The Quantity of G-Quadruplex in Bacterial Genome affects G-Quadruplex Ligand Sensitivity in Hypertension associated Tongue Coating Microbiota

Shiqin Zhang  
Peking University Third Hospital  
https://orcid.org/0000-0002-9847-5658

Yangong Liu  
Peking University Third Hospital

Jiang Zhou  
Peking University College of Chemistry and Molecular Engineering

Qinghua Cui (✉ cuiqinghua@bjmu.edu.cn)  
Peking University School of Basic Medical Sciences

Feng Chen  
Peking University School and Hospital of Stomatology: Peking University School of Stomatology

Ming Xu  
Peking University School and Hospital of Stomatology: Peking University School of Stomatology  
https://orcid.org/0000-0003-1680-3628

Research

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Abstract

Background

Recently, more and more attention has been paid to the role of oral microbiota in hypertension. It was reported that the disorder of tongue coating microbiota would significantly increase systolic blood pressure, yet the characteristics of tongue coating microbiota of hypertensives remain unknown. Microbiota is regulated by genes, G-quadruplex (G4) is a secondary structure of nucleic acid, which plays an important role in the regulation of microbial biologic features. there are many G4 ligands in oral administration, but how G4 ligands affect the bacterial biological phenotype needs further exploration.

Results

We used metagenomics for analysis of 58 subjects including 23 healthy subjects and 35 hypertensives, and bioinformatics technology for detecting G4 characteristic sequences, finally verified by biological and chemical experiments. We found that *Actinomyces* decreased significantly in the hypertension group with the highest average maximum putative G-quadruplex forming sequences (PQS) and GC quantity. We also screened out two species with significantly different abundance between two groups, *Actinomyces odontolyticus* and *Acinetobacter baumannii*. *A. odontolyticus* had higher GC% and frequency of PQS per 1000bp in the genome, which led to differential inhibition of bacterial growth, metabolism, biofilm formation by G4 ligand, sanguinarine. Major Facilitator Superfamily (MFS) was found to be involved in these biological phenomena, we found that sanguinarine could bind and stabilize the G4 structure related to MFS and further inhibited the expression of MFS.

Conclusions

Different quantities of G4 sequences in the bacterial genome affect G4 ligand sensitivity in hypertension-associated tongue coating species, *A. odontolyticus* and *A. baumannii*. G4 ligand (sanguinarine) can bind and stabilize the G4 characteristic sequence of the MFS gene to inhibit the expression, and then inhibit the growth, biofilm formation and metabolism. This study provides a theoretical basis for the selection of G4 based drugs in the oral cavity.

Introduction

Hypertension is one of the cardiovascular diseases that seriously threaten people's health. The number of disability-adjusted life years and deaths caused by high systolic blood pressure in China ranks the first in the world[1,2], hypertension is also considered to be the most serious risk factor of death in China[3]. It has been suggested that aberrant gut microbiota would contribute to the pathogenesis of hypertension, which emphasized the importance of bacterial homeostasis[4].

Oral microbiota, as the second-largest microbial community in the human body, also plays an important role in cardiovascular disease, especially hypertension. It has been reported that frequent use of broad-
spectrum bactericidal mouthwash may cause the disorder of tongue coating microbiota, and decrease the production of nitric oxide, which results in a significant increase in systolic blood pressure\cite{5}. It demonstrated that the homeostasis of tongue coating microbiota is closely related to the regulation of blood pressure. However, the current research on tongue coating microbiota is mostly limited to 16S rRNA technology. Therefore, further study of the characteristics of tongue coating microbiota in patients with hypertension, especially those hypertension-associated species with metabolic functions, is helpful to explore the effect of oral microbiota in hypertension.

Human oral microbiota is an intricate ecosystem, in healthy individuals, oral microbiota maintains oral homeostasis and is usually in dynamic balance, but external stimulus (such as exposure to drugs, host lifestyle)\cite{6,7} and genomic diversity will disturb this balance. It is reported that the genomic sequence and structure determine the expression of genes. G-quadruplex (G4) is a special nucleic acid secondary structure which is formed by several G-tetrads, accumulating evidence indicates that G4 serves as a regulatory role in the biological process\cite{8}, the stabilization of G4 may be linked to genome instability and diseases, such as cancers\cite{9,10} and cardiovascular diseases\cite{11}. For bacteria, the analysis showed that G4 was widespread in bacterial genomes\cite{12}, and was crucial for bacterial DNA replication, transcription\cite{13–15} and translation\cite{16}. The formation of G4 inhibits the expression of related genes\cite{17} and further affects the biological phenotype of bacteria\cite{18}. It should be noted that many G4 ligands are applied locally in the oral cavity, for example, oral mouthwash contains sanguinarine\cite{19}, herbal toothpaste contains nitidine, oral local anti-inflammatory drugs contain berberine\cite{20}. Nevertheless, the effect of G4 ligands on different species is still unclear.

Therefore, we utilized metagenomics to explore the characteristics of tongue coating microbiota in patients with hypertension, took bioinformatics analysis to detect G4 sequences of the oral bacterial genome, and verified the biological effects of G4 ligand, sanguinarine on hypertension associated strains with different G4 characteristics in the genome.

**Materials And Methods**

**Study subjects**

58 subjects were recruited in this study from a cohort of Jixian, Tianjin. They were divided into two groups according to the global hypertension practice guidelines\cite{21}, 23 subjects in the control group with SBP $\leq$ 140 mmHg and DBP $\leq$ 90 mmHg, 35 subjects in the hypertension group with SBP $\geq$ 140 mmHg or DBP $\geq$ 90 mmHg, the blood pressure was measured in a sitting posture with medical electronic sphygmomanometer after 5 minutes rest, and the mid-arm was at heart level, it took 3 times with an interval of 1min and used the average data.

This study was approved by the ethical committee of Peking University Third Hospital, and the informed consent were obtained from all subjects before the study. The exclusion criteria of subjects were as follows: A history of drug treatment (such as antihypertensive drugs, lipid-lowering drugs, anticoagulants,
etc.); Suffering from other cardiovascular diseases (such as coronary heart disease), diabetes and kidney diseases; Suffering from gastrointestinal diseases and cancers; Suffering from oral diseases (such as dental caries, etc.); A history of antibiotics or mouthwash treatment within three months; Suffering from respiratory infection within one month;

**Tongue coating sample collection and DNA extraction**

Tongue coating was collected using oral sterile swabs before breakfast, the tongue was scraped from the root of the tongue to the tip 10 times, the swab was snapped into DNA preservation solution and stored at -80°C in 4h. Lysozyme (10 mg/mL, 100 μL/sample) was pretreated before DNA extraction, and DNA was extracted from each tongue coating sample following the protocol of QIAamp DNA Mini Kit (Qiagen, Germany).

**Metagenomic sequencing and Library construction**

The DNA libraries were constructed following the Illumina TruSeq DNA Sample Prep v2 Guide (Illumina, Inc., San Diego, CA, USA), and all samples were subjected to 150 bp paired-end sequencing on an Hiseq X-ten platform (Illumina, Inc., San Diego, CA, USA).

**Quality control of Illumina reads**

Illumina raw reads should be screened as following criteria: Removing reads containing adaptor contamination, more than three ambiguous N bases, low quality (Q<20) bases or less than 60% of high-quality bases; Abandoning the host genomes and selecting the bacterial genomes with SOAPaligner (version 2.21)[22] for further analysis.

**Microbial relative abundance profiling**

Bacterial data were aligned to the NCBI database (National Center for Biological Information, http://www.ncbi.nlm.nih.gov) for detection of known microbiota by SOAPaligner 2.21[22], and then classified as Kindom, Phylum, Class, Order, Family, Genus, Species to count classification and taxonomic relative abundance, the total abundance of each species in a single sample is 1.

**Genome assembly, gene prediction and gene catalog construction**

The assembly of reads was carried out with a series of k-mer (51, 55, 59, 63) by SOAPdenovo (version 2.04)[23], and contigs longer than 500bp were kept for further analysis. Software MetaGeneMark[24] was used to predict open reading frames (ORFs) not less than 100bp. CD-HIT (version 4.5.7) [25] was used for pairwise comparison of predicted ORFs which were used for construction of a non-redundant gene catalog set[26].

**Gene functional annotation and functional profiling**
BLAST (Version 2.2.28+) was used to align the non-redundant gene catalog set with KEGG (Kyoto Encyclopedia of Genes and Genomes) and eggNOG (evolutionary genealogy of genes) database for the annotation information. And then the relative abundance of all orthologous genes was accumulated to generate the relative abundance of each KO/NOG.

**Quantitative Real-time PCR**

*A. odontolyticus* were grown at 37 °C in 3mL BHI broth supplemented with sanguinarine (10 μg/mL) until the OD570 reached 0.15. Total RNAs were extracted by RNAprep pure Cell / Bacteria Kit (DP430, Tiangen, China) according to the manufacturer's protocol. 1 μg total RNAs was used in 20 μL reaction volumes to generate cDNA by using a FastKing RT kit (KR116, Tiangen, China). QRT-PCR was performed using a Talent qPCR PreMix (SYBR Green) kit (FP209, Tiangen, China) with forward primer (5′-CGAAGTACTGCTGCGCCATC-3′) and reverse primer (5′-TCAACGTTGGCCTCGTCTAC-3′) primers for the AO-13 related MFS gene, forward primer (5′-CAGACCGCCATGACGATGAT-3′) and reverse primer (5′-ACGTATGCCGATGTCGATGG-3′) for the AO-19 related MFS gene. The internal control is forward primer (5′-CTTTGGGATAACGCCGGGAAC-3′) and reverse primer (5′-CTACCCGTCAAAGCCTTGGT-3′). The data analysis was done through 2−ΔΔCt method.

**Bacterial strains**

This study contained two strains, *A. baumannii* (JCM 6841) was purchased from China General Microbiological Culture Collection Center (CGMCC) which was grown in LB broth at 37°C, *A. odontolyticus* (ATCC 17929) was purchased from American Type Culture Collection (ATCC) which was grown in BHI broth at 37°C.

**Bioinformatics Analysis**

According to the five common bacteria of tongue coating, including *Actinomyces*, *Streptococcus*, *Veillonella*, *Neisseria*, and *Prevotella*, and ten differential strains with high relative abundance respectively enriched in two groups, all complete bacterial genomic DNA sequences was downloaded from eHOMD. If the strain had several complete genomes, the largest genome was selected for G4 analysis. To further analyze the G4 sequence of *A. baumannii* and *A. odontolyticus* which were considered as hypertension-associated bacteria, 244 complete genomes of *A. baumannii* and 2 genomes of *A. odontolyticus* were downloaded from National Center for Biotechnology Information.

Putative G4 sequences were predicted by G4RNA screener[27] (http://gitlabs.cottgroup.med.usherbrooke.ca/J-Michel/g4rna_screener). The sliding window of 30 nucleotides (nt) moving with steps of 15 nt was used to search potential G4s. The thresholds were as follows: cGcC score >4.5, G4Hunter score >0.9, G4NN score > 0.5. Putative G4 sequences that met the scoring threshold and had gene annotation function remained.

**Circular dichroism and thermal stability studies**
CD spectroscopy was recorded on a J-815 CD spectrometer (JASCO, Japan) at 20°C, over 220-330 nm with the scanning speed at 100 nm/min. Each sample was measured three times. DNAs with 5nM final concentrations were prepared in 30mM Tris-HCl and 0-150 mM KCl. The mixtures were then heated at 95°C for 15 min and gradually cooled to room temperature. For CD thermal stability studies, spectra were recorded with a temperature range from 20 to 95 °C, and the temperature rising rate was 3°C/min.

**ESI mass spectrometry (ESI-MS)**

The ESI-MS experiments were performed on Bruker SolariX-XR mass spectrometer (Bruker, Billerica, MA, USA) with an ESI source. All samples were tested in a negative ion mode, with a capillary voltage of 2.7 kV, and the samples were injected with the flow rate at 120 μL/h. To test the binding properties, the samples were prepared in the solutions with 25% CH3OH to have better efficiency, the sanguinarine and DNA sequences were at 4:1 ratio.

**Bacterial growth assays**

To monitor the effect of sanguinarine on *A. odontolyticus* and *A. baumannii*, the bacterial suspension containing agents (5, 10, 20 µg/mL sanguinarine) was grown under the anoxic condition at 37°C, the control was free of sanguinarine. At designated time points (0, 2, 4, 6,10,12,14 h), the cell broths were measured the OD with a microplate reader at 570 nm.

**Biofilm formation assay**

*A. odontolyticus* (10^6 CFU/mL) was inoculated in culture dishes with transparent bottom to form biofilms, containing sanguinarine (5 µg/mL, 10 µg/mL or 20 µg/mL) at 37°C and in anaerobic condition. The control was free of sanguinarine. After 24h culture, the dishes were washed two times with PBS. Subsequently, 1.5 µL SYTO 9 dye from LIVE/DEAD BacLight Bacterial Viability Kit were diluted in 1mL PBS and then the diluent was added into each dish. All wells were incubated for 30 min in the dark. After the staining, the wells were washed twice with PBS and each well was added 1mL 4% paraformaldehyde for 15 min for fixation. The images were visualized by laser confocal microscope (Carl Zeiss, LSM-780, Germany) at wavelengths of 480 nm excitation and 500 nm emission.

**Assessment of nitrite concentration**

The overnight cultured *A. odontolyticus* (OD570 of 0.15) was diluted into BHI broth (1:100 dilutions) supplemented with 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL sanguinarine, meanwhile, KNO3 was added at the final concentration of 1 mM as the substrate. After 24h under anoxic condition, the bacterial solution was vortexed and centrifuged at 12,000 rpm for 5 min to collect the supernatant. Using a nitrite colorimetric assay kit (Elabscience, E-BC-K070-S, China), supernatant nitrite levels were determined. The absorbance was read at 550 nm.

**LC-MS/MS for quantitation of TMA**
Overnight cultured *A. odontolyticus* (OD570 of 0.15) was diluted into BHI broth (1:100 dilutions) supplemented with 10 μg/mL sanguinarine and 10-4 mM γ-Butyrobetaine at 37°C under anoxic condition for 24h. Samples (20 μL bacterial suspension) were mixed with 80 μL of 10 μM d9-TMA in methanol to precipitate protein. LC–MS/MS analysis was conducted on a Q TRAP5500 mass spectrometer. The following settings were selected: curtain gas, 20 psi; source temperature, 600 °C; gas 1 and gas 2, 35 and 50 psi; spray voltage, 4.5 ESI+ kV; collision activation parameter, medium. Supernatants (10 μL) were analyzed by injection onto a silica column (Luna 5u Silica 100A, 2.0*150 mm) at a flow rate of 0.5 mL/min. The temperature was set at 35°C. Mobile phases A consisted of 1‰ Propionic Acid in LC–MS grade water and Mobile phases B consisted of 1‰ FA in MeOH. And the Gradient (B %) was 2% for 1 min, 95% for 11 min, 2% 6.5 min, and stop at 7 min. The internal standard d9-TMA was used for quantification.

**Biolog ECO microplates assay**

*A. odontolyticus* were prepared in BHI solid broth for biolog assays. Monoclonal colonies were mixed in IF-A liquid until reaching 90-98% turbidity. Then the IF-A liquid (150 μL) was added to ECO microplates, which were cultured at 37°C in the anaerobic condition. After 48h, the ECO microplates were read at absorption 590 nm using a BIOLOG microplate reader (MOLECULAR DEVICES, United States). And AWCD was used to test the carbon source utilization capability of *A. odontolyticus*.[28]

**Statistics.**

R software was used to perform all statistical analyses in metagenomics. Wilcox rank-sum test was used to calculate the significance of different taxonomic (phylum, class, order, family, genus, species). Other data were expressed as mean ± SEM. Statistical analysis between two groups used the Student’s t-test. Statistical analysis was performed on SPSS 23.0.

**Results**

**The presence and frequency of G4 Sequences in tongue coating microbiota**

To identify the characteristics of tongue coating microbiota in hypertensive patients, 58 tongue coating samples were collected from the hypertension cohort in Jixian county of Tianjin for metagenomics detection. According to the diagnostic criteria of hypertension[21], 58 subjects were divided into control group (n=23) and hypertension group (n=35). The systolic and diastolic blood pressure of the hypertension group were significantly higher than those of the control group, but there was no significant difference in other variables (including age, BMI, fasting blood glucose, blood lipids) (Table 1).

To explore the difference of microbial composition on phylum, class, order, family and genus levels, 58 tongue coating samples were used for DNA extraction, and all samples were subjected to 150 bp paired-end sequencing on the Illumina platform, average raw data per sample is 14.00 ± 1.90 Gb, average clean data per sample is 7.01 ± 3.43 Gb (Fig. S1, Table S1), all clean data were used for analysis. Figure 1a and
Figure S2a-d gave an overview of the relative abundances, Bacteroidetes, Bacteroidia, Bacteroidales, Prevotellaceae, Prevotella showed the highest relative abundance, yet with no significant difference between the two groups. The five most abundant genus were Prevotella, Neisseria, Veillonella, Streptococcus, Actinomyces (Fig. 1a), in both groups, however, only actinomyces have a significant change (Fig. 1b). In addition, the relative abundance of Actinomycetales, Actinomycetaceae also showed a significant decrease in the hypertension group compared to controls on order and family level (Fig. S2).

We analyzed the quantity of putative G-quadruplex forming sequences (PQS) in 33 oral bacterial species including 8 species of Actinomyces, 3 of Neisseria, 6 of Prevotella, 15 of Streptococcus and 1 of Veillonella. The mean length of the five species varied from 2.13 Gb to 3.14 Gb, and the mean GC% varied from 38.63% to 68.75%. Using G4 screener to analyze the genomes of five species, we found that the most abundant species was Actinomyces with an average quantity of 5144 PQS, the minimum was Veillonella with an average quantity of 130 PQS (Fig. 1c, Table S1). We further analyzed the relationship between PQS frequencies per 1000 bp and GC%, we found that high PQS frequencies corresponded with high GC%, in five most abundant oral bacterial species, Actinomyces had the highest GC content and PQS frequency (Fig. 1d, Table S2).

**The characteristics of G4 Sequences of hypertension-associated species**

Wilcoxon rank-sum test was used to calculate P values for the differences of tongue-coating microbiota between the control and hypertension groups. We found that 147 species were significantly enriched in the control group and 42 species significantly enriched in the hypertension group (Table S3). Respectively, twenty of them with higher relative abundance were further shown (Fig. 2a, b). Most importantly, the relative abundance of A. odontolyticus decreased in the hypertension group, which was considered as an effective nitrate-reducing species\cite{29}, and the relative abundance of A. baumannii increased in the hypertension group which produced TMA, then TMA could be oxidized into TMAO which promoted age-related endothelial dysfunction via oxidative stress\cite{30}. Meanwhile, the heatmap showed that A. odontolyticus was negatively correlated with systolic blood pressure, and A. baumannii was positively correlated with systolic blood pressure (Fig. 2d). Therefore, A. odontolyticus and A. baumannii were considered hypertension-associated bacteria because of their metabolic function.

Furthermore, we analyzed the characteristics of genomic G4 of 20 enriched species, only those with complete genomic sequences from expanded Human Oral Microbiome Database (eHOMD) were analyzed, including A. odontolyticus, Bifidobacterium longum and Eubacterium sulci of the control group, Porphyromonas gingivalis and A. baumannii of hypertension group. The results showed that A. odontolyticus had 2647 PQS, significantly higher than A. baumannii with 304 PQS (Fig. 2c). Further, we analyzed 244 complete genomes of A. baumannii and 2 complete genomes of A. odontolyticus from NCBI, similarly, A. odontolyticus had higher GC% and frequency of PQS per 1000bp (Fig. 2e, f, Table S3).

**Differential inhibition of bacterial growth and metabolism by G4 ligand sanguinarine**
Growth curves were measured to assess the impact of different concentrations of G-quadruplex ligands on the growth of *A. ondontolyticus* and *A. baumannii*. As shown in Fig. 3a and c, 5 μg/mL sanguinarine slightly reduced the growth of *A. ondontolyticus* during the phase 8-16h, and 20 μg/mL sanguinarine inhibited the growth completely, yet 20 μg/mL sanguinarine had no effect on the growth of *A. baumannii*. The results of nitidine were similar to sanguinarine (Figure S3a, b). Meanwhile, sanguinarine demonstrated different potency over the same concentration for bacterial metabolism. Sanguinarine dose-dependently inhibited *A. ondontolyticus* nitrite metabolism (Fig. 3b), but TMA metabolism wasn’t affected at the concentration of 10 or 20 μg/mL for *A. baumannii* (Fig. 3d).

**Inhibition of biofilm formation and carbon source metabolism of *A. odontolyticus* by sanguinarine**

Except for the inhibition of bacterial growth and nitrate metabolism, sanguinarine was also found to dose-dependently reduce biofilm formation of *A. odontolyticus*, 10 and 20 μg/mL sanguinarine markedly reduced biofilm densities (Fig. 4a). Based on the metagenomic analysis, Fig. 4b and c showed the microbial functions of tongue coating samples, mainly associated with metabolism, especially carbohydrate and amino acid metabolism. ECO microplate contains 31 different carbon sources, using carbon sources would make a color reaction, so average well color development (AWCD) is used to reflect metabolic functions of microorganisms[28]. At 48h, the AWCD was significantly inhibited with 10 μg/mL sanguinarine, which indicated that sanguinarine influenced the carbon source metabolic capability of *A. odontolyticus* (Fig. 4d).

**The bind and stabilization of G4 structure by sanguinarine inhibits the expression of MFS**

The MFS family is one of the largest group of secondary membrane transporters and is present in bacteria and mammals[31, 32]. MFS transporters contribute to small molecule transport especially uptake of sugars, and are related to bacterial survival, bacterial communication and biofilm formation[33]. The MFS family is important for biological phenotypes of bacteria, we further analyzed the characteristic of G4 sequences in the genome of *A. odontolyticus*, we found that there were 26 G4 sequences annotated as MFS (Table S5). We carried out two G4 sequences with the highest score (AO-13) and the lowest score (AO-19).

Circular dichroism (CD) spectroscopy was used to determine G-quadruplex configuration, as shown in Fig. 5a, the values at 260nm increased with the increasing concentration of KCl, which indicated that KCl induced the formation of a parallel G-quadruplex, yet the sequence (AO-19) showed KCl independent which formed an unconventional structure (mixed-type conformation) with a positive peak at 275 nm and a negative peak at 240 nm (Fig. 5b). In ESI-MS spectrum, the solution concluded 50 mM NH₄OAc and sanguinarine (C_sequence: C_sanguinarine =1:4), the peaks of G-quadruplex complex ions with one, two and three ligands ([S+L]⁵⁻, [S+2L]⁵⁻, [S+3L]⁵⁻) emerged with the [S]⁵⁻ as the base peak which demonstrated that there were two binding sites (Fig. 5c), as for the sequence of AO-19 (Fig. 5d), it showed four binding sites. In CD melting assay, the Tm value increased from 61.33°C to 74.34°C of AO-13 sequence (Fig. 5e) and from 59.51°C to 72.23°C of AO-19 sequence (Fig. 5f) with 100 mM KCl. These results proved that
sanguinarine could promote the formation and stability of the G-quadruplex of MFS related sequence (AO-13 and AO-19). Furthermore, the expression of MFS was significantly inhibited with 10 μg/mL sanguinarine (Fig. 5g, h).

**Discussion**

Previous studies have shown that the disorder of tongue coating microbiota caused an increase in blood pressure\[^{34}\], but limited studies have reported the correlation in hypertensives. And several external factors have an impact on bacterial relative abundance, such as diet, medication, it should be noted that many G4 ligands are applied locally in the oral cavity, such as sanguinarine, however, it remains unknown about the effect of G4 ligands on hypertension associated tongue coating microbiota.

Here, we collected 58 tongue coating samples which were subsequently detected by metagenomics. All subjects were from the cohort of Tianjin to lower the influence of diet. Subjects who suffered from cardiovascular diseases, oral diseases, digestive diseases such as gastritis\[^{35}\] and pancreatitis\[^{36}\] were excluded, and all of them didn't receive any treatment, including antihypertensive drugs, antibiotics. All the exclusion criteria were set to reduce the impact of external factors. In the present study, we found the five most abundant tongue coating genus were *Prevotella*, *Neisseria*, *Veillonella*, *Streptococcus* and *Actinomyces*. *Actinomyces* were enriched in the control group which was in contrast to gut microbiota\[^{4}\], probably because the environment from the oral to the colon is inconsistent\[^{37}\]. Meanwhile, we analyzed the number of G-rich sequences by G4 screener, we found that G-rich sequences were common in the genomes of tongue coating genus, and the quantities of G-rich sequences varied greatly among different genus which is consistent with a previous study\[^{12}\]. Particularly, *Actinomyces* had the highest GC content and PQS frequency among the five genus.

According to the metabolic functions, we screened out two hypertension-associated bacteria, *A. odontolyticus* and *A. baumannii*. Concomitant with the characteristics of G-rich sequences of *Actinomyces*, we observed that *A. odontolyticus* had higher GC% and frequency of PQS per 1000bp than *A. baumannii*. However, it was still unknown whether the quantity of G-rich sequences would affect the sensitivity of G4 ligands. In this study, sanguinarine was chosen as a typical G4 ligand, 10 μg/mL sanguinarine could significantly inhibit the growth and nitrate metabolism of *A. odontolyticus*, but had no impact on the growth and TMA metabolism on *A. baumannii*. The results of nitidine were similar, nitidine was regarded as a G4 ligand for promoting the formation of G4 structure in telomere\[^{38}\]. It has been reported that\[^{39,40}\] sanguinarine could intercalate DNA and thus inhibit bacterial replication and transcription, but it wasn't enough to explain the differential inhibition. Therefore, the number of genomic G4 may affect the response of bacteria to G4 ligands, but it calls for further verification.

To further explore the antibacterial mechanism of sanguinarine, the results demonstrated that sanguinarine inhibited the growth, nitrate metabolism, formation of biofilm and carbon source metabolism of *A. odontolyticus*. It has been reported that MFS transporters are related to the transport of small carbon source molecules such as sugars and amino acids, as well as biofilm formation\[^{41}\] and
bacterial virulence\cite{42}. Furthermore, chemical experiments were performed to verify that sanguinarine could bind and stabilize the G4 sequence of the MFS gene to inhibit the expression of the MFS gene, thereby inhibiting the biological phenotype of *A. odontolyticus*. However, the effect of sanguinarine on other G4 sequences still needs to be further verified, which is the limitation of this study.

**Conclusions**

This study indicated the characteristics of genomic G4 sequences of hypertension-associated tongue coating microbiota, the quantities of G4 sequences was significantly different among the five most abundant genus, and G4 ligands sensitivity differed between *A. odontolyticus* and *A. baumannii* with significantly different genomic G4 quantities. Moreover, sanguinarine, which was identified as one of the G4 ligands, could affect the biological phenotype of *A. odontolyticus* by promoting the formation and stability of G4 in the MFS gene. Our findings point out that it is necessary to pay more attention to the role of G4 ligands in oral local drug use.

**Abbreviations**

**G4**: G-quadruplex

**MFS**: Major Facilitator Superfamily

**PQS**: Putative G-quadruplex forming sequences

**eHOMD**: Expanded Human Oral Microbiome Database

**AWCD**: Average well color development

**CD**: Circular dichroism

**ORFs**: Open reading frames

**KEGG**: Kyoto Encyclopedia of Genes and Genomes

**EggNOG**: Evolutionary genealogy of genes

**ESI-MS**: ESI mass spectrometry

**CFU**: Colony-forming unit

**SAN**: Sanguinarine

**OD**: Optical density

**Declarations**
Ethics approval and consent to participate

This study was approved by the ethical committee of Peking University Third Hospital, and the informed consent were obtained from all subjects.

Consent for publication

Not applicable.

Availability of data and material

Raw data or further details can be obtained upon request from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MX, FC, QHC initiated, supervised this study, and designed the experiments. SQZ conducted most of the experiments; YGL collected the samples and clinical details; JZ assisted with chemical experiments; QHC performed bioinformatic analysis. All authors read and approved the final manuscript.

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Authors' information

Department of Cardiology, Institute of Vascular Medicine, Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Peking University Third Hospital, Beijing, China.

Shiqin Zhang; Yangong Liu and Ming Xu

Beijing National Laboratory for Molecular Sciences, Analytical Instrumentation Center, College of Chemistry and Molecular Engineering, Peking University, Beijing, China

Qinghua Cui

Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, 100081, China

Feng Chen
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Figures
Figure 1

Variation of G4 Sequences in tongue coating bacteria
Figure 2

The relative abundance and genomic G4 features of hypertension associated species.
Figure 3

G4 abundance of bacterial genomes affected sanguinarine sensitivity.
Figure 4

Inhibitory effects of sanguinarine on A. odontolyticus biofilm and carbon source formation.
Figure 5

Sanguinarine could bind and stabilize G4 structure of A. odontolyticus to inhibit the expression of MFS.

Supplementary Files

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