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ORIGINAL ARTICLE

A preliminary study on the relationship between iron and black extrinsic tooth stain in children

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Significance and Impact of the Study: In this study, we have confirmed the existence of iron in black extrinsic tooth stain by ICP-MS. It was the first time the functional genes of bacteria in black stain were accessed and the genes associated with iron were found. These findings provided clues on the research of aetiology of black stain, which troubled millions of children. It also revealed the association between metabolic pathway of microbiota and oral phenomenon.

Keywords
bacteria, black stain, children, genes, iron.

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Abstract

Black extrinsic tooth stain, which has long troubled many people, is common among children and influences the aesthetics of teeth. The pigment was proposed to be a black insoluble ferric compound, but this is controversial. To determine whether iron exists in black stain, we collected 10 samples of black stain and 10 samples of plaque separately from children with and without black stain using sterile titanium implant curettes, and analysed the samples by inductively coupled plasma-mass spectrometry. Iron was present in both black stain and plaque, with concentrations ranging from 76·12 to 1116·88 $\mu g\ g^{-1}$. The contents of iron in black stain were significantly higher than in plaque. Because bacteria may be involved in the aetiology of black stain, we assessed the functional genes of bacteria in black stain based on 16S rRNA gene sequencing results obtained using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. Of 253 Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways tested, 56 differed in abundance between samples from children with and without black stain. Genera altered in black stain were related to many of the pathways. Some KEGG Orthology groups showed differences between black stain and plaque of control group were found to be related to iron.

Introduction

Black extrinsic tooth stain is common among children; the prevalence is between 1 to 20% (Ronay and Attin 2011). It is characterized by dark lines or incomplete coalescence of dark dots on the cervical third of enamel, which can occur in both primary and permanent dentition (Koch *et al.* 2001). Black stain influences the aesthetics of teeth but is difficult to remove with a toothbrush, and tends to recur after scaling (Hattab *et al.* 1999). The research into the composition of black stain is rare and its source remains uncertain.

Reid *et al.* (1977) suggested that the pigment in children's teeth was probably ferric sulphide, formed by the reaction between hydrogen sulphide produced by bacterial action, and iron in the saliva or gingival exudate. This point of view was widely accepted but the method of chemical reagents to examine the chemical composition lacks in accuracy. Parnas *et al.* (2013) questioned that previous report of the presence of metallic ions was probably due to contamination by metal instruments. They collected black stain by graphite curettes and analysed the samples by energy dispersive spectrometry (EDS), but did not detect metallic ions in samples. The EDS enabled

main element identification with a precision of ±1%. We do not fully accept this opinion, because metallic ions such as iron widely exist in food as minor elements and inevitably have trace amounts of residual on the teeth. Another research analysed the stains in six extracted human permanent teeth by wavelength-dispersive spectrometry, found the stains were highly calcified and contained a significant amount of organic matter, with traces of iron and copper. Corresponding areas of high concentrations between sulphur and iron/copper were observed, suggesting complexes of sulphur and metal ions as possible colour-forming species (Tantbirojn *et al.* 1998). Whether iron/copper exists in black stain of deciduous teeth is obscure.

Many oral diseases are correlated with oral microbiome. For example, in subjects with halitosis, the hydrogen sulphide-related metabolic pathways are over-represented (Ren *et al.* 2016). Previous studies including traditional bacteriological culture examinations and real-time PCR have indicated a unique microbiota in dental plaque with black stain (Slots 1974; Saba *et al.* 2006; Heinrich-Weltzien *et al.* 2014).

In this study, we first used inductively coupled plasma-mass spectrometry (ICP-MS) to analyse the metallic traces in black tooth stain. Second, to reveal microbiota functions especially those related to iron in black stain, we predicted the functional genes of bacteria in black stain based on 16S rRNA gene sequencing results produced using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille *et al.* 2013).

Results and discussion

Iron and copper contents in black stain and plaque

Iron and copper were observed by ICP-MS in all samples, both black stains and dental plaque of control group. The concentrations of iron ranged from 76·12 to 1116·88 $\mu g\ g^{-1}$. The iron levels were significantly higher in the BS than in the PC ($P < 0{\cdot}001$), which were in accord with Reid, suggesting a role for iron in the black material of the teeth. The copper contents of the samples were much lower than the iron contents, ranging from 3·36 to 22·00 $\mu g\ g^{-1}$. No statistically significant difference was found between the groups in terms of the amount of copper ($P = 0{\cdot}912$) (Fig. 1). Copper is likely not involved in the formation of black stain.

Several epidemiological studies have supported the role of iron of black stain. A cross-sectional study in Nepal found a positive correlation between iron levels in water and stain distribution (Pushpanjali *et al.* 2004). In a survey of black stain in Spain, a statistically significant

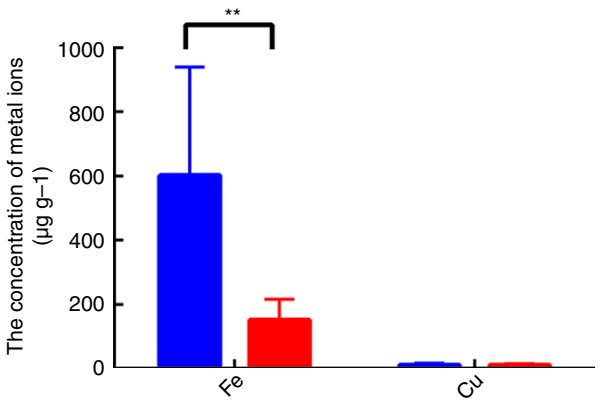

Figure 1 Comparison of Fe and Cu in (■) black stain and (■) plaque (**$P < 0{\cdot}01$). [Colour figure can be viewed at wileyonlinelibrary.com]

relationship was found between black stain and the regular consumption of foods rich in iron (Garcia Martin *et al.* 2013). A dentist made a small survey in dental practice, and found that the diet of patients with extrinsic black stains on their teeth was rich in cheese, which contains lactoferrin, a protein known for its high affinity for iron (Mesonjesi 2012). Iron existed extensively in the diet, the residue on the teeth may participate in the formation of black stain.

As one of the most important metal element in human, it is not surprising to find iron in both black stain and dental plaque. However, the concentration of iron in these samples was quite low, perhaps for the following reasons. First, the amounts of impurities involved in the samples, including food residue and salivary proteins, may have been significant. Second, the iron may bind with proteins and form large molecular metal complexes. Parnas *et al.* (2013) did not find traces of metallic ions, perhaps due to the lower sensitivity of EDS. As far as we know, it is the first time that ICP-MS was used to detect metal ions in the black material of the teeth. The quantitative analysis had quite high sensitivity, whose minimum detectable concentration of iron was 0·12 $\mu g\ g^{-1}$. By the method, we confirmed the existence of iron in the black stain and the level was significantly higher than that in plaque.

Functional genes predicted by PICRUSt based on 16S rRNA gene sequences

After data trimming, a total of 372 971 reads were generated from 26 samples, with an average of 14 345 reads per sample (ranging from 4311 to 22 656). The reads were clustered into 724 operational taxonomic units (OTUs) by picking closed-reference OTUs at 97% similarity using Qiime (Table S1).

Then, the microbiota functions were predicted based on inferred metagenomes using PICRUSt. Of 253 Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways tested, 56 differed in abundance between BS and PC ($P < 0{\cdot}05$) (Table S2). Genera altered in black stain were related to many of the pathways (Fig. 2, Table S3). For example, *Actinomyces*, which was more abundant in black stain, was positively associated with pyruvate metabolism and lipoic acid metabolism, and negatively associated with DDT degradation.

Pathways related to glycolysis/gluconeogenesis, starch and sucrose metabolism, pyruvate metabolism, vitamin B6 metabolism, nicotinate and nicotinamide metabolism, lipoic acid metabolism, tyrosine metabolism, were more abundant in BS. The results indicated a more active metabolic state in teeth with black stain. The deposits on the teeth may provide bacteria nutritional substances and the metabolic products may participate in the formation of black stain.

In addition, the pathway of inorganic ion transport and metabolism and other transporter, which contain sodium channel, zinc transporter and iron transporter, were increased in black stain. Some of them participate in energy transformation, while some of them may participate in cell-to-cell communication between bacteria (Lau *et al.* 2016).

To assess more about the role of iron in the bacteria of black stain, those KEGG Orthology (KO) groups that showed differences between BS and PC and were related to iron were selected (Fig. 3). It is interesting that some KO enriched in black stain were composite of ferrous iron (K04758, K04759), while KO enriched in plaque of control group were composite of ferric iron (K02010, K02011, K02012). Iron primarily exists as two cations, the oxidized ferric (Fe^{3+}) form and the reduced ferrous (Fe^{2+}) form (Cassat and Skaar 2013). It was inferred that some bacteria in black stain were anaerobic, iron existed as ferrous form in them.

Iron plays an important role in transporter system. For example, 'efeO', the gene which was increased in the BS, has functions in binding with Cu^{2+} and Fe^{3+} and iron transport. In KEGG Modules, M00317 is called Manganese/iron transport system, and composite of Mn^{2+} and Fe^{2+} substrates. It, in combination with other ion transport systems, may contribute to acquisition of iron and manganese, and resistance to oxidative stress (Sabri *et al.* 2006). M00319 is called Manganese/zinc/iron transport system, and composite of Mn^{2+}, Zn^{2+} and Fe^{2+} substrates. It involves in the uptake of manganese and/or zinc, and plays an important role in the virulence of pathogen (Diep *et al.* 2014). It was interesting to find the Magnesium/iron transport system group (M00317) shows increased activity in the BS group, while the Magnesium/iron transporter (M00319) and the Iron (III) transporter (M00190) showed increased activity for the PS group. Different bacteria use different iron sources through different iron transporters (Lau *et al.* 2016). The type of iron source used affects biofilm formation (Hayrapetyan *et al.* 2016). The differences of KO groups related to iron between BS and PC also indicated a characteristic microbiota in black stain. But we cannot confirm these differences were related to the formation of black stain so far.

When we explored the relationship between iron and black stain, there were two hypotheses. First is that black stain is the outcome of microbiological metabolism. The

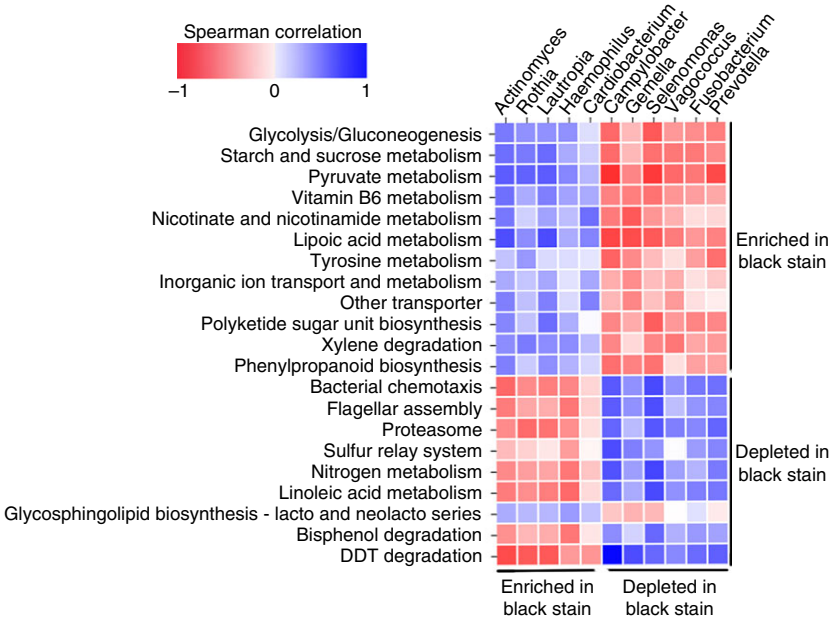

Figure 2 Bacterial taxa associated with black stain are related to some Kyoto Encyclopedia of Gene and Genomes (KEGG) pathways. Spearman's correlation coefficients were estimated for each pairwise comparison of genus abundances and KEGG pathway abundances. Only bacterial taxa and KEGG pathways with significantly different abundances between BS and PC are included in the heatmap ($P < 0{\cdot}05$). Full lists of genera and KEGG pathways associated with black stain can be found in Table S3. [Colour figure can be viewed at wileyonlinelibrary.com]

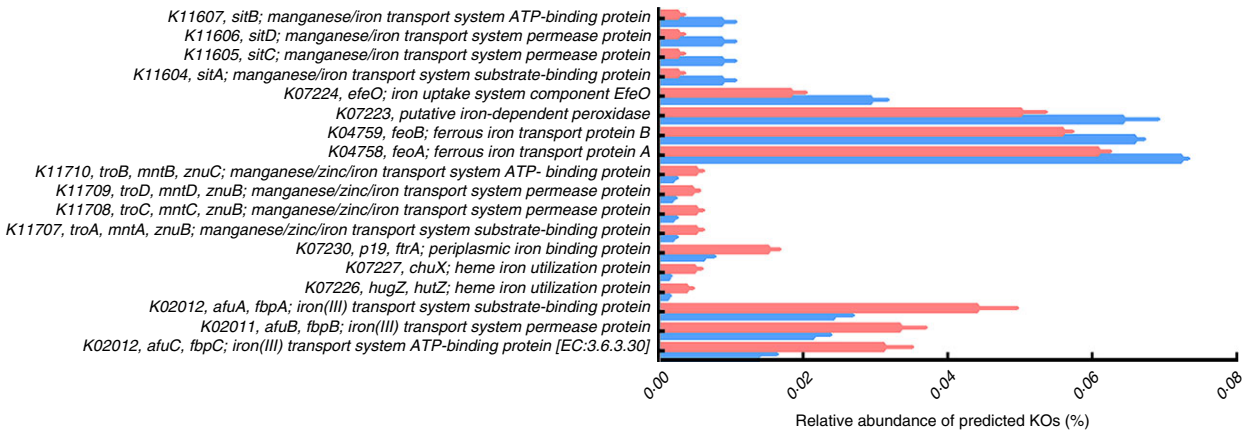

Figure 3 The Kyoto Encyclopedia of Gene and Genomes (KEGG) orthology (KO) groups related to iron that have significantly different abundances between (■) BS and (■) PC. Among K11607, K11606, K11605 and K11604 formed a KEGG Module called Manganese/iron transport system (M00317). While K11710, K11709, K11708, and K11707 formed a KEGG Module called Manganese/zinc/iron transporter (M00319) and K02012, k02011, k02010 formed a KEGG Module called Iron(III) transporter (M00190). [Colour figure can be viewed at wileyonlinelibrary.com]

microbiome in the plaque produces iron compounds and creates black sedimentation. Second is that environmental factors (as may be diet for example) create black sedimentation through chemical reactions while altering the microbiome in the plaque. Before we took samples, we learned the dietary habits of the children with black stain from their parents or caregivers. None of the children was on a specific diet. Most of their family members and classmates who had the same diet with these children did not have black materials in their teeth. So, we thought the cause of black stain was not only diet.

Many previous studies found the association between black stain and bacteria (Slots 1974; Saba *et al.* 2006; Heinrich-Weltzien *et al.* 2014; Li *et al.* 2015) and consider microbiome as one of the important aetiologies. Our research cannot make a certain conclusion that black stain is the outcome of microbiological metabolism but provide some clues that these bacteria metabolic pathway may associated with black stain in children's teeth. It was the first time the functional genes of bacteria in black stain were explored.

This study was limited by a lack of metagenomics data to determine the actual gene contents of the bacteria in plaque with black stain. Future studies should investigate the metagenomics of plaque with black stain to confirm the role of iron in the formation of black stain.

Materials and methods

Subject selection

This study was approved by the Ethics Committee of Stomatological Hospital of Peking University (PKUSSIRB-201311085). Parents and/or caregivers of the recruited children signed informed consent forms and provided details on the children's dietary habits.

In total, 46 systemically healthy children, aged 3–6 years, were recruited from the Third Kindergarten of the Chinese Academy of Sciences and Experimental Kindergarten of Beijing Normal University. None of the children was on a specific diet or have taken iron supplements, and children taking medicines for longer than 6 months were excluded (Heinrich-Weltzien *et al.* 2014). None had used antibiotic drugs within the 2-week period before sample collection. Due to the limited amount of sample that could be collected from each child, the children were divided into two groups.

Group A included 10 children with at least six black-stained teeth and 10 children free of black stain. The samples from this group were used to assess the metal ion contents of black stain and plaque.

Group B included 26 caries-free children aged 4–5 years, 10 children with at least six black-stained teeth and 16 children without black stain. The samples from this group were used to analyse oral micro-organisms by next-generation sequencing.

No significant difference was found between children with and without black stain involving age and gender, dentition and caries status.

Sample collection

Samples were collected in the morning at the kindergarten. The children were asked to rinse their mouths with water before sample collection.

In group A, to avoid contamination by iron instruments, we used sterile titanium implant curettes (titanium mini curettes GRXST 13-14; Stoma, Emmingen-Liptingen,

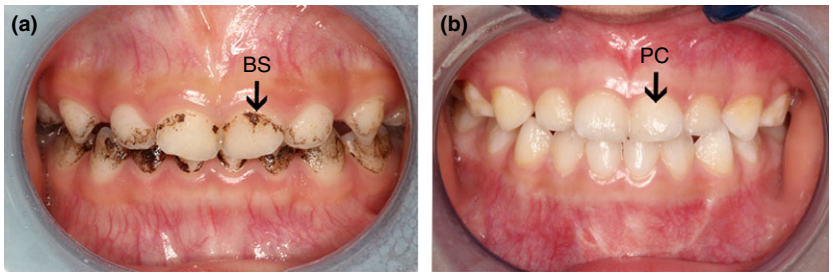

Figure 4 Primary dentition with and without black stain. (a) For children with black stain, samples of black stain (BS) were obtained from dental surfaces with black material. (b) For children without black stain, samples of plaque (PC) were collected. [Colour figure can be viewed at wileyonlinelibrary.com]

Germany) to scrape samples from the teeth. The tip of the curette does not contain iron and is more suitable for scraping black stain, which attaches firmly to teeth, than a graphite curette because the latter is softer and easier to break. All samples were collected from tooth surfaces without caries. For children with black stain, we collected black stain from the black area of the teeth surfaces. For children without black stain, we obtained normal dental plaque from buccal and lingual surfaces. Samples were collected into 1·5-ml microcentrifuge tubes containing 400 μl of sterile water, and then transferred on ice to the laboratory and centrifuged (13 400 ***g***, 10 min) to remove the supernatant. The precipitates were dried thoroughly in a vacuum freeze dryer and weighed with an analytical balance.

In group B, samples were collected with metal curettes. For children with black stain, samples of black stain (BS) were scraped from the black area of the dental surface. For children without black stain, samples of plaque (PC) were collected from normal surfaces (Fig. 4). The methods of sample collection and processing are described in Li *et al.* (2015).

Quantitative analysis by ICP-MS

Samples of black stain and plaque from group A were analysed by ICP-MS (DRC-II; Perkin-Elmer, Antigo, WI) at the Center of Medical and Health Analysis of Peking University. The ICP-MS operation conditions were optimized for the measurement of Fe and Cu.

Bioinformatic analysis

Raw sequence data of the bacterial 16S rRNA gene from group B were submitted to the NCBI SRA (accession number SRP063716).The raw data generated were analysed by QIIME (ver. 1.9.1). The multiplexed samples were deconvoluted based on the unique barcode assigned to each sample. The barcodes and primers were then trimmed off and sequences with a quality value <20 and low-quality sequences were removed. High-quality reads were clustered into OTUs at 97% similarity corresponding to the GreenGenes (ver. 13.5) reference database.

PICRUSt (ver. 1.0.0) [17] was used to predict the functional gene content in the microbiome based on the KEGG database. PICRUSt was used online in the Galaxy workflow framework.

Statistical analysis

The data were analysed using SPSS software (ver. 22). The iron and copper contents between the samples of black stain and plaque from group A were compared using the Mann–Whitney *U* test. Differences in the relative abundances of genes within the BS and PC were analysed using the same test. Significance levels were set at $P < 0{\cdot}05$.

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/Dfrgsg

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The OTU lists clustered from each sample at 97% similarity.

Table S2. The lists of KO groups counts and KEGG pathway counts of each sample.

Table S3. Spearman's correlation coefficients between bacterial taxa and KEGG pathways with significantly different abundances between BS and PC.