	266	145	28	155	110	40	5	0.315	0.5747
		rs1799946	G/A	439	175	189	75	155	64	69	22	0.236	0.6268

HWE: Hardy-Weinberg equilibrium, it was evaluated using the likelihood-ratio chi-square test. P-values were presented. P<0.05 was considered representative of a departure from HWE; SNP: Single nucleotide polymorphism; WH: Wild/homozygote; HT: Heterozygote; MH: mutational/homozygote.

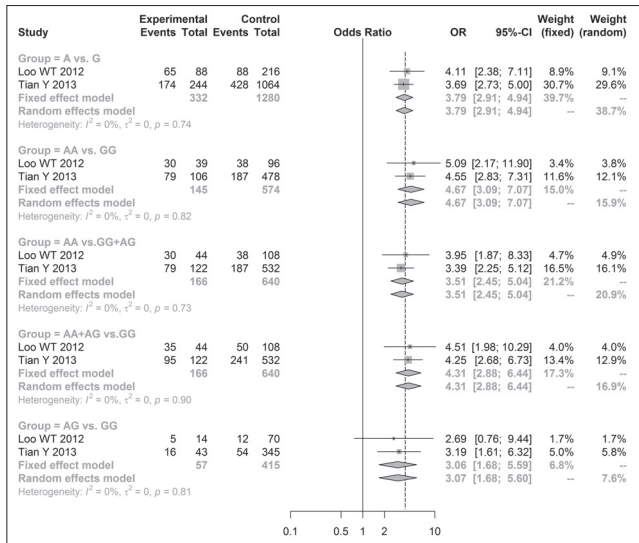


Fig. 2: Meta-analysis of *DEFB1*-G1654A

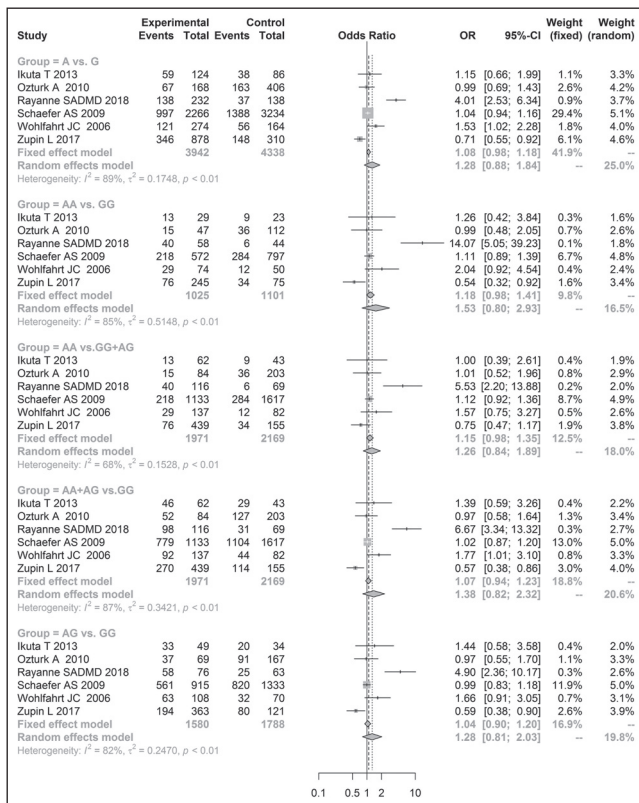


Fig. 3: Meta-analysis of *DEFB1*-rs11362

accuracy evidence about the functional role of *DEFB1* genetic variants in the susceptibility of periodontitis development.

2. Investigations and results

2.1. Study characteristics

The flow chart of the selected literature is shown in Fig. 1. A total of 161 papers (16 from PubMed, 19 from Embase, 2 from Cochrane Library and 108 from Springer) were included. After removing 13 duplicate papers, the 148 studies remained. Among these, 121 obviously not conforming to the title were excluded, and 18 papers (3 letter/editorial/comment; 4 case series/report; 3 review; 6 cannot extract data; 2 duplicated populations) were also excluded. In the end, a total of 9 related studies were included (Cimoes et al. 2014; Ikuta et al. 2015; Loo et al. 2012; Ozturk et al.

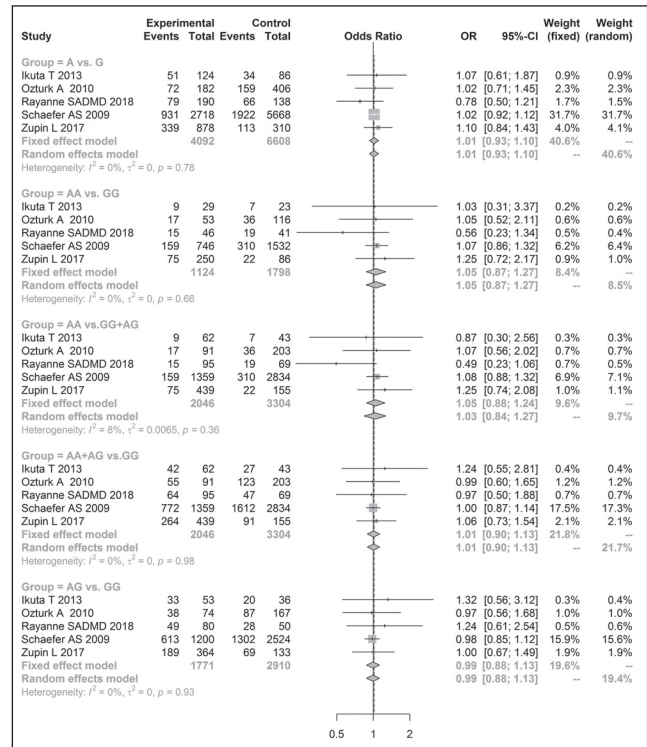


Fig. 4: Meta-analysis of *DEFB1*-rs179946

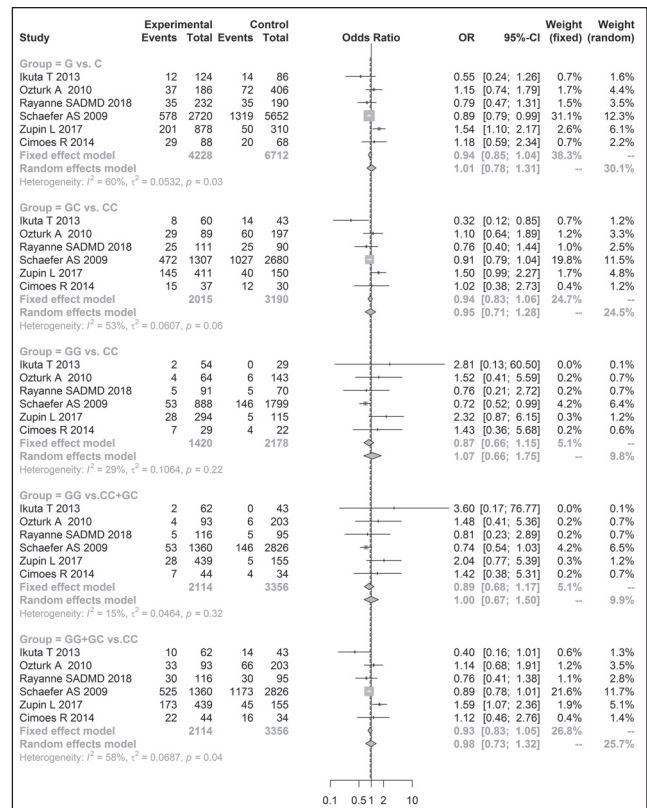


Table 3: Meta-analysis results

Gene	Gene model	Sample size			Test of association			Test of heterogeneity ^{ab}			Egger's test ^c		
		K	Cases	Control	OR (95%CI)	Z	P	Model	Q	P	I (%)	t	P
G1654A	A vs. G	2	332	1280	3.7876 [2.9051; 4.9382]	9.8399	<0.0001	Fixed	0.11	0.74	0.0	-	-
	AA vs. GG	2	145	574	4.6743 [3.0900; 7.0710]	7.3022	<0.0001	Fixed	0.05	0.82	0.0	-	-
	AA vs. GG+AG	2	166	640	3.5131 [2.4496; 5.0384]	6.8299	<0.0001	Fixed	0.12	0.73	0.0	-	-
	AA+AG vs. GG	2	166	640	4.3087 [2.8827; 6.4402]	7.1230	<0.0001	Fixed	0.02	0.90	0.0	-	-
	AG vs. GG	2	57	415	3.0639 [1.6804; 5.5863]	3.6537	0.0003	Fixed	0.06	0.81	0.0	-	-
	A vs. G	6	3942	4338	1.2768 [0.8844; 1.8433]	1.3043	0.1921	Random	44.63	<0.01	88.8	0.8162	0.4602
rs11362	AA vs. GG	6	1025	1101	1.5256 [0.7954; 2.9259]	1.2712	0.2037	Random	33.06	<0.01	84.9	0.6977	0.5238
	AA vs. GG+AG	6	1971	2169	1.2601 [0.8400; 1.8902]	1.1174	0.2638	Random	15.66	<0.01	68.1	0.5730	0.5973
	AA+AG vs. GG	6	1971	2169	1.3790 [0.8206; 2.3172]	1.2135	0.2249	Random	39.82	<0.01	87.4	0.9459	0.3978
	AG vs. GG	6	1580	1788	1.2804 [0.8070; 2.0316]	1.0495	0.2940	Random	27.18	<0.01	81.6	0.9762	0.3843
	A vs. G	5	4092	6608	1.0144 [0.9315; 1.1047]	0.3286	0.7424	Fixed	1.77	0.78	0.0	0.4047	0.7128
	AA vs. GG	5	1124	1798	1.0527 [0.8745; 1.2673]	0.5427	0.5874	Fixed	2.39	0.66	0.0	0.7169	0.5252
rs1799946	AA vs. GG+AG	5	2046	3304	1.0464 [0.8796; 1.2448]	0.5120	0.6087	Fixed	4.36	0.36	0.0	0.9705	0.4034
	AA+AG vs. GG	5	2046	3304	1.0064 [0.8957; 1.1309]	0.1073	0.9146	Fixed	0.37	0.98	0.0	1.1752	0.3247
	AG vs. GG	5	1771	2910	0.9946 [0.8793; 1.1250]	-0.0861	0.9314	Fixed	0.84	0.93	0.0	2.1236	0.1238
	G vs. C	6	4228	6712	1.0143 [0.7834; 1.3133]	0.1077	0.9142	Random	12.51	0.03	60.0	0.5404	0.6176
	GC vs. CC	6	2015	3190	0.9528 [0.7102; 1.2782]	-0.3225	0.7471	Random	10.58	0.06	52.7	0.1464	0.8907
	GG vs. CC	6	1420	2178	0.8742 [0.6640; 1.1510]	-0.9581	0.3380	Fixed	7.01	0.22	28.7	2.1975	0.09291
rs1800972	GG vs. CC+GC	6	2114	3356	0.8894 [0.6774; 1.1678]	-0.8436	0.3989	Fixed	5.90	0.32	15.3	2.5852	0.06099
	GG+GC vs. CC	6	2114	3356	0.9795 [0.7269; 1.3199]	-0.1361	0.8917	Random	11.98	0.04	58.3	0.2037	0.8485

^aRandom-effects model was used when the P for heterogeneity test < 0.05, otherwise the fixed-effect model was used. ^bP < 0.05 is considered statistically significant for Q statistics. ^cEgger's test to evaluate publication bias, P < 0.05 is considered statistically significant. OR: Odds ratio; CI: confidence interval.

case group and 4113 in the control group. The patients in the control were all healthy participants. The methods for SNP detection were mainly RT-PCR and PCR-RFLP. The diagnostic criteria of periodontitis were issued by the American Academy of Periodontology in 1999. The probing depth, clinical attachment loss, and bleeding on probing index were recorded. There were no statistically significant differences in demographic characteristics, age and gender between the two groups (Table 1). In addition, the gene distributions of *DEFBI*-G1654A, rs11362, rs1799946 and rs1800972 were included in each literature analysis and are listed Table 2.

The NOS score was 6-9 (Table 1 and supplementary material available from the authors on request), indicating that the overall quality of literature was relatively high. According to the results of HWE test, the others were in accordance with HWE except the ones by Loo et al. (2012) and Tian et al. (2013).

2.2. Merging quantitative data by meta-analysis

In this paper, different gene models of *DEFBI*-G1654A, rs11362, rs1799946 and rs1800972 were analyzed by meta-analysis. The gene models were listed as following: allele genetic model (*DEFBI*-G1654A, A vs. G; rs11362, A vs. G; rs1799946, A vs. G; rs1800972, G vs. C); codominant genetic model (*DEFBI*-G1654A, AG vs. GG, AA vs. GG; rs11362, AG vs. GG, AA vs. GG; rs1799946, AG vs. GG, AA vs. GG; rs1800972, GC vs. CC, GG vs. CC); recessive genetic model (*DEFBI*-G1654A, AA vs. GG+AG; rs11362, AA vs. GG+AG; rs1799946, AA vs. GG+AG; rs1800972, GG vs. CC+GC); and dominant genetic model (*DEFBI*-G1654A, AA+AG vs. GG; rs11362, AA+AG vs. GG; rs1799946, AA+AG vs. GG; rs1800972, GG+GC vs. CC).

Firstly, a heterogeneity test was carried out. The appropriate effect model was selected to calculate the combined effect value according to the *P* values and *I*² statistics of Q test. The heterogeneity test results showed that there were significant differences in all models of rs11362, and in G vs. C, GC vs. CC and GG + GC vs. CC models of rs1800972 gene (*P* < 0.05, *I*² > 50%). Therefore, random effect model was used to calculate the combined OR value and 95% CI. The fixed effect model was used to merge in the rest other models used (Figs. 2-5 and Table 3).

Meta-analysis of *DEFBI*-G1654A gene polymorphisms showed that there were significant differences in model A vs. G (OR = 3.7876, 95%CI = 2.9051-4.9382, *P* < 0.001), AA vs. GG (OR = 4.6743, 95%CI = 3.0900-7.0710, *P* < 0.001), AA vs. GG + AG (OR = 3.5131, 95%CI = 2.4496-5.0384, *P* < 0.001), AA + AG vs. GG (OR = 4.3087, 95%CI = 2.8827-6.4402, *P* < 0.001) and AG vs. GG (OR = 3.0639, 95%CI = 1.6804-5.5863, *P* = 0.003). These results implied that *DEFBI*-G1654A may be a risk factor gene of the susceptibility of periodontitis (Fig. 2 and Table 3). However, the results of meta-analysis of rs11362, rs1799946 and rs1800972 gene polymorphisms showed no statistical difference among all the results (Figs. 3-5 and Table 3). It was proven that rs11362, rs1799946 and rs1800972 were not associated with periodontitis.

2.3. Results of publication bias

Publication bias test was performed on all models of *DEFBI* gene (G1654A could not be performed with publication bias test because only two literatures were included). The results showed that there was no publication bias in all the results, confirming that our conclusions were reliable (Table 3).

2.4. Results for sensitivity analysis

Sensitivity analysis of all models was analyzed by ignoring each study. All outcomes were not reversed in *DEFBI*-G1654A, rs11362, rs1799946 and rs1800972. The sensitivity analysis showed that the combined results were stable (data not shown).

3. Discussion

In the present study, we systematically performed a meta-analysis about the association between *DEFBI* gene polymorphisms and

the susceptibility to periodontitis. Our meta-analysis of 9 case-control studies showed that significant associations were observed in all models of *DEFBI*-G1654A including A vs. G, AA vs. GG, AA vs. GG + AG, AA + AG vs. GG and AG vs. GG. However, no significant differences were found between *DEFBI* rs11362, rs1799946 and rs1800972 and periodontitis.

A growing number of studies have confirmed that genetic mutations are considered as an important risk factor in the pathogenesis of periodontitis (Borba et al. 2019; Cavalla et al. 2018; de Vries et al. 2017), even though the inherited risk variants have fundamentally remained unsolved. In addition, periodontitis has been well-acknowledged as an inflammatory disease. hBD-1 is constitutively expressed in the oral mucosa, playing imperative roles in the antimicrobial activities including against viruses, fungi, gram negative and positive bacteria (Weinberg et al. 1998). *DEFBI* was the first defensin to be revealed and is one of the different SNPs within the hBD-1 encoding gene. Not surprisingly, *DEFBI* also displays broad-spectrum activities against infectious agents (Wilson et al. 2014). *DEFBI* has been reported to be expressed in numerous cell types, including immune cells and epithelial cells (Carter et al. 2010; Huang et al. 2015). More importantly, *DEFBI* is not only expressed in the health body but also under specific conditions, such as in inflammation (Andresen et al. 2011; Huang et al. 2015). Consequently, *DEFBI* gene and its polymorphisms are linked to the human health (Casalichio et al. 2014). Currently, there are several studies supporting that *DEFBI* gene and its polymorphisms function as potential roles in periodontal diseases (Boniotto et al. 2004; Schaefer et al. 2010; Shao et al. 2019). For instance, Wohlfahrt et al. (2006) suggested that there was no noticeable association between *DEFBI* rs11362 polymorphism and the risk of severe CP in Caucasians; In addition, a study conducted by Ozturk et al. (2010) also did not see a significant effect of *DEFBI* genetic markers on periodontal disease. Shao et al. (2019) confirmed that *DEFBI* rs11362 polymorphism may not be associated with the risk of CP, either according to unadjusted or adjusted data. Schaefer et al. (2010) demonstrated that there were no associations between rs11362, rs1800972 and rs1799946 and CP development. Similarly, our meta-analysis of nine case-control studies also specified no noteworthy association between rs11362, rs1800972 and rs1799946 and the susceptibility of periodontitis. Surprisingly, we found that *DEFBI*-G1654 was associated with the risk of periodontitis. In addition to the reported *DEFBI* common promoters, this is the first meta-analysis systematically accomplished to explore the relationship between *DEFBI*-G1654 and the risk of periodontitis. In contrast, Zupin et al. (2017) revealed that rs11362 and rs1800972 SNPs in *DEFBI* gene are significantly related to periodontitis. The reason may be heterogeneity. In the genetic models we included, the results of the heterogeneity test showed that some models had statistical significance. The possible sources of heterogeneity were as follows: differences caused by different regions, such as the differences of living habits, living environment and economic development level; the influence of other mixed factors, such as gender and age. The strength of our meta-analysis was that this study was the first meta-analysis exploring the relationship between *DEFBI* gene and periodontitis. *DEFBI*-G1654A may be a risk factor of development of periodontitis.

However, there were also some deficiencies. Firstly, due to the incomplete data of some studies and the relatively small number of studies included, no covariate correction and subgroup analysis were carried out in this study. They may affect the results of meta-analysis as potential confounding factors. Secondly, the results of HWE test showed that G1654A of the control groups in the studies of Tian et al. (2013) and Loo et al. (2012) were not in accordance with HWE, indicating that the representativeness of G1654A population was poor. Thirdly, few publications discussed G1654A, only two studies could not be included for publication bias test. Forth, only four genotypes of *DEFBI* was carried out, and other *DEFBI* genes was not considered because of the small number of studies included.

In conclusion, our results showed that *DEFBI* rs11362, rs1799946 and rs1800972 were not associated with periodontitis, while *DEFBI*-G1654A may be a risk factor gene. However, larger-scale, high-quality studies or updated meta-analysis with larger sample size still need to verify our results.

4. Experimental

4.1. Search strategy and study selection

A systematic search of studies was performed to identify the relevant published papers addressing the association between DEFB1 gene polymorphisms and periodontitis, up to February 13th, 2019. The databases include PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>), Embase (<http://www.embase.com/>), Springer (<https://link.springer.com/>) and Cochrane Library (<http://www.cochranelibrary.com/>). The search keywords were listed as following: periodontitis OR “dental arthritis” OR “periodontal inflammation” OR “Peridentitis” OR “pericementitis”, AND *DEFB1* OR “ β -defensin-1” OR “hBD-1” OR “Human beta-defensin-1” OR “human beta-defensins-1” OR “Human β -defensin 1” AND “polymorphi” OR “genetic” OR “variant” OR “gene”.

4.2. Inclusion and exclusion criteria

Inclusive criteria: (1) the original literature were about the distribution of mutation gene frequencies of *DEFB1* gene (including *DEFB1*-G1654A, rs11362, rs1799946 and rs1800972) in periodontitis patients and non-periodontitis patients; (2) the studies should be case-control studies; and (3) the studies should provide precise genotype or allele frequency data.

Exclusion criteria: (1) data are incomplete and cannot be used for statistical analysis; (2) non-monographic literature such as reviews, letters and comments; and (3) repeated publications or the same population data for multiple studies.

4.3. Data extraction and quality assessment

Two investigators (Shichun Zhong and Caijun Wang) independently extracted the following data from each included study: the first author of the literature, the year of publication, the research area, the diagnostic criteria of periodontitis, the detection method of gene polymorphism, the gene type of *DEFB1*, the source of control group, the number of cases group (periodontitis group) and control group (non-periodontitis group), sex ratio, age, and the gene distribution of *DEFB1*-G1654A, rs11362, rs1799946 and rs1800972 analyzed in each literature. The methodological quality of the included studies was evaluated according to Newcastle-Ottawa Scale (NOS) recommended by the Agency for Health Care Research and Quality (AHRQ) (Wells 2001). Any divergent opinions in the process of document data extraction and quality evaluation were solved by discussion with the third author.

4.4. Statistical analyses

Hardy-Weinberg equilibrium (HWE) tests were performed and the Chi-squared test was used to evaluate the deviation of genotype distribution from HWE tests among controls (Schaid and Jacobsen 1999). Meta-analysis was carried out by using R 3.12 software (<http://www.R-project.org/>; R Foundation for Statistical Computing, Beijing, China). The OR value and 95% CI were selected as the effect indicators (Liu et al. 2013). Heterogeneity tests were based on Chi-square Q-test (Lau et al. 1997) and *I*² statistics. If heterogeneity test has statistical differences ($P < 0.05$ or $I^2 > 50\%$), random effect model is used to calculate the combined effect value; otherwise, fixed effect model is used to merge data ($P > 0.05$ and $I^2 < 50\%$) (Feng et al. 2011). In addition, Egger's test was used to evaluate the potential publication bias. If the *P* value > 0.05 , there was no publication bias (Egger et al. 1997). The robustness of the results was evaluated by sensitivity analysis.

Conflicts of interest: None declared.

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