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Investigation of gastric microbiota in patients with coronary atherosclerosis disease infected by *Helicobacter pylori*

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Abstract: Gastric microbiota may be involved in the pathogenicity of *Helicobacter pylori*(*Hp*). In the present study, 30 male patients with coronary atherosclerosis disease (CAD) infected with *Hp* and 30 healthy male volunteers with *Hp* infection as the control group were detectedby macrogenomic sequencing for gastric microbiota. According to the diversity of gastric microbiota, the CAD group was further divided into two subgroups: CAD treatment (CAD-T) and CAD fellow-up (CAD-F). Shannon index of CAD-T was significantly lower than that of the control group and CAD-F (P<0.05), Simpson index was significantly higher than that of the control group and CAD-F (P<0.05), and there was no statistical difference between CAD-T and the control group and CAD-F patients in Chao1 and ACE index (P>0.05). There is a difference in the dominant flora between the CAD group and the control group. After *Hp* eradication, Shannon index of gastric microbiota increased, Simpson index decreased, and there was statistical difference before and after *Hp* eradication in CAD-T group (P<0.05). There was no significant difference in Chao1 and ACE index between before and after *Hp* eradication (P>0.05). There is a significant difference in the dominant flora before and after eradication in CAD-T group. There were significant differences in clinical manifestations, endoscopic manifestations and pathological results among the three groups (P<0.05). The diversity of gastric microbiota is closely related to the pathogenicity of *Hp,*, regardless of dominant flora.

Keywords: *Helicobacter pylori*, coronary atherosclerosis disease, gastric microbiota, gastroscopic displays.

INTRODUCTION

With the aggravation of aging in China, the incidence of coronary atherosclerosis disease is increasing year by year. At the same time, China is also a large country infected with *Helicobacter pylori* (*Hp*), more than half of the population infected with *Hp*, which leads to more and more coronary atherosclerosis disease patients infected with *Hp* (Jiang *et al*., 2017) . Since the triple plan was established in 1990s, large-scale clinical eradication of *Hp* has brought more and more severe drugs resistance, so that the current radical plan has to be upgraded to quadruple plan, and the course of treatment has also been extended from 7 days to 10-14 days. For example, although Japan has standardized the eradication of *Hp*, the *Hp* resistance brought about by antibiotic exposure makes Japanese have to develop a concomitant therapy to deal with *Hp* infection. At present, the treatment of *Hp* needs a new and breakthrough management.

Helicobacter pylori infection can cause many kinds of gastric diseases, including gastritis, gastric ulcer and gastric cancer, but most of patients have no clinical manifestations. Why there are different clinical outcomes is still unclear. More and more evidences show that the pathogenicity of *Hp* is not only related to *Hp* strains and host immunity, but also to the occurrence of gastric microbiota (Yu Yang, Feng Wang & Dong 2017). As an important part of gastric microecology, gastric microbiota maintains the stability and balance of gastric micro ecology through a variety of regulatory systems and ways (He Yang & Lu, 2016). For patients with coronary atherosclerosis disease infected with *Hp*, because most of them need to take aspirin and other antiplatelet drugs for a long time, it will increase the probability of upper gastrointestinal bleeding (Guo *et al*., 2020), but this situation does not always occur and some patients can tolerate aspirin for a long time. In order to reduce the bias caused by confounding factors, this study investigated gastric microbiota only in male patients with coronary atherosclerosis disease and healthy men with *Hp* infection, and analyzed the influence of gastric flora on prognosis, so as to find a new target for the management of *Hp* infection.

MATERIALS AND METHODS

Clinical data

Inclusion criteria: Patients with *Hp* infected coronary atherosclerosis disease: patients with positive 14C breath test and four items test of UreA, UreB, CagA and VacA of *Hp* serum antibody, regardless of clinical manifestations

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of upper gastrointestinal tract; healthy volunteers with *Hp* infected: No clinical symptoms, positive 14C breath test and four items test of UreA, UreB, CagA and VacA of *Hp* serum antibody, no abnormality or patients with chronic superficial gastritis in gastroscopy; men aged 40-60 who have lived in Liuzhou city of China for more than 10 years; Informed consent to this study and able to insist on participating in the whole follow-up; Clinical manifestations of upper digestive tract: upper abdominal pain, epigastria distention, acid regurgitation, belching, nausea, vomiting, early satiety.

Exclusion criteria: proton pump inhibitor (PPI), H2 receptor antagonists, antibiotics admission in the last 4 weeks; anticoagulant or anti-platelet drugs admission in the last 1 week, coronary atherosclerosis disease patients with abnormal coagulation. Finally, this study included 30 male patients with coronary atherosclerosis disease (CAD group, age 40-60 years old) who had *Hp* infection in our hospital between January 2017 and February 2018 and 30 male healthy volunteers (without symptoms of upper digestive system) who had *Hp* infection in the same period, as the control group, age 40-60 years old.

The diversity of gastric microbiota in the CAD group was quite different, while the diversity in the control group was relatively consistent. According to the diversity of gastric microbiota, the CAD group was further divided into two subgroups. Twenty one patients with CAD had different diversity of gastric microbiota compared with the control group, which was set as CAD treatment (CAD-T) group; nine patients with CAD had similar diversity of gastric microbiota as the control group, which was set as CAD follow-up (CAD-F) group. In CAD-F group, 14 C breath test and gastroscopy were performed at the same time every 6 months, with a total follow-up of 1 year; in CAD-T group, standard quadruple regimen containing bismuth for *Hp* was used for 2 weeks (rabeprazole sodium enteric coated capsule 10mg Bid + amoxicillin potassium clavulanate tablet (4:1) 937.5mg Bid + left oxygen 0.5g QD of fleroxacin capsule and 0.2g Bid of colloidal bismuth pectin capsule. After 4 weeks of eradication, 14C breath test was used to determine whether the eradication was achieved. After that, 14C breath test and gastroscopy were performed at the same time every 6 months for a total follow-up of 1 year.

Follow up termination criteria

If the clinical symptoms are serious or gastrointestinal bleeding occurs, the patient follow-up in CAD-F shall be stopped immediately, and the patients shall be hospitalized or outpatient for treatment.

Clinical evaluation index: Whether there is upper gastrointestinal symptoms. Gastroscopy evaluation index: hyperemia, erosion and ulcer of gastric mucosa. Pathological evaluation index: Whether there is precancerous lesions (chronic atrophic gastritis and intestinal metaplasia/dysplasia).

Instrument and materials

Instrument

Low temperature ultracentrifuges (Sigma Company) and ultralow temperature refrigerators (Thermo Scientific Company of the United States), etc.

Kit

1. *Hp* antibody typing test kit based on immunoblotting technology is provided by Shenzhen Broot Biological Products Co., Ltd. for the detection of *Hp* IgG antibody in patients' serum, including cytotoxic (CagA), vacuolating (VacA), urease subunit A and B antibodies.
2. DNA Extraction Kit: Dneasy Blood and Tissue Kit 250t, Qiagen;

Microbiome detection methods

DNA extraction of bacteria in the stomach: three pieces of gastric mucosa biopsy (one piece of gastric antrum, one piece of gastric angle, one piece of gastric body), immediately put into the sterilized Eppendorf tube, put into the ice box and send to the real laboratory to extract DNA. Strict aseptic operation was carried out during biopsy and DNA was extracted rapidly after biopsy.

Macrogenomic sequencing: take the bacterial 16S rRNA V4 region as the target sequence (primer: 515f: gtgccagcmgccgcggtaa; 806r: ggactachvggtwtctaat), use Illumina miseq PE250 for high-throughput sequencing, and then conduct bioinformatics analysis. The content of information analysis is as follows: tags are clustered into out (operator national taxonomic units), PCA (principal component analysis), Wayne diagram, rank curve and other analysis are carried out for OTU, species annotation is carried out for OTU through database comparison, species profiling histogram, heat map and phylogenetic tree are drawn; alpha diversity analysis is carried out for samples based on the results of OTU clustering analysis. Sequencing and analysis of flora provided by Beijing Genomics Institution (BGI).

Microbiota evaluation index

Diversity analysis of microbial flora: α diversity index of flora includes flora diversity (Shannon and Simpson index) and flora abundance index (Chao1 and ACE). Among them, the larger the Shannon value, the higher the community diversity; the larger the Simpson index value, the lower the community diversity; the larger the Chao1 and ACE values, the more the total number of species.

STATISTICS ANALYSIS

SPSS 22.0 statistical software (Inc., Chicago, IL, USA) was used for statistical analysis. Mean ± standard deviation was used for measurement data, and t-test was

used for two sample measurement data; One-way analysis of variance was used for multiple groups of homogeneous measurement data, Kruskal Wallis test was used for multiple groups of uneven measurement data, and least significant method was used for comparison between the two groups; Chi-square test was used for measurement data and correlation analysis Fisher's exact probability method was used to compare the data of four grid tables with small sample size; $P<0.05$ was statistically significant.

RESULTS

According to the diversity of gastric microbiota, the CAD group was further divided into two subgroups: CAD treatment (CAD-T) and CAD fellow-up (CAD-F).

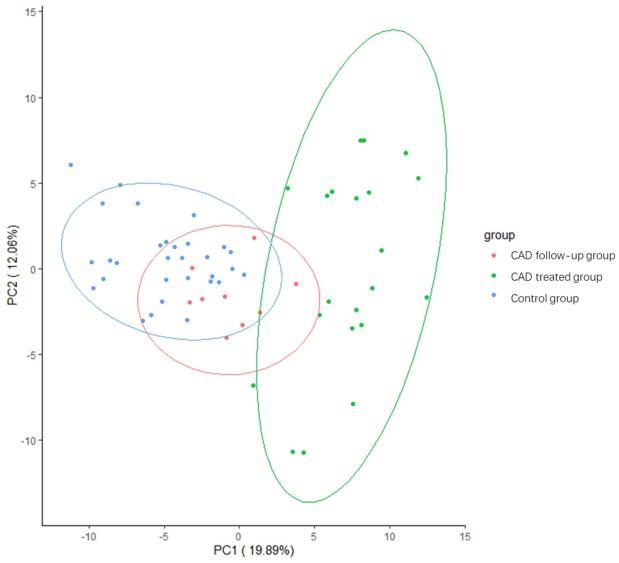

Fig. 1: The β diversity of gastric flora in different patients.

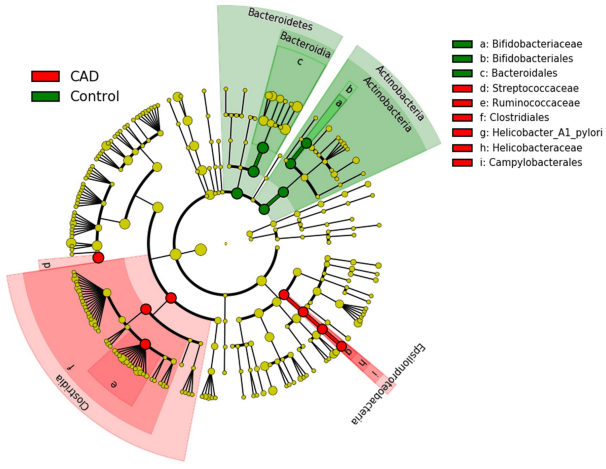

Fig. 2: Cladogram of gastric flora in patients of two groups.

There was no statistical difference in Chao1 and ACE index of gastric flora between CAD-T group and control group and CAD-F patients (P>0.05). The Shannon index of gastric flora in CAD-T group was significantly lower than that in control group and CAD-F and Simpson index was significantly higher than that in control group and CAD-F, with statistical difference ($P<0.05$), as shown in table 1.

Compared with that before treatment, the Shannon index of gastric flora increased and Simpson index decreased after treatment in CAD-T. The difference was statistically significant ($P<0.05$). There was no statistical difference between the indexes of Chao1 and ACE ($P>0.05$), as shown in table 2.

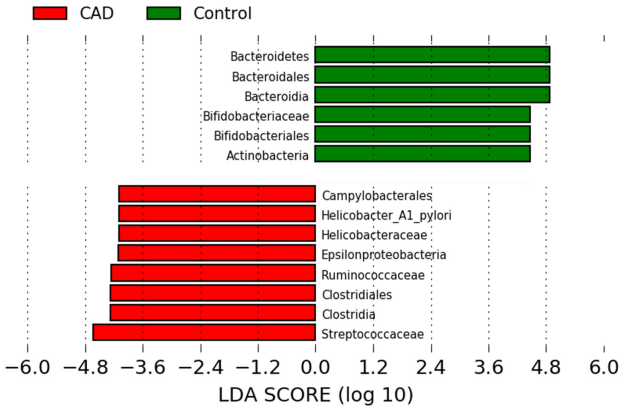

Fig. 3: LDA value distribution histogram of gastric flora in patients of two groups.

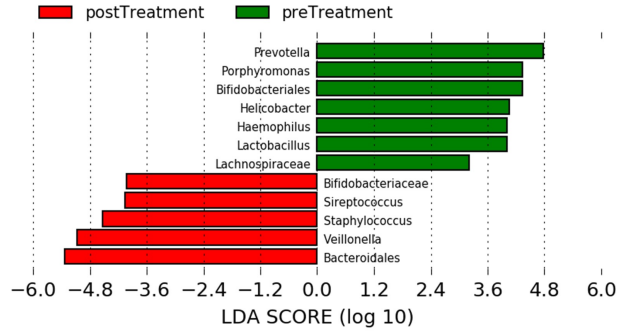

Fig. 4: LEfSe analysis of gastric flora between pre-treatment and post-treatment in CAD-T group.

The β diversity of gastric flora in CAD group is divided into CAD-T and CAD-F. The β diversity of gastric flora in CAD-F group is similar to that in control group.

The results of macro genomic sequencing showed that *streptococcaceae*, *ruminococcaceae*, *clostridiales*, *helicobacter pylori*, *helicobacteraceae* and *campylobacter ales* were the dominant bacteria in the stomach of CAD group and *bifidobacteriaceae*, *bifidobacter ales* and *bacteroidales* were the dominant bacteria in the stomach of control group, as shown in fig. 2.

Lefse analysis showed that *streptococcaceae*, *clostridia*, *clostridiales*, *ruminococcaceae*, *ε-proteobateria*,

Table 1: Comparison of α diversity index of gastric flora between two groups

Group	Diversity of flora		Abundance of bacteria	
	Shannon index	Simpson index	Chao1 index	ACE index
CAD treatment group (*n*=21)	1.41±0.39	0.37±0.14	473.66±65.38	481.22±68.34
CAD follow-up group (*n*=9)	1.89±0.43#	0.25±0.09#	481.45±78.36	499.33±76.59
Control group (*n*=30)	2.06±0.55#	0.22±0.07#	497.41±91.23	504.40±82.73
F	5.280	5.249	1.159	1.183
P	0.000	0.000	0.251	0.242

Note: Comparison with CAD treatment group, #*P*<0.05.

Table 2: Comparison of α diversity index of gastric flora in CAD treatment group before and after eradication (*n*=21).

Group	Diversity of flora		Abundance of bacteria	
	Shannon index	Simpson index	Chao1 index	ACE index
Before eradication	1.41±0.39	0.37±0.14	473.66±65.38	481.22±68.34
After eradication	2.04±0.37	0.22±0.09	492.77±54.82	509.95±61.27
t	5.370	4.130	1.026	1.434
P	0.000	0.000	0.311	0.159

Table 3: Correlation analysis between gastric microflora, clinical manifestations, endoscopic manifestations and pathological results in each group before treatment

Gastric microflora	Clinical manifestations (no / yes)	Endoscopic findings (congestion / erosion / ulcer)	Precancerous lesions (no / yes)
CAD-T (21)	5/16	2/17/2	12/9
CAD-F (9)	8/1	8/1/0	9/0
Control (30)	30/0	28/2/0	30/0
x^2	36.861	8.576	19.664
P	0.000	0.000	0.000

Table 4: Comparison of clinical manifestations between two subgroups of CAD group before and after follow-up

Mucous morphology under gastroscopy	Before follow-up				At the last follow-up			
	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*
Without clinical manifestation in upper digestive tract	8	5	-	0.004	8	19	-	>0.05
With clinical manifestation in upper digestive tract	1	16			1	2		

Table 5: Comparison of gastroscopy results between two subgroups of CAD group before and after follow-up.

Mucous morphology under gastroscopy	Before follow-up				At the last follow-up			
	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*
Congestion	8	2	3.039	0.014	9	20	0.683	0.711
Erosion	1	17			0	1		
Ulcer	0	2			0	0		

helicobacteraceae, *helicobacter pylori*, *campylobacter ales* were the main advantages in the stomach of patients in CAD group, while *bacteroidetes*, *bacteroidales*, *bacteroidia*, *bifidobacteriaceae*, *bifidobacterales* and *actinobacteria* were the main advantages in the control group (LDA >log10), as shown in fig. 3.

There were statistical differences in clinical manifestations, endoscopic displays and pathological results among the three groups (*P*<0.05).

The patients with clinical symptoms of CAD-T were significantly higher than CAD-F and control, and the patients with mucosal erosion and ulcer under were also higher than CAD-F and control. The patients with precancerous lesions were also higher than CAD-F and control, as shown in table 3.

The results showed that there were statistical differences in clinical manifestations, macroscopic displays and pathological results between CAD-T and CAD-F (*P*<

Table 6: Comparison of pathological results between two subgroups of CAD group before and after follow-up

Mucous morphology under gastroscopy	Before follow-up				At the last follow-up			
	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*	Follow-up group (*n*=9)	Treatment group (*n*=21)	x^2	*P*
Without local intestinal metaplasia and atrophy	9	12	-	<0.05	9	19	-	>0.05
With local intestinal metaplasia and atrophy	0	9			0	2		

0.05); there was no statistical difference in after treatment (*P*>0.05), as shown in table 4-6.

Lefse analysis showed that *prevotella, porphyromonas, bifidobacterales, helicobacter, haemopyilus, lactobacillus* and *lachnospiraceae* were the main advantages in the stomach of patients before treatment, while *bifidobacteriaceae*, *streptococcus*, *staphylococcus, veilonella* and *bacteroidales* were the main advantages after treatment (LDA >log10), as shown in fig. 4.

DISCUSSION

People always thought that the high acid environment in the stomach made it impossible for microorganisms to colonize until *Hp* was found, which broke the traditional concept. In recent years, with the development of high-throughput gene sequencing technology, more and more microbial communities in the stomach have been found. In addition to *Hp*, there are hundreds of bacteria, mainly including *proteobacteria*, *firmicutes*, *actinobacteria*, *bacteroidetes*, *fusobacteria* and *gemmatimonadetes*. In addition, more and more evidences show that *Hp* infection can changes the microbial flora through influencing the biological environment and mucosa immune of the stomach. But *Hp* is not the absolute cause of many diseases in the stomach. The developments of gastric mucosal diseases are affected by the host immune, the gastric microecology and other factors (Schulz Schutte & Malfertheiner, 2016). There is an interactive relationship between *Hp* and other microorganisms in the stomach. They compete with each other and maintain a dynamic balance in the healthy population. Once the structure of gastric microbiota changes, this balance will be broken, which will promote various gastric diseases, such as gastric cancer (Wang *et al*., 2018). Therefore, how does the change of gastric microbiota affect the pathogenicity of *Hp*? The solution of the above problems will help to regulate the gastric microbiota to treat *Hp* infection, and will also bring new solutions for the management of *Hp* infection.

A foreign autopsy report pointed out that, the incidence of coronary atherosclerosis disease in patients with peptic ulcer aged 40-50 years was 2.5 times higher than that in patients without ulcer (Wang *et al*., 2018). In recent years, a number of studies have shown that (Sung *et al*., 2016), *Hp* infection and coronary atherosclerosis disease have a significant correlation, mainly reflected in *Hp* can promote inflammatory response *in vivo* and lead to dysfunction of coronary artery endothelial cells; *Hp* can lead to abnormal oxidative stress response *in vivo,* thus increasing the incidence of cardiovascular events (Muhammad Zaidi, Saeed & Ishaq, 2017). In addition, as we all know, the vast majority of patients with coronary atherosclerosis disease need long-term oral aspirin and this drug itself has certain damage to the gastric mucosa. Some studies have pointed out that (Schulz, Koch, Schutte, Pieper & Malfertheiner, 2015) the invasion of aspirin on the gastric mucosa, further increased the possibility of *Hp* reproduction in the stomach, the interaction between the two, formed a vicious circle and then increased the rate of bleeding. In clinical practice, only a small part of the population with coronary atherosclerosis disease will have gastric ulcer and bleeding, but how to screening this part of the population and effectively eradicate *Hp* treatment is still a clinical problem. This study found that the pathogenicity of *Hp* increased when the diversity of gastric microbiota decreased in patients with coronary atherosclerosis disease. It is speculated that long-term use of aspirin and other drugs to treat coronary atherosclerosis disease may affect the diversity of gastric microbiota and lead to the dysbiosis of gastric flora. In clinical practice, it is possible to increase the diversity of gastric microbiota by adding suitable dominant flora, so as to achieve the purpose of inhibiting *Hp* pathogenicity. After all, long-term use of PPI drugs will bring many side effects, such as intestinal micro biota dysbiosis, gastric gland hyperplasia. But in the specific treatment, it is necessary to achieve the accurate sequencing of the gastric microbiota, and accurately supplement the strains (Nakae, Tsuda, Matsuoka Mine, & Koga, 2016).

After *Hp* eradication in patients with coronary atherosclerosis disease, the gastric microbiota reached a new balance, the diversity of flora increased, but the dominant flora was still different from that of healthy volunteers. In the follow-up group of coronary atherosclerosis disease and the control group, the gastric microbiota was balanced, and the dominant flora of the two groups was also different. This result suggests that the dominant flora may be different in different population due to the influence of diet, medicine and environment. Different studies on the balance of gastric microbita have different dominant flora (Gantuya *et al*.,

2019; Guo *et al*., 2019; Zhou & Fang, 2018). But one thing is the same, the diversity of gastric microbiota is high. The diversity of gastric microbiota may be the key to the balance of gastric microbiota. Specific to a certain group of people, what kind of bacteria should be used to regulate the microbiota in the stomach or adjust the microbiota in the stomach with drugs and diet, it is necessary to accurately sequence, accurately supplement the bacteria or drugs needed and change the diet. Although some people do not have *Hp* infection, due to improper diet and drug effects, they will still have symptoms of upper gastrointestinal tract and severe inflammation of gastric mucosa, which may be related to the dysbiosis of gastric microbiota (He *et al.,* 2018; Zhang *et al*., 2019).

The causal relationship between the dysbiosis of gastric microbiota and *Hp* colonization in the stomach can produce many toxic factors related to inflammation and immune deficiency, and activate immune response in many ways(Guo *et al*., 2019). Through synergistic action, it can cause local inflammatory damage, which is manifested in edema, congestion, exudation and microcirculation disorders in the mucosa, and can prevent normal bacterial colonization in the stomach, which is mainly reflected in the number of other bacterial colonization decreased, which broke the microecological balance in the stomach, resulting in symptoms of upper gastrointestinal tract and damage of gastric mucosa. Therefore, the diversity of microbiota in the stomach decreased, the clinical symptoms of upper gastrointestinal tract were obvious, and the inflammation of gastric mucosa was serious. However, *Hp* infection does not always lead to the dysbiosis of gastric microbiota, such as the balance of gastric microbiota in the control group. In addition to *Hp* infection, there are also improper diet, bad living habits and drugs (Tyma, Epstein, Whitfield-Cargile, Cohen, & Giguere, 2019). *Hp* infection may increase the possibility of gastric microbiota dysbiosis and may aggravate this process. However, if we keep a good diet and living habits and do not take harmful drugs, the pathogenicity of *Hp* will be significantly reduced. It is also an evidence that clinical symptoms rarely appear in adolescents infected with *Hp*. Teenagers often study and live in school, have parents' supervision, can maintain good living and eating habits, and have no basic diseases, and do not need to take drugs (Llorca *et al*., 2017).

CONCLUSION

To sum up, the diversity of gastric flora is closely related to the pathogenicity of *Hp,* regardless of dominant flora.

Ethics approval and consent to participate

The research was conducted after obtaining an ethical clearance letter from Ethical Review Committee of Liuzhou People's Hospital. A permission letter was obtained from Liuzhou People's Hospital. Written informed consent was not applicable.

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