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Association of gene polymorphisms for plasminogen activators with alveolar bone loss

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Background and Objective: The plasminogen activating system is a protease/inhibitor system central to extracellular matrix remodeling with a suggested role in periodontal disease pathology. A few studies have reported polymorphisms in the genes of plasminogen activator inhibitors to be associated with periodontal disease severity. Two gene polymorphisms – a *Bam*HI restriction fragment length polymorphism in the urokinase plasminogen activator gene (*uPA*) and a *Hin*dIII restriction fragment length polymorphism in the plasminogen activator inhibitor type 1 gene (*PAI-1*) – have been associated with conditions having a vascular component, and our objective was to assess the association of these gene polymorphisms with alveolar bone loss in chronic periodontal disease of adults.

Material and Methods: Genotype was determined by polymerase chain reaction amplification of whole blood, pertinent histories were obtained by interview, and alveolar bone loss was assessed from current radiographs.

Results: In 77 elderly patients with a normal distribution of alveolar bone loss, we demonstrated a significant association between levels of alveolar bone loss and these polymorphisms in the *uPA* and *PAI-1* genes. Controlling for the contributions of smoking or diabetes to periodontal bone loss, estimated odds ratios for predicting lower levels of alveolar bone loss, associated with a greater degree of periodontal health, were strongest when defined by the concurrent presence of a homozygous urokinase plasminogen activator genotype and the nuclease-sensitive plasminogen activator inhibitor type 1 (*Hin*dIII) allele (odds ratio = 2.6; 95% confidence interval: 5.8–1.3).

Conclusion: The urokinase plasminogen activator (*Bam*HI) and plasminogen activator inhibitor type 1 (*Hin*dIII) genotypes may serve as useful markers for heritability of bone loss associated with periodontal disease.

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The pathogenesis of chronic periodontal disease in adults appears to be related to plasminogen activators and plasminogen activator inhibitors, which are involved in the activation of plasmin and are known to play a significant role in vascular events and tissue remodeling (1,2).

Plasminogen activators and plasmin can be measured in gingival crevicular fluid and gingival tissues. Plasmin activity levels and plasminogen activator activity levels in both gingival crevicular fluid and homogenized gingiva are increased in accordance with disease severity (3,4), reportedly being present at a concentration of up to 200-fold higher in gingival crevicular fluid than in plasma (5,6). The urokinase plasminogen activator has been immunohistochemically identified in inflamed periodontal tissues (7), where intense urokinase plasminogen activator staining was associated with the

blood vessels with greatest intensity in the small vessels near the epithelial pocket lining. Plasminogen activator inhibitor type 1 mRNA in the diseased periodontium has been localized to blood vessels in the connective tissue (8), evidence which supports its role in vascular remodeling relative to chronic periodontal disease.

A 4G/5G promoter polymorphism in the gene for plasminogen activator inhibitor type 1 has been shown to have a significant association with periodontal disease severity (9). Within the Pima Indian tribe, which presents with higher-than-average levels of periodontal disease, the prevalence of a plasminogen activator inhibitor type 2 allelic combination within the coding region (Ser/Cys 413) was reported as less frequent than in a control population (10).

A *Hin*dIII restriction fragment length polymorphism in the 3′ end of the plasminogen activator inhibitor type 1 gene (*PAI-1*), and a *Bam*HI restriction fragment length polymorphism at the 3′ end of the urokinase plasminogen activator gene (*uPA*), have been linked to diseases with a vascular component (11–16). In this cross-sectional analysis, we report a significant relationship between levels of alveolar bone loss in periodontal disease and these polymorphisms in the *uPA* (*Bam*HI restriction fragment length polymorphism) and *PAI-1* (*Hin*dIII restriction fragment length polymorphism) genes.

Material and methods

A convenience sample of adult patients presenting for treatment to a University Health Center was recruited over the course of 2 years with informed consent. The study accepted all candidates over the age of 21 years without bias to race or gender. Those unable to donate blood or undergo radiographic examination were excluded from participation. For this analysis, those with evidence or history of rapidly progressive periodontitis were excluded.

Loss of alveolar bone during the natural history of periodontal disease is cumulative, and genetic predisposition of adults to chronic periodontal disease can therefore best be evaluated in older adults. Therefore, in this analysis, only adults over the age of 47 years were included. A score for alveolar bone loss severity was determined by radiographic examination of each participant by one investigator. In determining a score for alveolar bone loss severity, all present teeth were assigned a designation of none, mild, moderate, or severe, based on the greatest radiographic extent of alveolar bone loss for each tooth, where the designations of mild represented $< 25\%$ crestal bone loss, moderate represented 25–50% crestal bone loss, and severe represented $> 50\%$ crestal bone loss, relative to the distance from the apex to a point 2 mm apical to the cemento–enamel junction. The designation of mild alveolar bone loss had the criterion that one or more sites demonstrated radiographic evidence of at least, but no more than, mild crestal alveolar bone loss. The designation of moderate had the criterion that at least one site demonstrated between 25 and 50% crestal bone loss, but had no teeth with a designation of severe. The designation of severe alveolar bone loss had the criterion that at least one site demonstrated $> 50\%$ crestal alveolar bone loss.

Smoking history and diabetes status were obtained verbally, and glycated hemoglobin (HgA1c) values were used to corroborate diabetes status. After obtaining informed consent and verbal history, venous blood was collected from the participants, and genomic DNA was extracted from peripheral blood cells. Polymerase chain reaction amplification was performed with the addition of 12.5 pmol of forward and reverse primers, as described previously for the *Bam*HI urokinase plasminogen activator polymorphism (17) and the plasminogen activator inhibitor type 1 (*Hin*dIII) polymorphism (18). Polymerase chain reaction products were digested with restriction endonucleases *Bam*HI and *Hin*dIII, as indicated.

Genotypes for *uPA* (*Bam*HI) and *PAI-1* (*Hin*dIII) were determined for each participant from ethidium bromide-stained gels under ultraviolet light. Considering the ordinal designations of periodontal disease severity, analyses were performed by ordinal logistic regression. Where indicated, association with the dependent outcome, alveolar bone loss, was modeled with the inclusion of periodontal disease risk factors of smoking (yes/no) and diabetes status (none, or type 2), as covariates (type 1 diabetics were excluded from the analysis).

Analysis of the International HapMap Project database was performed using the available NCBI Build 35, release 20, with the HapMap Population Dataset of European ancestry.

Results

We recruited 93 participants over the age of 21 years for the study, but it became clear, during genotype analysis, that inclusion of younger participants skewed the disease distribution towards health and that the long-term consequences of a given genetic predisposition towards periodontal bone loss were not represented adequately in the younger participants. Therefore, the analysis was limited to those participants > 47 years of age. These 77 participants ranged in age from 47 to 83 years, with a normal distribution and a mean and median of ≈ 62 years. Levels of alveolar bone loss in this cohort, as determined radiographically, were evenly distributed, with 32.4% having only mild bone loss, 37.6% demonstrating moderate levels of alveolar bone loss, and 30.0% demonstrating severe levels of alveolar bone loss. Our sample population comprised 41 men and 36 women, with 77% self-reported as non-Hispanic White, 13% as Hispanic, 5% as Black, and 5% as Asian. Because both a history of smoking and diabetes are each significant risk factors in periodontal disease, and because we are interested in the possible interplay between complications arising from these risk factors and gene polymorphisms of the plasminogen activating system, approximately equal numbers of self-reported smokers (49%) and nonsmokers (51%) were included in the analysis, and 69% self-reported a history of type 2 diabetes. No type 1 diabetics were included in the analysis. Out of the 78 participants,

43 (55%) of the cohort reported the use of diabetes control medication, while another nine (11%) denied the use of diabetes control medication but presented with an elevated HgA1c level. The median self-reported duration of diabetes in the diabetic subset was ≈9 years (± 7), and the median HgA1c level in the diabetic subset was 6.9% ± 1.4%. Of the smoking subset, the median pack/years smoked during the participant's life was 20 ± 32.

Our sample population included a balanced sampling of disease severity, gender, smokers, and diabetic participants, while the cohort was overwhelmingly non-Hispanic White. Genotype distributions for the *PAI-1* (*Hin*dIII) and *uPA* (*Bam*HI) polymorphisms were balanced and not significantly different from a Hardy–Weinberg distribution ($p = 0.11$ and 0.20, respectively) (Fig. 1).

Those participants who had the *PAI-1* (*Hin*dIII) nuclease-resistant allele (allele '1') or the urokinase plasminogen activator (*Bam*HI) heterozygous allele pair (genotype '1/2') were found to have higher levels of alveolar bone loss than the remainder of the cohort, and this is depicted in the mosaic plots of Fig. 2.

When the participant's genotype was heterozygous for the *uPA* (*Bam*HI) allele pair, the estimated odds ratio for having more severe alveolar bone loss was 2.0 (3.2–1.2) ($p = 0.004$). When the participant's genotype included a *PAI-1* (*Hin*dIII) nuclease-resistant allele and the heterozygous *uPA* (*Bam*HI) allele pair, the estimated odds ratio for having more severe alveolar bone loss associated with chronic periodontal disease was 2.1 (3.7–1.3) ($p = 0.005$). When the genotype did not include either the *PAI-1* (*Hin*dIII) nuclease-resistant allele or a heterozygous *uPA* (*Bam*HI) allele pair, the estimated odds ratio for less advanced alveolar bone loss was 2.6 (5.8–1.3) ($p = 0.01$). Therefore, in this analysis, the strongest statistical association with advanced alveolar bone loss in chronic periodontal disease was the concomitant presence of the *PAI-1* (*Hin*dIII) nuclease-resistant allele and the heterozygous *uPA* (*Bam*HI) allele pair (odds ratio = 2.1), while the lowest risk for alveolar bone loss was associated with absence of both the *PAI-1* (*Hin*dIII) nuclease-resistant allele and the heterozygous *uPA* (*Bam*HI) allele pair (odds ratio = 2.6) (Table 1).

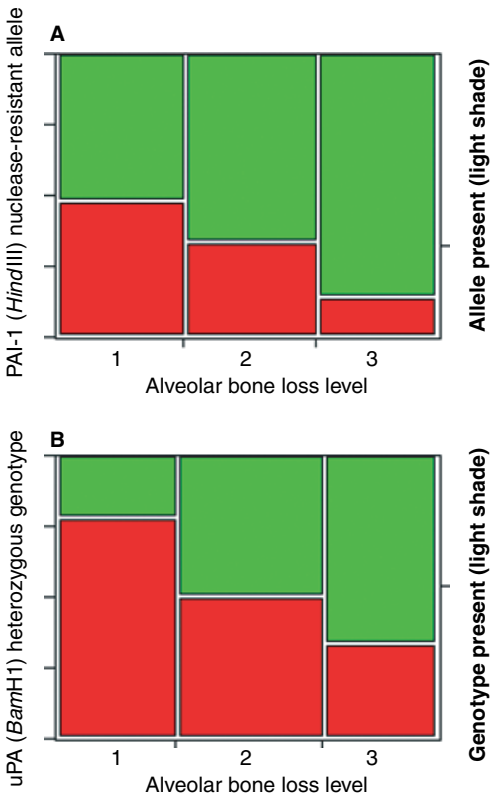
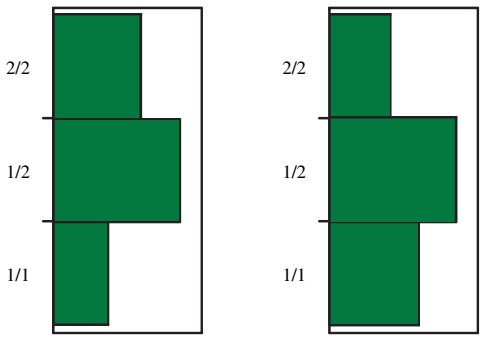


Fig. 2. Mosaic plot depiction of the alveolar bone loss levels (1–3 on the *x*-axis) vs. the presence or absence of (A) the *PAI-1* (*Hin*dIII) nuclease-resistant '1' allele, and (B) the *uPA* (*Bam*HI) heterozygous '1/2' allelic pair genotype in the sample population. Level 3 represents advanced, level 2 represents moderate, and level 1 represents mild alveolar bone loss, as described in the Material and methods. Dimensions in each axis represent relative proportion. PAI-1, plasminogen activator inhibitor type 1; uPA, urokinase plasminogen activator.



Frequencies *PAI-1* (*Hind*III)

Level	Count	Prob
1/1	14	21%
1/2	32	47%
2/2	22	32%
Total	68	100%

Frequencies *uPA* (B*am*H1)

Level	Count	Prob
1/1	23	32%
1/2	33	46%
2/2	16	22%
Total	72	100%

Fig. 1. Frequency distributions for the three allele combinations (genotype) for the *PAI-1* (*Hin*dIII) polymorphism and the *uPA* (*Bam*HI) polymorphism. Each genotype is designated with an allele pair, where the '1' allele represents the restriction endonuclease-resistant allele, and the '2' allele represents the restriction endonuclease-sensitive allele. PAI-1, plasminogen activator inhibitor type 1; uPA, urokinase plasminogen activator.

Table 1. Integrated risk analyses for alveolar bone loss

Genotype	Odds ratio[a] for increased ABL	*p*-value	Odds ratio[a] for decreased ABL	*p*-value	Statistical risk level for ABL
+ PAI-1 (*Hin*dIII) resistant allele + uPA (*Bam*HI) heterozygous	2.1 (3.7–1.3)	0.005			Highest
+ uPA heterozygous	2.0 (3.2–1.2)	0.004			High
+ uPA homozygous			2.0 (3.2–1.2)	0.004	Low
(–) PAI-1 (*Hin*dIII) resistant allele (–) uPA (*Bam*HI) heterozygous			2.6 (5.8–1.3)	0.01	Lowest

[a]Estimated odds ratios (OR) with 95% confidence intervals in parentheses.
ABL, alveolar bone loss; PAI-1, plasminogen activator inhibitor type 1; uPA, urokinase plasminogen activator.

Discussion

In the *uPA* gene (gene name *PLAU*, gene ID 5328, chromosome 10 at 10q24, gene accession no.: AF377330) the *Bam*HI restriction fragment length polymorphism is located within the *Bam*HI site (sequence GGATCC) surrounding base 8928A/C, where a nuclease-sensitive allele is hydrolyzed by *Bam*HI activity, thereby producing differential restriction enzyme digestion fragments of the genomic DNA in this region. This polymorphism lies outside the protein coding sequence (bases 2207–3055) in the 3′ untranslated region of the *uPA* gene (bases 3056–10 075). In the *PAI-1* gene (gene name *SERPINE1*, gene ID 5054, chromosome 7 at 7q21.3-q22, gene accession no.: AC004876), the gene polymorphism on one or both alleles also lies outside the protein coding region (bases 23 159–34 338) within the 3′ untranslated region of the *PAI-1* gene (bases 34 339–126 462) and is located within the *Hin*dIII site (sequence AAGCTT) within bases 37 873–37 878, allowing differential restriction enzyme digestion fragments of the genomic DNA in this gene region. At this point, we cannot speculate on a mechanism of action for either of these polymorphisms because each lies in the 3′ untranslated region. Alhough neither may be functional, it is highly possible that the polymorphisms function as *cis*-acting regulatory regions, contributing to transcriptional control (for a current review, see ref. 19). However, while serving as potentially useful and relevant markers for periodontal disease susceptibility, they may simply be in linkage disequilibrium with other, more relevant, genomic polymorphisms.

Within the coding regions of the *PAI-1* and *uPA* genes there are several nonsynonymous coding single nucleotide polymorphisms that would be expected to be very closely linked to the *Hin*dIII or *Bam*HI polymorphisms in the 3′ untranslated regions. These nonsynonymous coding single nucleotide polymorphisms would result in primary sequence changes, and any one or combination of these coding single nucleotide polymorphisms could have accounted for the significant clinical relationships to the polymorphisms reported here.

Downstream of the *uPA* gene, only 80 000 bases on chromosome 10, lies the vinculin gene (*VCL*) coding for a cytoskeletal protein involved in cell–cell and cell–matrix junctions. There is one nonsynonymous coding single nucleotide polymorphism known to be located within this gene, which could significantly alter the amino acid sequence and character of the gene product. It is expected that the proximity of *VCL* to *PLAU* would result in significant linkage disequilibrium between the two. Analysis of the International HapMap Project database for linkage disequilibrium revealed that the log of the odds for linkage disequilibrium between single nucleotide polymorphisms within *PLAU* and *VCL* was as high as 2.0. Interestingly, urokinase plasminogen activator and vinculin are shown to colocalize on the endothelial cell at focal contacts (20).

Upstream of *PLAU*, within a 40 million base range, lie several other known genes with known nonsynonymous coding single nucleotide polymorphisms. One is a gene for fucosyltransferase (*FUT11*), with three nonsynonymous coding single nucleotide polymorphisms, which are of particular interest because urokinase plasminogen activator is known to be modified with a single fucose monosaccharide (21). HapMap database analysis revealed a log of the odds of 13.2 reported between single nucleotide polymorphisms within *PLAU* and *FUT11*.

In *PAI-1* linkage analysis, the *SERPINE1* gene was determined to be closely linked to the erythropoietin gene (*EPO*) in nine informative families (22). Since then, several genes with single nucleotide polymorphisms in the coding regions have been mapped within the distance of approximately 0.4 million bases between *EPO* and *PAI-1* in the long arm of chromosome 7. Among these are two mucin genes – *MUC3B* (10 nonsynonymous coding single nucleotide polymorphisms) and *MUC17* (three nonsynonymous coding single nucleotide polymorphisms). HapMap database analysis revealed a log of the odds of 1.2 between single nucleotide polymorphisms within *MUC17* and *SERPINE1*.

Downstream, a comparable 0.4 million bases from the *PAI-1* gene, lies a gene encoding collagen lysyl hydroxylase enzyme (*PLOD3*) with one nonsynonymous coding single nucleotide polymorphism and a protein with homology to a member of the major histocompatibility complex class 1 receptor complex, with five nonsynonymous coding single nucleotide polymorphisms (LOC4426060). HapMap database analysis revealed a log of the odds of 4.3 between single nucleotide

polymorphisms within *PLOD3* and *SERPINE1*.

With an estimated odds ratio of 1.2×10^{10} for linkage between *PAI-1* and *EPO* reported by Klinger *et al.* (22), there would be a similarly high probability that these contiguous genes of equal or less distance from *PAI-1* are also closely linked. Of particular note in periodontal disease would be possible changes in lysl hydroxylase activity, leading to diminishment of collagen cross-linking, alterations of mucin, or changes to the major histocompatibility complex I receptor complex.

An interleukin-1β genomic polymorphism has been shown to be associated with severity of adult periodontitis (23). Other genomic polymorphisms have been examined for interaction with periodontal disease severity, such as two in the human toll-like receptor-4 gene, a gene which encodes a lipopolysaccharide-binding protein (24). Another, pertaining to the Fc-gamma receptor, may also be significant relative to periodontal disease (for a review of genomic considerations in periodontal disease see ref. 25).

In the gingiva, age-dependent intimal thickening of arteries and arterioles has long been documented (26). Accordingly, the microvasculature of the gingiva accumulates peri-lumenal extracellular matrix in association with periodontal disease severity in adults (27–29). The balance between processes which promote extracellular matrix degradation and those that inhibit is therefore critical in maintaining homeostasis and health. Because vascular wall remodeling is dependent upon the expression and activation of the plasminogen activators and plasminogen activator inhibitors (1,2), this mechanism of action for these serine proteases and inhibitors may be as important in periodontal disease pathology as it is in other diseases with a vascular component, such as coronary artery disease and certain complications of diabetes.

In our analysis, it was the heterozygous *uPA* (*Bam*HI) genotype that was significantly associated with more advanced alveolar bone loss. In coronary artery disease, it was reported that the same heterozygous *uPA* genotype was significantly associated with a decreased level of heart disease (11). While it is not known why the heterozygous alleles of the *Bam*HI *uPA* genotype should partition together relative to heart disease or periodontal disease parameters, the opposing relationship of this genotype in heart disease vs. periodontal disease is reasonable considering contrasting mechanisms that may be at play in macro- and microvascular disease processes, respectively (30). Interestingly, an opposing *PAI-1* (*Hin*dIII) genotype relationship also exists between data reported for coronary artery disease (12) and our data relating to periodontal disease presented herein.

Level of oral hygiene, current or lifelong, practiced by the participants was not evaluated. However, with a larger study population, possible group differences in oral hygiene levels should be negligible, where the average dental hygiene history might be assumed to be similar for each group. In this study, with small numbers of participants in each of three genotype groups, it is possible, though not probable, that significant differences in oral hygiene levels existed between the genotype groups, which could have contributed to the genotype associations found here. Also, in a complex disease, there are other host and microbiologic characteristics which must also be considered. While host immune parameters are considered to play a key role in periodontal disease susceptibility and progression, it is interesting to note that there is a reported link between interleukin-1β and urokinase plasminogen activator expression in the gingival tissue (31).

The effect of the *uPA* (*Bam*HI) and *PAI-1* (*Hin*dIII) genotypes on alveolar bone loss was strongest in never-smokers and nondiabetics, but insufficient numbers of smokers and diabetics at this time prevents a more robust analysis that considers pack/years smoked, current smoking, years since last smoked, duration of diabetes, and long-term level of diabetes control. Similarly, insufficient data are available to address race in these analyses, although current and future data collection at several sites will allow inclusion of these covariates in the analysis.

In conclusion, there is sufficient reason to implicate the plasminogen activator system in periodontal disease progression. In this genomic analysis, the *uPA* (*Bam*HI) heterozygous genotype was strongly associated with more advanced levels of alveolar bone loss in chronic periodontal disease. Furthermore, the *PAI-1* (*Hin*dIII) nuclease-resistant allele, when present concurrently with the heterozygous *uPA* (*Bam*HI) genotype, provided a slightly stronger risk interval for more advanced alveolar bone loss in our sample population. These data further implicate the role of the microvasculature and plasminogen activator system in periodontal disease progression and may help provide a growing basis for understanding genetic contributions to the disease process.

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