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Immunogenicity and Prediction of Epitopic Region of Antigen AgⅠ/Ⅱ and Glucosyltransferase from *Streptococcus mutans**

Xi-xi CAO (曹茜茜)[1†], Jian FAN (樊 华)[2†], Jiang CHEN (陈 江)[2], Yu-hong LI (李宇红)[1#], Ming-wen FAN (樊明文)[1#]
[1]*The State Key Laboratory Base of Basic Science of Stomatology (Hubei-Most) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan 430079, China*
[2]*Department of Oral Implantology, Affiliated Stomatological Hospital of Fujian Medical University, Fuzhou 350002, China*

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Summary: The levels of *Streptococcus* (*S.*) *mutans* infections in saliva were evaluated and a comparison for specific antibody levels among children with different levels of *S. mutans* infection was made. The promising epitopic regions of antigen AgⅠ/Ⅱ (PAc) and glucosyltransferase (GTF) for potential vaccine targets related to *S. mutans* adherence were screened. A total of 94 children aged 3–4 years were randomly selected, including 53 caries-negative and 41 caries-positive children. The values of *S. mutans* and those of salivary total secretory immunoglobulin A (sIgA), anti-PAc and anti-Glucan binding domain (anti-GLU) were compared to determine the correlation among them. It was found the level of s-IgA against specific antigens did not increase with increasing severity of *S. mutans* infection, and the complete amino acid sequence of PAc and GTFB was analyzed using the DNAStar Protean system for developing specific anti-caries vaccines related to *S. mutans* adherence. A significantly positive correlation between the amount of *S. mutans* and children decayed, missing, and filled teeth index was observed. No significant difference was detected in specific sIgA against PAc or GLU between any two groups. No significant correlation was found between such specific sIgA and caries index. A total of 16 peptides from PAc as well as 13 peptides from GTFB were chosen for further investigation. *S. mutans* colonization contributed to early children caries as an important etiological factor. The level of sIgA against specific antigens did not increase with increasing severity of *S. mutans* infection in children. The epitopes of PAc and GTF have been screened to develop the peptide-based or protein-based anti-caries vaccines.
Key words: anti-caries vaccine; secretory immunoglobulin A; *Streptococcus mutans*; bioinformatics

Dental caries is a multifactorial disease that remains a prevalent global health concern. Despite treatment improvements in the last three decades, caries represent one of the most severe global health burdens, affecting 60%–90% of children and the vast majority of the adults. However, effective methods against caries have not yet been discovered or practically utilized[1]. The etiology of dental caries has been associated with the acid by-products of bacterial metabolism. Important microorganisms in this group that are found in human beings include *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrin*us (*S. sobrin*us). Antigen AgⅠ/Ⅱ (PAc) and glucosyltransferase (GTF) are the two major virulent factors related to *S. mutans* adherence.

Although studies on anti-caries vaccine have been carried out for many years, e.g., the development of the subunit anti-caries vaccines and the DNA vaccines targeting the PAc or GTF, none of them have ever been clinically applied. Impediments of clinical utilization might be low vaccine immunogenicity and unsatisfactory immune responses through mucosal administration, which results in the production of variable, transient, and low magnitude secretory immunoglobulin A (sIgA)[2].

In order to explore a new type of mucosal anti-caries vaccine, we screened the promising epitopic region of antigen PAc and GTF to predict potential vaccine targets. First, we evaluated the levels of *S. mutans* infection in saliva and made comparison for the anti-PAc and anti-Glucan binding domain (anti-GLU) specific antibody levels among children with different levels of *S. mutans* infection. Both anti-PAc and anti-GLU specific antibody level were relatively low, thereby the anti-PAc and anti-GLU are not effective antibody upon the infection of *S. mutans*. Therefore, the development of PAc or GTF peptide-based anti-caries vaccine might be a promising candidate for future clinical application. Second, we screened and evaluated epitopes of PAc and GTFB using DNAStar Protean, which uses combined prediction methods and considers the basic fundamental properties corresponding to what should be an ideal epitope, as follows: surface accessibility, hydrophilicity and T cell epitopes. Moreover, linear B cell epitopes were predicted using ABCpred prediction server. All these efforts were made to induce mucosal immunity, which can ultimately produce effective, relatively permanent, and high magnitude sIgA to prevent the onset or progress of dental caries.

Xi-xi CAO, E-mail: caoxixi891125@hotmail.com
†The authors contributed equally to this work.
#Corresponding authors, Yu-hong LI, liyuhong_an@yahoo.com.cn; Ming-wen FAN, fmwmail@whu.edu.cn
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1 MATERIALS AND METHODS

1.1 Subjects and Clinical Examination

A total of 94 children aged 3 and 4 years old were randomly selected, including 53 caries-negative (decayed, missing, and filled teeth, dmft=0) and 41 caries-positive children (25 children with low caries: 0<dmft<4; 16 children with high caries: dmft≥4)[2]. Normal patterns of growth and development, absence of congenital or systemic diseases, absence of dental abscess and absence of any recent medication therapy were required for all children. There were no statistically significant differences between the groups in terms of age and gender (Mann–Whitney *U* test, *P*>0.05 and Chi-square test, *P*>0.05). Clinical examinations were performed by one calibrated dental epidemiologist according to the World Health Organization criteria[3]. Informed consent was obtained from all the subjects or their guardians.

1.2 Saliva Sample Collection

All children were instructed in advance not to eat or drink at least 2 h before saliva collection. About 1–2 mL of unstimulated saliva from each subject was collected in sterile tubes. Children were undisturbedly seated in the chair. Saliva was gently collected from the floor of the mouth of children using a sterile syringe without a needle. Saliva collection was always performed between 9:00 and 11:00 am. All saliva samples were transferred on ice in the laboratory within 1 h after collection, and centrifuged at 11 000 g at 4°C for 10 min. Both the supernatant and pellet were collected and frozen at −70°C until laboratory analysis.

1.3 Genomic DNA Isolation and PCR Assay

Total genomic DNA of salivary bacteria was extracted using a DNA purification kit (Epicentre, USA), with modifications, according to LI *et al*[4]. Real-time quantitative PCR (qPCR) was performed using an Opticon Monitor Real-time PCR system 7900 (Applied Biosystems, USA) with a specific forward primer (5'-GCC TAC AGC TCA GAG ATG CTA TTCT-3') and a reverse primer (5'-GCC ATA CAC CAC TCATGA ATT GA-3').

1.4 Salivary Antibody Study

The levels of total salivary sIgA and specific IgA against two virulence antigens of *S. mutans*–PAc and GLU were evaluated by enzyme linked immunosorbent assay (ELISA) according to the protocol of our laboratory[5]. The total protein content was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific) with bovine albumin as the standard. All analyses were performed in duplicate.

1.5 Epitope Prediction

To predict the epitopes on PAc and GTF, the complete amino acid sequence of PAc and GTFB was analyzed using the DNAStar Protean system. The secondary structures of these two proteins were predicted by the methods of Garnier–Robson and Chou–Fasman. The surface properties of the structural proteins, such as hydrophilicity, flexibility, accessibility, antigenicity and T cell epitopes prediction were analyzed by the methods of Kyte–Doolittle, Karplus–Schulz, Eminiand Jameson–Wolf, respectively. Linear B cell epitopes were predicted using ABCpred prediction server (http://www.imtech.res.in/raghava/abcpred/). Based on results of these methods, the peptides with good hydrophilicity, high accessibility, outstanding flexibility and strong antigenicity were chosen for further investigation. The peptides that are located in α-spiral and β sheet regions and do not readily form epitope regions were excluded.

1.6 Statistical Analysis

Data management and analyses were performed with SPSS 13.0 software. The $\log_{10}$ values of *S. mutans* and antibodies concentration were used for data analysis, which were expressed as $\bar{x}\pm s$ or median±interquartile range (IQR). Differences between groups were estimated by *T*-test and Analysis of Variance (ANOVA) or Mann-Whitney *U*-test and Kruskal-Wallis *H*-test depending on the data nature: parametric or non-parametric respectively. The values of *P*<0.05 were considered statistically significant for all analyses.

2 RESULTS

2.1 Levels of *S. mutans* and sIgA Antibodies in Children

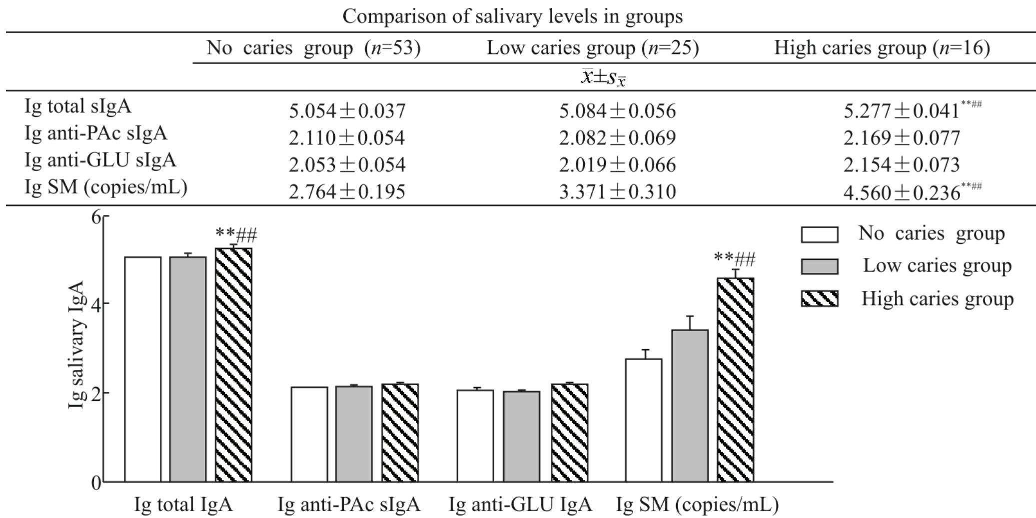
The $\log_{10}$ values of *S. mutans* and antibodies concentration of total sIgA, anti-PAc sIgA and anti-GLU sIgA level in the saliva of children were shown in fig. 1. The total IgA secretion in high caries group was significantly higher (*P*<0.01) than in no caries group or low caries group. And *S. mutans* copies in high caries group were also significantly increased as compared with those in no caries or low caries groups. However, no significant difference was observed among the three groups in anti-PAc or anti-GLU sIgA. The results revealed that anti-Pac and anti-GLU specific antibody level were relatively low.

2.2 Secondary Structure of PAc and GTFB

The first class to be considered for PAc or GTFB is the type of secondary substructure of residues, as predicted using the usual Chou-Fasman methods and the Gamier-Robson methods in fig. 2. Only the β-turns and coil conformations are favorable for antigenicity. Thus, the residues predicted in these conformations are the most likely to be antigenically predicted. As for PAc, Chou-Fasman and Gamier-Robson methods showed that residues 201–474 typically consisted of 4 alanine-rich repeats (82 residues each) with 23%–30% alanine content and 7 β-turns occurring between residues 550 and 1000. By contrast, Chou-Fasman and Gamier-Robson methods showed that β-turns occurred frequently among residues 100–250, 600–800, and 1000–1400 of GTFB.

2.3 Hydrophilicity of PAc and GTFB

The second class of parameters is the hydropathy of residues in fig. 3. Three scales were used as follows: Kyte-Doolitle (fig. 3a), Hopp-Woods (fig. 3b), and Goldman-Engleman-Steitz (fig. 3c). The residues of PAc (272–313, 355–368, 437–483, 494–509, 808–816, 832–862, 866–895, 914–934, 949–967, 1141–1174, 1187–1208, 1269–1289, 1304–1321, 1376–1401 and 1485–1519) achieved relatively high hydrophilicity values according to the results, as well as the GTFB residues 164–183, 219–235, 260–273, 276–287, 293–308, 378–402, 491–516, 604–621, 693–711, 733–745, 813–832, 841–854, 898–919, 970–986, 1134–1161, 1197–1236, 1263–1295, 1332–1359, 1390–1435 and 1456–1474.



Comparison of salivary levels in groups

	No caries group (n=53)	Low caries group (n=25)	High caries group (n=16)
		$\bar{x}\pm s_{\bar{x}}$	
Ig total sIgA	5.054±0.037	5.084±0.056	5.277±0.041$^{**\#\#}$
Ig anti-PAc sIgA	2.110±0.054	2.082±0.069	2.169±0.077
Ig anti-GLU sIgA	2.053±0.054	2.019±0.066	2.154±0.073
Ig SM (copies/mL)	2.764±0.195	3.371±0.310	4.560±0.236$^{**\#\#}$

Fig. 1 Levels of *S. mutans* and salivary secretory IgA (sIgA) antibodies in children

No caries group (NCG): dmft=0, low caries group (LCG): 0<dmft<4, high caries group (HCG): dmft≥4; *T*-test: $\bar{x}\pm s$. $^{**}P<0.01$ *vs*. no caries group, $^{\#\#}P<0.01$ *vs*. low caries group

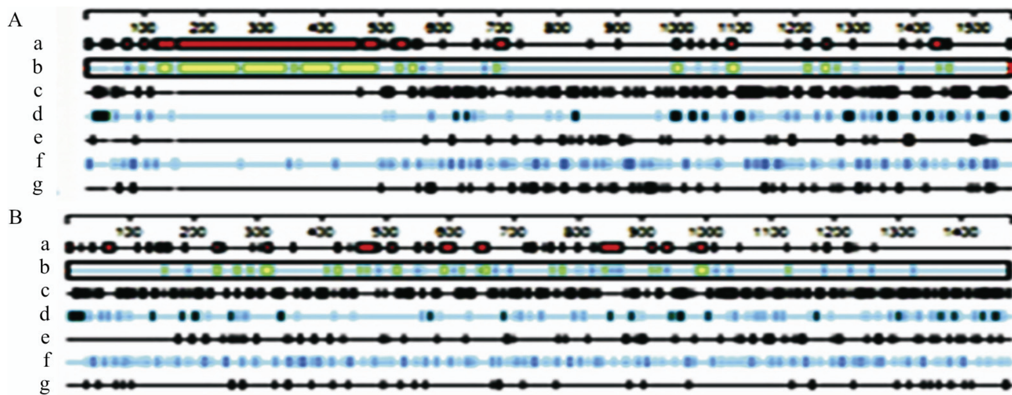


Fig. 2 Secondary structure of PAc (A) and GTFB (B) analyzed by software protean

Alpha region was predicted by Gamier-Robson (a) and Chou-Fasman (b). Beta region was predicted by Gamier-Robson (c) and Chou-Fasman (d). Turn region was predicted by Gamier-Robson (e) and Chou-Fasman (f). Coil region was predicted by Gamier-Robson (g).

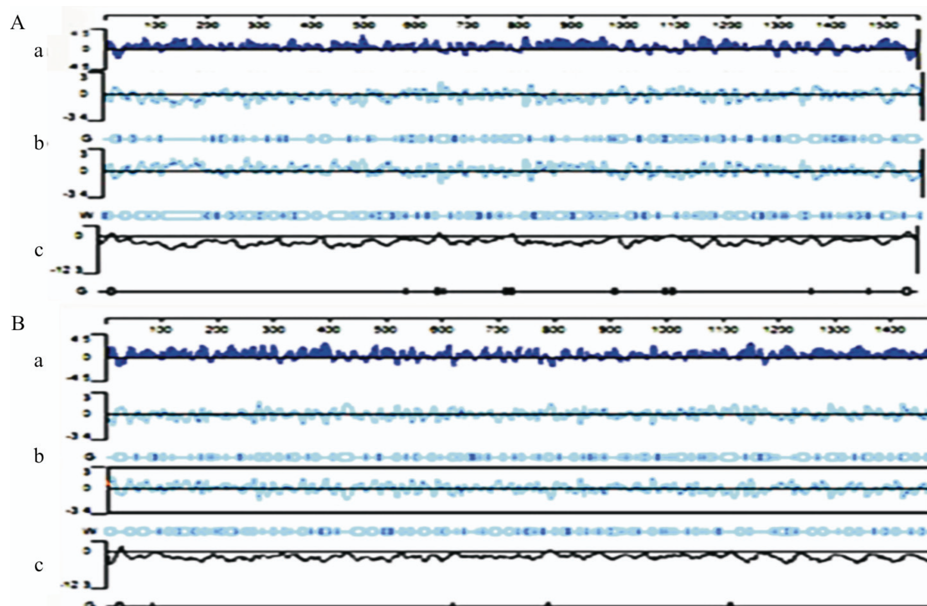


Fig. 3 Hydrophyilicity analysis of the PAc (A) and GTFB (B)

Hydrophilicity plot according to the scales of Kyte-Doolitle (a). Hydrophobicity and hydrophilicity plot according to the scales of Hopp-Woods (b). Hydrophilicity plot according to the scales of Goldman-Engleman-Steitz (c)

2.4 Surface Probability, Flexibility, Antigenic Index and General Analysis Results of PAc and GTFB

The third class is the surface accessibility of residues. One scale was used, e.g., the fractional probabilities of Emini *et al* (fig. 4a). Each prediction of surface accessibility was first treated independently, and then, the PAc residues 274–291 and 357–368 were predicted as surface accessible and more likely to be antigenic, along with the GTFB residues 115–127 and 135–146. The last class concerns the flexibility of residues (fig. 4b). One scale was used, e.g., Karplus-Schulz. The flexible PAc residues 496–515 and 530–554, as well as the GTFB residues 93–182, were more likely to be antigenic. The Jameson–Wolf method produced an index of antigenicity by combining values for hydrophilicity, surface probability and flexibility (fig. 4c). The AMPHI method identifying regions with high amphipathicity potential (fig. 4d) was utilized for calculating the intensity of hydrophobicity. The Sette MHC algorithm searched for sequence motifs commonly found in peptides presented by murine MHC molecules (fig. 4e). The I-A motif is based on similarity to the ovalbumin peptide Ova (327–332), VHAAHA. On one hand, based on these methods, the high antigenic index of PAc included the residues 125–194. On the other hand, 107–156 and 1133–1346 residues from GTFB achieved relatively high antigenic index.

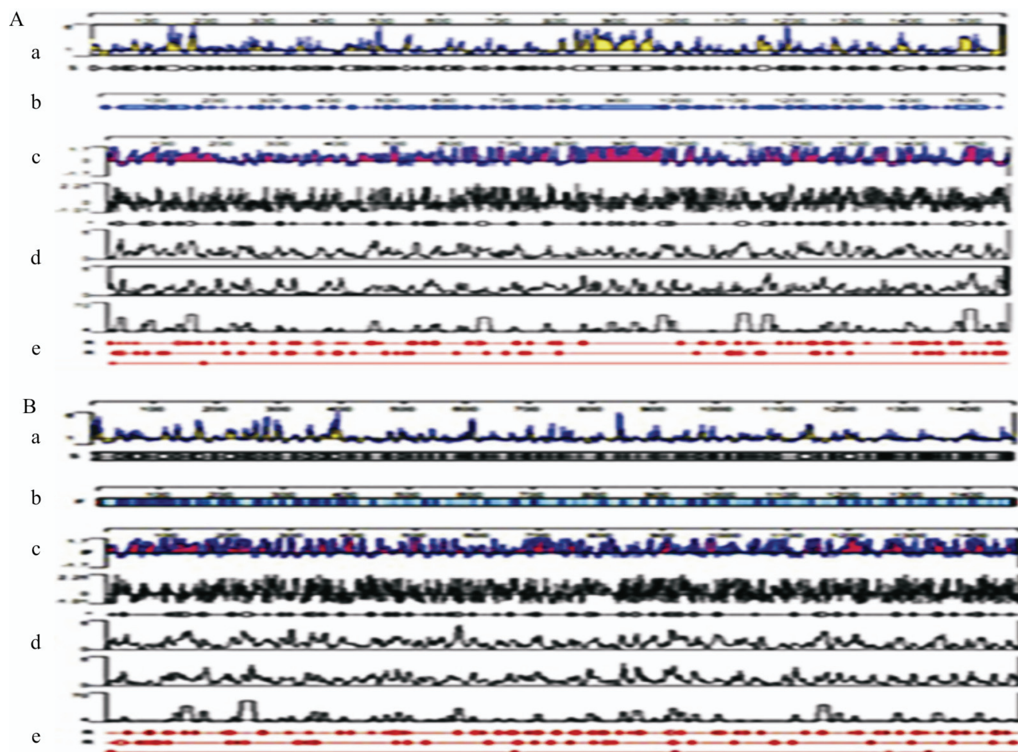


Fig. 4 Surface probability, flexibility, antigenic index and general analysis results of PAc (A) and GTFB (B) surface probability plot predicted by Emini (a). Flexibility region predicted by Karplus-Schulz (b). Antigenic index assessed by Jameson-Wolf (c). Antigenic index assessed by AMPHI, including FaucherPliska Hydrophobicity Plot, AMPHI region, Alpha Helix Plot, 3–10 Helix Plot and Intensity Plot (d). T cell motif and MHC Motifs (e)

2.5 Putative B Cell Epitope Prediction

Linear B cell epitopes were predicted using ABCpred prediction server (http://www.imtech.res.in/raghava/abcpred/). For this prediction, conserved sequences with ≥0.4 VaxiJen scores and exomembrane topology were applied in prediction server by setting cut-off value at 0.51, and the length of the epitopes was fixed as a decamer. Overlapping sequences were also filtered. The nonamers, which were significantly superimposed (≥7 amino acid overlaps) on putative B cell epitope (decamer peptides), were considered for interpretation.

3 DISCUSSION

In this study, we explored the characteristics and features of changes in caries-related microflora, salivary immunity, and their association with severe early caries in children. Our research detected a significantly positive correlation between the amount of *S. mutans* and children dmft index, thereby suggesting that *S. mutans* colonization contributes to the early children caries as an important etiological factor. However, no significant difference was detected in specific sIgA against PAc or GLU between any two groups. No significant correlation was found between such specific sIgA and caries index. These results indicated that even in a circumstance with more intensive insults from *S. mutans* infection, the level of sIgA against specific antigens was not increased simultaneously. As specific sIgA antibody in saliva has been proven to be a specific protection for dental caries against *S. mutans* infection, it is necessary to apply anti-caries vaccine to enhance the level of specific sIgA against PAc or GLU to protect children from caries.

It was recently suggested that effective screening for candidate vaccine antigens should include epitope analysis followed by *in vitro* and *in vivo* evaluations of antigenicity to reduce time and cost[6–8]. Therefore, we screened and evaluated the epitopes of PAc and GTFB

by DNAStar Protean. Linear B cell epitopes were predicted using ABCpred prediction server. Our results revealed that PAc residues 352–370, 381–397, 746–770, 830–990, 1139–1173 and 1481–1515, as well as GTFB residues 253–289, 365–403, 888–914 and 1061–1107 were candidates for anti-caries peptide vaccine development in the future (tables 1 and 2). These candidate residues include PAc A region (186–464), PAc P region (840–963), and GTFB variable region (V region, 360–400).

Table General analysis results of PAc

Prediction items	Prediction results (Iocation of deduced peptides)
Hydrophilicity	127-158, 272-313, 355-368, 437-483, 494-509, 808-816, 832-862, 866-895, 914-934, 949-967, 1141-1174, 1187-1208, 1269-1289, 1304-1321, 1376-1401, 1485-1519
Accessibility	171-186, 274-291, 357-368, 439-450, 463-472, 494-503, 538-549, 830-843, 849-858, 864-901, 910-934, 952-962, 1040-1053, 1142-1171, 1189-1206, 1270-1288, 1383-1405, 1489-1516, 1521-1531
F1exibility	496-515, 530-554, 612-630, 824-859, 866-993, 1144-1172, 1183-1210, 1378-1404, 1479-1518
Antigenicity	125-194, 443-513, 645-664, 759-768, 873-882, 866-933, 958-987, 1138-1158, 1375-1410, 1491-1517
General analysis results	127-158, 352-370*, 381-397*, 436-486, 490-510, 535-550, 602-644, 673-686, 746-770*, 802-816, 830-990*, 1139-1173*, 1184-1223, 1295-1325, 1374-1408, 1481-1515*

Sixteen peptides with good hydrophilicity, high accessibility, and flexible and strong antigenicity were screened. Asterisks on the upper-right corner of the peptides indicate that the residues contain screened linear B cell epitopes.

PAc functions as an adhesive that mediates attachment of *S. mutans* to the tooth surface. It is essential for colonization *in vivo* and binds to salivary receptors *in vitro*[9–11]. Based on our analysis, the candidates of the residues include a series of three alanine-rich tandem repeats called A region and a series of three tandem proline-rich repeats called P region. The PAc (361–386) peptide was confirmed as an ideal peptide antigen for inducing the inhibiting antibody to *S. mutans* in 151 healthy human subjects. This finding indicated that the coupled PAc (361–386) peptide from residues 361–377 and 370–386 may be a minimum antigen of PAc that induces the inhibiting antibodies for adherence of the tooth surfaces coated by salivary components in humans. The PAc (361–386) was employed as potential vaccine target regions in previous attempts to develop a component vaccine against PAc because of their association with enzyme function and the high degree of sequence conservation among *streptococci*. Consistently, DNAStar Protean analysis from our study indicated relatively high immunogenicity within the A region from residues 350–400. PAc [(352–370) and (381–397)] exhibited prominent antigenic peaks according to our data, indicating that residues 350–400 within PAc A region should be considered when selecting epitope peptide candidates. Immunodominant T-cell and B-cell epitopes from our research were identified within PAc P region from residue 830 to residue 990, and these residues were separated from the adhesion epitopes (residues 1005–1044) in the linear sequence of PAc. Moreover, PAc residues 1139–1173 and 1481–1515, which have been neglected by previous studies, were surprisingly selected from our results. Therefore, the residues beyond A or P region from PAc should also be studied in our future work.

GTF produced by the *S.mutans is* recognized as virulence factors in dental caries, and the inhibition of GTF by sIgA is predicted to provide protection against this disease. From the results of our study, the GTFB residues 253–289, 365–403, 888–914, and 1061–1107 were highly scored (table 2).

Table 2 General analysis results of GTFB

Prediction items	Prediction results (Iocation of deduced peptides)
Hydrophilicity	164-183, 219-235, 260-273, 276-287, 293-308, 378-402, 491-516, 604-621, 693-711, 733-745, 813-832, 841-854, 898-919, 970-986, 1134-1161, 1197-1236, 1263-1295, 1332-1359, 1390-1435, 1456-1474
Accessibility	115-127, 135-146, 168-182, 214-235, 242-254, 261-269, 277-287, 294-307, 345-356, 383-403, 599-618, 697-710, 733-743, 754-775, 842-854, 902-914, 970-986, 1143-1158, 1199-1232
F1exibility	93-182, 195-274, 313-403, 526-552, 664-711, 871-917, 946-987, 1044-1156, 1187-1284
Antigenicity	107-156, 189-295, 339-410, 805-955, 1133-1346
General analysis results	106-129, 158-184, 214-237, 253-289*, 343-359, 365-403*, 463-502, 597-617, 888-914*, 967-984, 1061-1107*, 1133-1157, 1180-1231

Thirteen peptides with good hydrophilicity, high accessibility, and flexible and strong antigenicity were screened. Asterisks on the upper-right corner of the peptides indicate that the residues contain screened linear B cell epitopes.

These contain both T- and B-cell epitopes. In fact, in the development of a component anti-caries vaccine, the catalytic region (CAT, residues 400–800) and glucan-binding domain (GBD, residues 1114–1452) from *S. mutans* have been employed as target antigens by many researchers because of their association with enzyme function and the high degree of sequence conservation among *streptococci*. The composition of this peptide construct, a major repeating sequence in the C-terminal region (residues 1293 to 1328) of GTFB, elicited high levels of serum immunoglobulin G antibody to GLU after subcutaneous injection into Sprague-Dawley rats[12]. Nevertheless, this specific residue was not predicted as a candidate epitope in our study. Investigation of a synthetic peptide of 19 amino acids from a conserved region in GTF (residues 435–453) revealed that it includes one of the major B-cell epitopes of GTF. The caries-negative volunteers exhibited significantly higher sIgA antibody levels to the peptide and to GTFB/C than the caries-active subjects. This region within residues 435–453 from GTF also exhibited prior linear B cell epitopes, but the relatively inferior surface accessibility, hydrophilicity,

mobility, and flexibility of this region veiled its general ability to be the candidate. Interestingly, the antigenic region of GTFB was reevaluated recently by using in silico analyses combined with *in vitro* and *in vivo* experiments. The results suggested that the ca. 360-amino acid in the N terminus of GTFB is more reactive than GBD. This result was consistent with our study as the GTFB residues 365–403 were selected as candidates. Thus, the non-conserved and the unknown functional region of GTF might be promising candidates for peptide vaccine development according to the results of bioinformatics analysis[13]. Nonimmunodominant regions are reportedly effective as building blocks in a streptococcal fusion protein vaccine. Although CAT and GBD were suggested to be nonimmunodominant in our study, they nevertheless may be attractive vaccine targets. Thus, fusion proteins of CAT and GBD may induce more effective antibodies to inhibit glucan synthesis of GTFB.

All these above-mentioned studies indicated that this topic has resulted in vast insights into structures and functions of the PAc and GTF proteins. To target these factors and fight dental caries, a fusion DNA vaccine was constructed using the GLU domain of the GTF enzymes and the A-P fragments of the *S. mutans* PAc genes in our previous study. The fusion vaccine was more effective in eliciting specific sIgA response that produced a significant anti-caries effect than either PAc vaccine or GLU vaccine. Some evidence has proven that the combination of both PAc and GTF epitopes for anti-caries vaccine utilization can be more effective than PAc or GTF alone[14]. Nevertheless, few studies have involved both PAc and GTFB epitopes in constructing the anti-caries peptide vaccines. In conclusion, we have screened the epitopes of PAc and GTF and these candidates might be verified *in vivo* and *in vitro*.

To enhance the immunogenicity of the vaccines, the combined peptide of both PAc and GTF regions might be used, and the nanoparticles or the self-assembly constructed vaccines might also be utilized for inducing mucosal immunity and producing steady, relatively permanent and high-magnitude sIgA. The development from a linear map of sequences that interacted with salivary glycoproteins or carry T- or B-cell epitopes to a 3D structure that now encompasses almost all linear functional sequences has provided substantial information. Among recent innovations, several natural proteins have shown the ability to form nanoparticles that are well-suited for antigen presentation and immune stimulation[15–19]. One such protein is ferritin, a ubiquitous iron storage protein that self-assembles into nanoparticles[20]. In the stage of our study, by using the self-assembling function of ferritin, the screened *S. mutans* epitopes, including both screened PAc and GTFB residues, will be linked to the N-exposed area of the cage shaped ferritin. Therefore, the new type of self-assembling anti-caries nanoparticle vaccine will be constructed. Ultimately, it could be adapted to create analogous vaccines for a wide variety of pathogens with the help of bioinformatics and nanotechnology. These methods will be compared *in vivo*, and the promising anti-caries vaccine for clinical application in the long run will be chosen.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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