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## DNA microarray analysis reveals that antibiotic resistance-gene diversity in human gut microbiota is age related

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**The human gut is a reservoir for antibiotic resistance genes. In this report, we used a DNA microarray chip covering 369 resistance types to investigate the relationship between antibiotic resistance-gene diversity and human age. Metagenomic DNA from fecal samples from 124 healthy volunteers of four different age groups (pre-school-aged children (CH), school-aged children (SC), high school students (HSS) and adults (AD)) were hybridized to the microarray chip. The results showed that 80 different gene types were recovered from the gut microbiota of the 124 individuals: 25 from CH, 37 from SC, 58 from HSS and 72 from AD. Further analysis indicated that the antibiotic resistance genes in the CH, SC and AD groups clustered independently, whereas the gene types in the HSS group were more divergent. Our results indicated that antibiotic resistance genes in the human gut microbiota accumulate from childhood to adulthood and become more complex with age.**

In recent decades, antibiotic resistance has become a global health problem<sup>1</sup>. The emergence of drug-resistance pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA)<sup>2</sup>, multidrug-resistant (MDR) and/or extensively drug-resistant (XDR) *Mycobacterium tuberculosis*<sup>3</sup> and New Delhi metallo-beta-lactamase (NDM-1) superbugs<sup>4</sup> has presented a significant challenge for the treatment of infectious diseases.

The tremendous number of indigenous bacteria (microbiota) in the human gut act as a reservoir of antibiotic resistance genes<sup>5</sup>. Due to the dense microbial population in this environment, the potential for antibiotic resistance-gene transfer is high. Significant evidence has accumulated from both *in vivo* and *in vitro* models that resistance genes can be exchanged among intestinal bacteria or even transferred to pathogens that pass through the colon<sup>5,6</sup>. The spread of antibiotic resistance genes in the human gut represents a serious threat to human health, and the selective pressure caused by antibiotic use greatly increases this risk. The earliest example of this phenomenon was a study using a human volunteer that showed that antibiotic resistance transfer occurred between *Escherichia coli* of animal origin and the indigenous *E. coli* in the gastrointestinal tract (GIT) after treatment with therapeutic levels of tetracycline<sup>7</sup>. In addition, selective pressure causing increased antibiotic resistance-gene transfer has been demonstrated in a clinical case in which ACC-1 AmpC was transferred from *K. pneumoniae* to indigenous *E. coli* during antibiotic treatment<sup>8</sup>.

Different approaches, such as the isolation of resistant strains, polymerase chain reaction, DNA microarray and metagenomic expression library screening, have been used to analyze antibiotic-resistance genes in human gut microbiota. Combining culture-independent and -dependent sampling strategies, Sommer *et al.* characterized a total of 210 antibiotic-resistance genes in the gut microbiome through functional screening of expression libraries<sup>9</sup>. Their study suggested that the human microbiome could constitute a mobile reservoir of antibiotic resistance genes that are accessed by pathogenic bacteria to acquire antibiotic resistance. In another study involving screening fosmid libraries of tetracycline-resistance genes from the guts of a healthy mother-infant pairs one month after childbirth, Lisbeth *et al.* revealed the likely transmission of antibiotic resistance in the GIT from mother to infant, and their study indicated the possibility of gene transfer among distantly related bacteria co-inhabiting the GIT of the same individual<sup>10</sup>. A recent study using microarray analyses of metagenomic DNA



from healthy volunteers in six European countries demonstrated that *tet(M)* and *tet(W)* were the most prevalent tetracycline resistance genes in the oral and fecal metagenomes, respectively<sup>11</sup>.

Recently, we analyzed the antibiotic resistance genes in the gut microbiota of 162 individuals from three different populations, and we found that Chinese individuals harbored the highest number and greatest diversity of antibiotic resistance genes<sup>12</sup>. However, little is known about the full profile of antibiotic resistance-gene diversity in different human age groups. In this study, we used a custom-designed DNA microarray containing most of the antibiotic resistance genes in the Antibiotic Resistance Genes Database (ARDB) to analyze the antibiotic resistance-gene diversity in the microbiota of 124 healthy Chinese volunteers belonging to four different age groups.

## Results

**Microarray design.** An oligonucleotide-based DNA microarray was custom designed (Roche NimbleGen) to detect antibiotic resistance genes in human gut microbiota. This microarray contained a total of 8,746 probes targeting 2,915 antibiotic resistance genes that were classified into 369 antibiotic resistance gene types according to the ARDB. These antibiotic resistance-gene types covered most known antibiotics, including aminoglycosides (64 types), penicillin beta-lactamases (50 types), other beta-lactamases (17 types), amphenicols (40 types), trimethoprim (22 types), macrolide lincosamide streptogramin\_B (MLS<sub>B</sub>) (52 types), sulfonamides (3 types), tetracyclines (40 types), vancomycin (37 types) and other antibiotics (50 types) (Supplementary Table 1).

**Microarray validation and determination of the detection threshold.** Microarray validation was performed through the hybridization of genomic DNA from two *E. coli* isolates that were either susceptible or resistant to 8 antibiotics. The results indicated that all of the genes conferring resistance to the 8 antibiotics in the resistant *E. coli* yielded hybridization signal values higher than 10. In contrast, the values were all below 9 in the sensitive strain (Supplementary Table 2). The presence or absence of selected resistance genes in the two *E. coli* isolates was further confirmed using specific PCRs; the results were consistent with the microarray detection results (Supplementary Table 3). The signal threshold of 10 for the detection of resistance genes was also validated using other 5 multidrug-resistant clinical isolates. These results suggested that the microarray was able to detect most of the antibiotic-resistance genes using a signal threshold of more than 10, which was adopted in the subsequent hybridization analysis. Because the hybridization signals were low (threshold values of 9 and 10 for the sensitive and resistant strains, respectively) for cephalixin, amoxicillin and ampicillin, three beta-lactam antibiotics, we analyzed the range of the hybridization signals for all 17 beta-lactamase-type genes in each sample when a threshold of 10 was used. The results showed that the signal values differed from type to type and were significantly higher than the threshold of 10 in most of the samples (Supplementary Figure 1), suggesting that beta-lactamase resistance genes can be reliably detected using this threshold.

**Detection of antibiotic resistance genes in human gut microbiota.** Fecal samples from 124 healthy volunteers were collected in Hangzhou, China. These volunteers were grouped based on their age: pre-school-aged children (CH, 3–5 years old), school-aged children (SC, 10–11 years old), high school students (HSS, 15–17 years old) and adults (AD, 26–55 years old) (Supplementary Table 4). Metagenomic DNA from the fecal samples of the volunteers was hybridized to the antibiotic resistance microarray. The results showed that a total of 80 different antibiotic resistance-gene types were recovered from the gut microbiota of the 124 individuals, including genes conferring resistance to aminoglycosides (17),

penicillin beta-lactams (11), other beta-lactams (8), amphenicols (5), trimethoprim (1), MLS<sub>B</sub> (12), sulfonamides (2), tetracyclines (10) and other antibiotics (14).

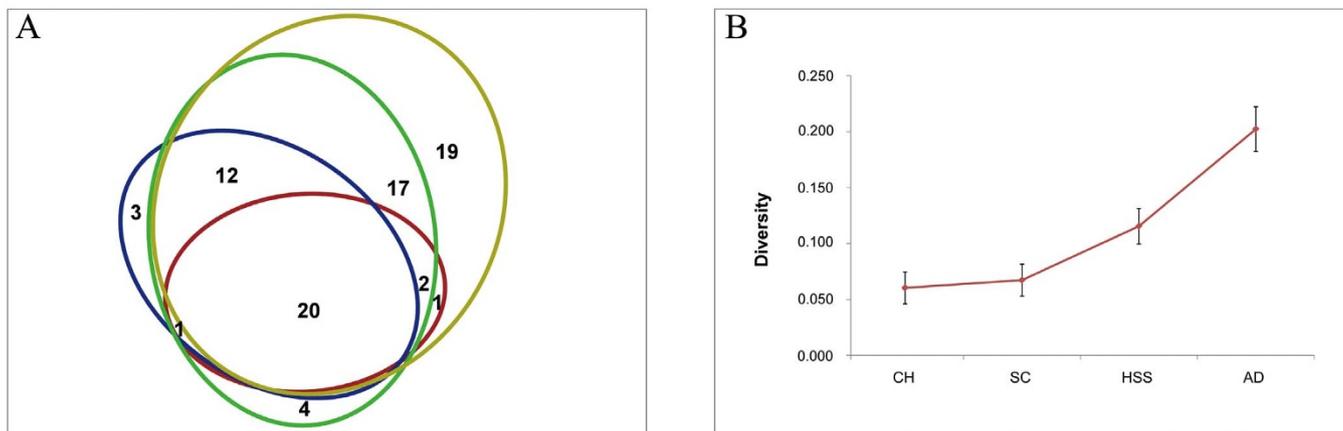
Interestingly, the diversity of antibiotic resistance-gene types in the human gut microbiota tended to increase with age; 25, 37, 58 and 72 gene types were identified in the CH, SC, HSS and AD groups, respectively (Supplementary Table 5). The average number of gene types in an individual of the CH, SC, HSS and AD groups was 8, 14, 15 and 24, respectively; the difference between any two groups was statistically significant, except for the difference between the SC and HSS groups. Moreover, 20 resistance genes were shared among all 4 groups, 12 were shared among the SC, HSS and AD groups and 17 were shared between the HSS and AD groups (Fig. 1A). A total of 0, 3, 4 and 19 unique genes were detected in the CH, SC, HSS and AD groups, respectively.

**Diversity of antibiotic resistance-gene types is age-associated.** We then calculated the antibiotic resistance gene-type diversity coefficients (Gini-Simpson index) for each group. The values for the CH, SC, HSS and AD groups were 0.061, 0.067, 0.116 and 0.202, respectively; the difference between any two groups was significant, except for the difference between the CH and SC groups (Fig. 1B), and the Spearman correlation coefficient between the age of each individual and the number of antibiotic resistance-gene types was 0.824 ( $p < 0.0001$ ) (Supplementary Figure 2), suggesting that the antibiotic resistance gene-type diversity gradually increased with age. Additionally, principal coordinate analysis (PCoA) showed that the individuals in the CH, SC and AD groups clustered independently, meaning that the antibiotic resistance gene-type profiles in these individuals were more similar within the group. The exception to this trend was the HSS group, whose individuals were scattered among the other three groups, but overall, this group clustered most closely to the SC group (Fig. 2A). Similar clustering results were reflected in the neighbor-joining (NJ) tree (Fig. 2B). Furthermore, the AD group in the NJ tree was very distant from the other three groups, suggesting that the antibiotic resistance gene-type profile in the AD individuals was highly different from the other three groups. Additionally, the CH group was the outlier in the NJ tree, followed by the SC group, the HSS group and the AD group, which constituted the innermost layer; the distance between individuals increased with age. These results indicated that the antibiotic resistance-gene diversity in the human gut microbiota was increasingly complex as individuals aged and that diversity likely accumulated from childhood to adulthood.

## Discussion

Microarray analysis has been widely used to detect antibiotic resistance genes in clinical isolates of metagenomic samples of various origins<sup>11,13–17</sup>. However, most of the microarrays used in these studies covered a limited number of genes and usually targeted the antibiotic resistance genes of a specific class of antibiotics, such as aminoglycosides, tetracyclines and/or erythromycins. In this study, we used a custom-designed antibiotic resistance-gene microarray that covered almost all known antibiotic resistance genes for the 9 major classes of antibiotics. To our knowledge, this is the most informative antibiotic resistance microarray to date. Additionally, this is the first study to investigate the antibiotic resistance-gene types and their diversity in the microbiota of healthy people at different ages.

We identified a total of 80 antibiotic resistance-gene types in the microbiota of the 124 samples that crossed our detection threshold. The development of antibiotic resistance in microbes can be attributed to many factors, among which antibiotic use is the most important. A direct correlation between antimicrobial use and the extent of antimicrobial resistance has been reported<sup>18</sup>. When antibiotics are administered, the balance between the human host and its associated

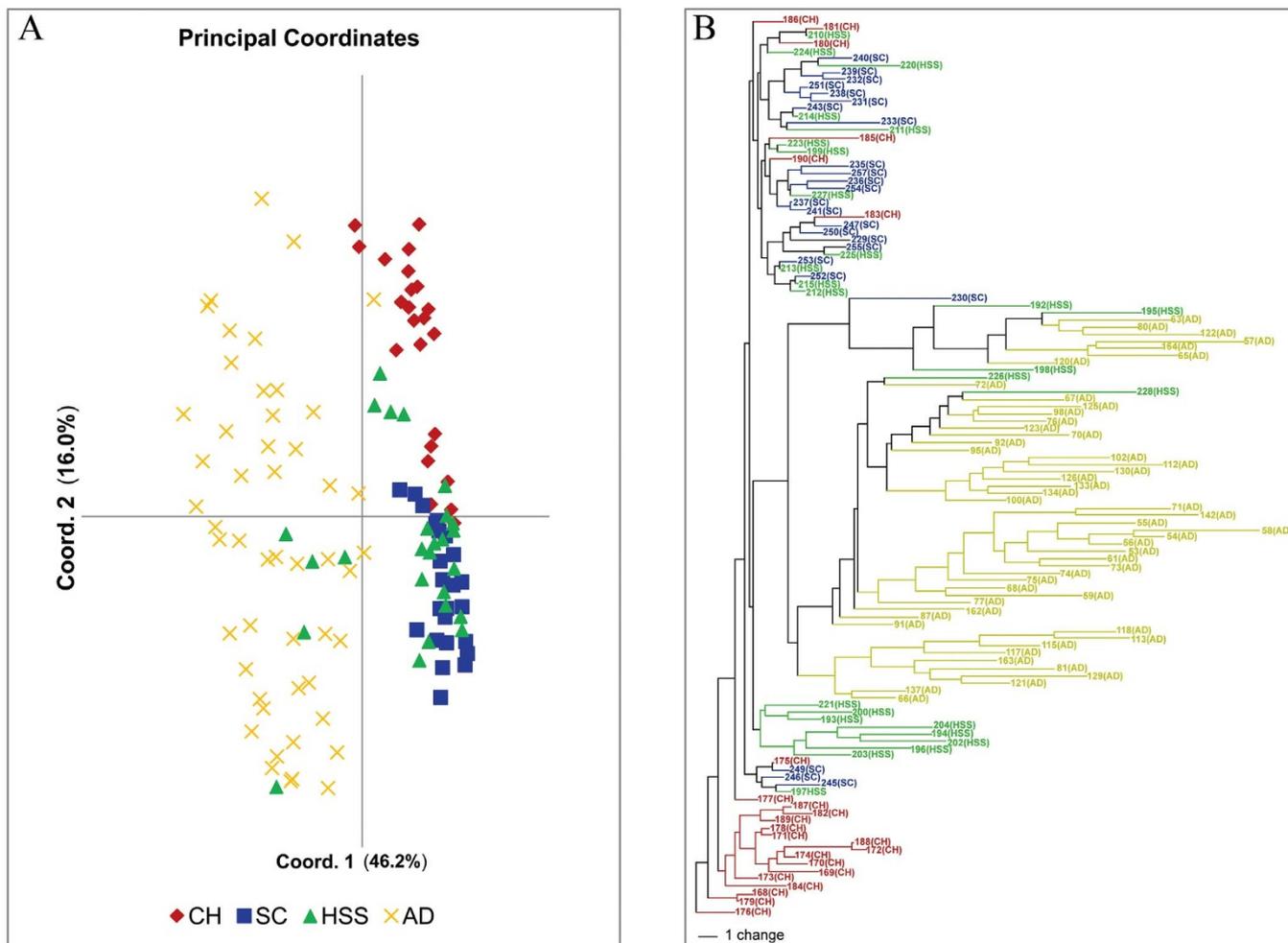


**Figure 1** | (A) Venn diagram of the antibiotic resistance-gene types shared between the groups and (B) Diversity analysis of the antibiotic resistance-gene types in each group. The following abbreviations were used: CH, pre-school-aged children; SC, school-aged children; HSS, high school students; and AD, adults. The different colors in panel (A) represent the different groups: red, CH group; blue, SC group; green, HSS group; and yellow, AD group; one antibiotic resistance-gene type shared among the CH, SC and AD groups is not presented. The Gini-Simpson index was calculated using GENALEX 6.

microorganisms can be dramatically disturbed, leading to the emergence of diseases as well as antibiotic-resistant strains<sup>19,20</sup>.

Recently, the large-scale characterization of the human gut microbiome in different age populations has shown that the complexity of gut microbiota increases after birth, matures during the first 3 years

of life at both the species and gene levels and decreases with age<sup>21</sup>. However, our results revealed that the number of antibiotic resistance-gene types in the human gut tended to increase with age; the older age groups possessed a higher diversity of antibiotic resistance-gene types than the younger groups. Additionally, the antibiotic



**Figure 2** | (A) Principal coordinate analysis and (B) the neighbor-joining tree of antibiotic resistance genes in each individual. The NJ tree was constructed based on the 0/1 data for each sample. The accession numbers are noted at the ends of each branch, and the group information is shown in brackets after the accession number. The four different groups are represented by different colors as in Figure 1.



resistance genes were more diverse between individuals in the older groups than in the younger groups. We hypothesize that this diversity may be caused by selective pressures, particularly exposure to antibiotics.

It has been reported that antimicrobial agents not only have short-term impacts on the human microbiota but also exert long-term influence. For example, in a study that involved 7 days of treatment with clindamycin and 2 years of follow-up sampling, the diversity of *Bacteroides* isolates declined sharply, and the *Bacteroides* community never returned to its original composition<sup>22</sup>. Additionally, a dramatic and persistent increase in the levels of specific resistance genes was observed after the clindamycin treatment. In another study, Sjölund *et al.* showed that resistant enterococci harboring *erm(B)* could be isolated immediately following a regimen (clarithromycin, metronidazole and omeprazole) that is widely used to eradicate *Helicobacter pylori*, and the resistant strains persisted for at least 3 years without further selection<sup>20</sup>. In our study, although the volunteers we used for sampling had not been exposed to any antibiotic agents in the past 3 months, we cannot exclude the possibility that they were previously exposed to antibiotics. Therefore, the increased diversity and accumulation of antibiotic resistance genes in the human gut may be partly attributable to gradual antibiotic exposure; in other words, the older individuals may have been exposed to a more diverse set of antibiotics or more antibiotic-resistant microorganisms than the younger individuals.

In this study, we determined the antibiotic resistance-gene diversity in the human gut microbiota of individuals of different ages using DNA microarray analysis. We found that antibiotic resistance genes were prevalent in the human gut, and older people harbored more complex and more diverse antibiotic resistance genes than younger individuals, which could potentially be explained by gradual exposure to different antibiotics. Although the microarray we used was validated using clinical isolates, it is worth noting that false-positive and false-negative hybridization signals could not be completely excluded when using metagenomic DNA from gut microbiota. Additionally, because the development of antibiotic resistance involves complicated mechanisms, the presence of these genes cannot be attributed only to antibiotic use; other factors such as bacterial intrinsic resistance (efflux pumps)<sup>23</sup> and heavy metal selection<sup>24</sup>, among others, can also increase resistance, and their role should be further investigated.

## Materials and methods

**Probe design.** To design the probes used to detect the antibiotic resistance genes in the human gut microbiota, all of the protein sequences of the antibiotic resistance genes in the ARDB (version 1.0)<sup>25</sup> were downloaded on Dec 20, 2008. We then determined the gene sequences of these antibiotic resistance proteins using a tBLASTn search against the NCBI nr database, and only sequences with more than 95% sequence identity were used; next, we classified these antibiotic resistance-gene sequences into 369 resistance types according to the ARDB, and the gene sequences corresponding to all 369 antibiotic resistance-gene types were used for probe design and microarray production.

The antibiotic resistance gene microarray was custom-designed (Roche NimbleGen) based on a single chip containing 3 internal replicated probe sets of 12 probes per resistance gene to cover the whole 315K 12-plex platform. The mean GC content and Tm of all the probes were 51.8 ± 0.1% and 80.7 ± 4.4°C, respectively. Random oligonucleotides were used as control probes. Thus, every antibiotic resistance gene was represented by twelve 60-mer probes that were synthesized *in situ* by photolithography on glass slides; a random positional pattern was used.

**Samples and DNA extraction.** Two clinical *Escherichia coli* isolates with different resistance phenotypes and five other multidrug resistance isolates from Hangzhou Yifu hospital were used to validate the new antibiotic resistance microarray. The minimal inhibition concentrations (MICs) for 8 antibiotics (tetracycline, cephalalexin, amoxicillin, ampicillin, streptomycin, kanamycin, chloramphenicol and gentamicin) were determined for these isolates using the broth microdilution method as described in the Clinical and Laboratory Standards Institute guidelines<sup>26</sup>. Bacterial genomic DNA was extracted following a standard protocol<sup>27</sup>. The presence or absence of selected resistance genes in the *E. coli* isolates was further confirmed by specific PCRs using the primers listed in Supplementary Table 3; the resulting PCR products were then sequenced using a 3730 DNA Analyzer. A total of 124 fecal samples were collected from healthy volunteers in Hangzhou, China. None of the volunteers had

taken any antibiotic agents in the prior 3 months. DNA from the gut microbiomes was extracted directly from each fecal sample using the QIAamp DNA Stool Mini kit according to the manufacturer's protocol. This research was approved by the Research Ethics Committee of the Institute of Microbiology, Chinese Academy of Sciences, and informed consent was obtained from each subject.

**Microarray hybridization and scanning.** After sonication, 1 µg of DNA fragments (500 to 2,000 bp) was labeled by Cy3-dUTP (TriLink Biotechnologies) incorporation using Klenow fragment polymerase (NEB). The labeled genomic DNA (2 µg) was hybridized to the arrays in NimbleGen Hybridization Buffer for 16 hours at 42°C using a MAUI hybridization system (BioMicro Systems, Inc., Salt Lake City, Utah). Then, the hybridized arrays were washed with NimbleGen wash buffer, spun dry in a microarray high-speed centrifuge (TeleChem International, Inc., Sunnyvale, CA) and stored until scanned. The Genepix® 4000B scanner (Axon Instruments, Union City, CA) was used to scan the microarrays at a 5-µm resolution.

**Data analysis.** The Burst Multiplex Image system was used to divide each array into 12 smaller arrays and then to extract the pixel intensities of each smaller array. All of the data in each array were normalized as described by Bolstad *et al.*<sup>28</sup>. The gene calls were generated using the Robust Multichip Average (RMA) algorithm<sup>29</sup>. These analyses were performed with the NimbleScan™ v2.4 software (NimbleGen). Because each probe had three internal replicates in the antibiotic resistance microarray, we derived three fluorescence intensity values for each gene after RMA analysis, and the average value was regarded as the antibiotic resistance-gene value. The antibiotic resistance-gene-type value was defined as the sum of all of the antibiotic resistance-gene values that belonged to one antibiotic resistance-gene type. Notably, the value we obtained for each gene was its fluorescence intensity signal divided by the background signal generated by the random oligonucleotides (control probes) and was, therefore, a fold value. Student's t-test was used for the statistical comparisons between any two groups. The microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus Database (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE54070.

The signal threshold value for detection was determined by comparing the results of the drug sensitivity tests and the antibiotic resistance-gene-type values. The antibiotic resistance-gene-type values above the threshold value were considered positive, while results below the threshold value were considered negative; the positive and negative antibiotic resistance-gene-type results in each sample were converted to 1 and 0, respectively, to generate the antibiotic resistance-gene-type profile. The antibiotic resistance-gene-type diversity (Gini-Simpson index) and the principal coordinate analysis (PCoA) of the gene-type profile in each individual were calculated using GENALEX 6<sup>30</sup>. The neighbor-joining tree was constructed using PUPL 4.0 and was based on the antibiotic resistance-gene-type profile.

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## Author contributions

B.Z., Z.M. and Y.H. conceived and designed the project. L.Z., Y.Y., F.L., Y.Z. and Q.D. collected the fecal samples from the volunteers and performed the DNA extractions. N.L. performed the microarray hybridization. Y.H., N.L. and X.Y. performed the data analysis, and Y.H. and N.L. wrote the manuscript. X.W. helped analyze the results. N.L., Y.H. and L.Z. contributed equally to the work. All of the authors read and approved the final manuscript.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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