Comparative study on the mutation spectrum of tissue DNA and blood ctDNA in patients with non-small cell lung cancer

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Background: To study the mutations detected in tissue DNA and blood circulating tumor DNA (ctDNA) of patients with advanced lung cancer and analyze the correlations between gene mutations, clinical features, and treatment.

Methods: Targeted next-generation sequencing (NGS) technology based on probe hybrid capture and Illumina high-throughput sequencing was used to detect the DNA of tumor tissue samples (n=24) and blood samples (n=15) of ctDNA from 28 lung cancer patients with a clear pathological diagnosis. For mutations, the detection range included 4 types of mutations (point mutations, small indels, copy number variations) in all exon regions and partial intron regions of the 556 genes panel.

Results: The most frequently detected mutant genes in 24 lung cancer tissue samples were TP53 (58.3%, 14/24), EGFR (33.3%, 8/24), LRP1B (25.0%, 6/24), KRAS (20.8%, 5/24), and ARID1A (16.7%, 4/24). The common mutant genes detected in 15 ctDNA samples were TP53 (60%, 5/15), EGFR (33.3%, 5/15), LRP1B (20.0%, 3/15), ERBB4 (3/15), and ARID1A (13.3%, 2/15). Among the 28 patients, 11 patients underwent both tissue DNA and ctDNA detection. The average co-mutation frequency of paired tissue DNA with ctDNA was 38.9% (0–83.3%), and the median value was 37.5%.

Conclusions: Tissue DNA and ctDNA samples could detect genetic mutations and be consistent. Therefore, ctDNA detection as a method for disease diagnosis and evaluation of tumor molecular status may be helpful for the clinical diagnosis and treatment of lung cancer patients.

Keywords: Non-small cell lung cancer (NSCLC); circulating tumor DNA (ctDNA); next-generation sequencing (NGS); epidermal growth factor receptor (EGFR)

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Introduction

Lung cancer is a malignant tumor with the highest mortality and second highest morbidity worldwide. In 2020, there were 2.2 million new cases of lung cancer worldwide and 1.8 million deaths (1). Non-small cell lung cancer (NSCLC) treatment methods include surgical resection, comprehensive adjuvant treatment, neoadjuvant radiotherapy, and chemotherapy. Surgery and puncture tissue samples to detect gene mutations are still the gold standard for diagnosing whether NSCLC patients are suitable for targeted therapy (2). The sooner the patients receive treatment, the better the treatment effect. However, most NSCLC patients are at the middle or advanced stage.
when the diagnosis is confirmed, losing the opportunity for surgical treatment. Traditional treatment uses platinum-containing dual-agent chemotherapy as the first-line treatment. The National Comprehensive Cancer Network (NCCN) treatment guidelines recommend that patients with advanced NSCLC take comprehensive treatment based on systemic therapy (3). Although chemotherapy has made significant progress, the overall therapeutic effect of NSCLC is still not satisfactory, and its 5-year survival rate remains low.

There are two types of mutations in the tumor genome: “passenger mutations”, which have little effect on the development of tumors, gradually disappear passively as the disease progresses. “Driver mutation” is another type of mutation that is crucial to oncogenesis and tumor progression. Cancer cells carrying driver mutations aberrantly activate/inhibit key signaling pathways and have selective advantages in survival or proliferation (4). Since tumors rely on driver mutations to maintain the malignant phenotype, it has also become a hot spot in current tumor treatment research. Recent studies have shown that NSCLC has a significant connection with gene mutations. The commonly NSCLC driver genes include epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), Kirsten rat sarcoma viral oncogene (KRAS), phosphatidylinositol 3-kinase catalytic subunit α (PIK3CA), and mesenchymal to epithelial transition factor (MET), which mutated may directly “drive” the oncogenesis and tumor progression of NSCLC (5-7). Currently, EGFR-TKIs are widely used in the treatment regimen of patients with EGFR-mutant NSCLC while benefiting patients (8).

The diagnosis of NSCLC mainly relies on pathological biopsy (9). Although this traumatic examination method can directly observe pathological cell changes, it has poor reproducibility and is not suitable for post-treatment monitoring. Many patients lose the opportunity for targeted therapy due to the inability to obtain tissue specimens or too few tissue specimens are obtained for molecular type testing. At the same time, due to the existence of tumor heterogeneity, mutations of different tumor sites and even different tumor cells at the same tumor site may be different (10), and the genetic testing of a single tissue biopsy cannot accurately reflect the overall characteristics of the tumor (11). With the evolution of tumors caused by treatment selection pressure and the emergence of drug-resistant genes, relying only on pre-treatment specimens to guide subsequent treatment may lead to treatment bias (12).

cDNA comes directly from the tumor or from circulating tumor cells and usually carries the gene mutation information of the tumor tissue. In recent years, cDNA-based liquid biopsy has provided new opportunities for molecular diagnosis and monitoring of tumors. cDNA gene mutation detection can be used for early cancer screening and real-time monitoring of cancer development, metastasis, and prognosis (13). Circulating tumor DNA (cDNA) is a tumor-specific gene carried by apoptotic tumor cells and is released into the blood, which can fully reflect the molecular composition of tumor tissue. Lebofsky et al. confirmed that 97% of mutation genes in metastasis biopsies could be found in cDNA of tumor-matched samples (14). Guo et al. revealed that NGS detection results of tumor tissue samples and cDNA samples are not entirely consistent. The concordance was 54.6% in patients with early-stage NSCLC and 80% in patients with advanced NSCLC, so indifferent patients, the intrinsic biological Mechanisms may affect the consistency of tumor biopsies and cDNA genomic profiles (15). Therefore, this study intends to use NGS panel testing to detect tissue samples and cDNA samples to analyze the mutation type and mutation frequency of differential genes and differences in the enrichment of biological functions of mutant genes. NSCLC patients with EGFR sensitive mutations respond well to EGFR-tyrosine kinase inhibitor (TKI) treatment, but most patients develop resistance 9–13 months after receiving treatment (16). With the emergence of new targeted drugs and the in-depth study of drug resistance mechanisms, a more comprehensive understanding of the composition of patients’ oncogenes is needed to guide treatment and evaluation.

This study analyzed the detection results of gene mutations in tissue samples and blood samples of patients with advanced NSCLC, explored the relationship between cDNA detection and prognostic judgment, and performed curative effect evaluation and recurrence monitoring of NSCLC. We present the following article in accordance with the MDAR reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-22-970/rc).

Methods
Patient enrollment

The cohort included 28 NSCLC patients from Guangzhou First People's Hospital. Among them, 11 patients underwent complete tumor staging in accordance with the seventh
edition of the NSCLC tumor, lymph node, and metastasis (TNM) standards. Tissue samples were obtained through prostate puncture or radical prostate cancer surgery, then tissues were paraffin-embedded, sliced, and submitted for examination. Blood samples were collected from the patient’s vein and stored in an EDTA anticoagulant tube for cryopreservation, then submitted for inspection. All samples submitted for inspection met the quality control requirements of gene sequencing and were sequenced for mutation analysis. This study has been approved by the relevant supervision and independent ethics committee of Guangzhou First People’s Hospital (No. K-2020-051-01). Every patient provided written informed consent for use of their samples. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Next-generation sequencing (NGS) and annotation

DNA was isolated from the FFPE samples and blood samples using the DNeasy Blood and Tissue Kit (69504, QIAGEN, Venlo, Netherlands). Multi-gene panel targeted NGS including 556 genes was performed on Ion Torrent (Tongshu BioTech, Shanghai, China). The targeted libraries were constructed using NGS Fast DNA Library Prep Set (Thermo Fisher, Waltham, MA, USA). BWA (Burrows-Wheeler-Alignment) software was used to compare the sequencing results. GATK (The Genome Analysis Toolkit) was used to correct the comparison quality. Mutect2 and VarDict software were used to detect somatic mutation sites from the comparison results, and the intersection of mutation sites detected by the software was used as the candidate somatic mutation sites. For the detected candidate somatic mutation sites, ANNOVAR software was used to annotate the population frequency in the ExAC (Exome Aggregation Consortium) and ESP (NHLBI Exome Sequencing Project) databases and the functional information of mutation function and mutation type. In order to further improve the credibility of mutations, we removed mutants with low support for reads according to the sequencing depth of the loci. In addition, mutation sites with a population frequency greater than 0.01 were also removed, and the remaining mutations were used as mutation sites with high confidence for subsequent analysis.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis

Oncoplot mutations were plotted for each sample and different groupings using the MAfTools R package. Mutations in cancer-related driver genes were also analyzed. The clusterProfiler R package was also used for GO and KEGG functional enrichment analysis of mutated genes in each grouping sample. In order to analyze the overlap between samples of different groups, the Venn diagram R package was also used to draw Venn diagrams between each group of mutations.

Statistical analysis

GraphPad Prism 8.0 software was used for statistical analysis. The t-test was used for continuous variables, while the chi-square test or Fisher’s exact test was used for categorical variables. Two-sided tests were used, and P<0.05 was considered statistically significant.

Results

Patient characteristics

From May 2018 to Dec 2020, we recruited 28 patients diagnosed with advanced or metastatic NSCLC and collected 39 samples (24 tumor tissues and 15 blood samples) from the patients. In addition, paired tissue samples and blood samples were tested in 11 patients. The median age of the 11 patients was 64 years (55–77 years). Six cases were females, and 5 cases were males. Four patients had a history of smoking, while 7 did not smoke. Nine patients were diagnosed with adenocarcinoma and 2 patients with squamous cell carcinoma. Three patients accepted EGFR-TKI treatment, and 2 patients accepted chemotherapy. There was a lack of treatment information for the other patients. Table 1 summarizes the clinical characteristics of the cohort.

Comparison of the mutational profiles of tissue DNA and ctDNA

We analyzed a total of 39 mutation profiles of tumor tissues and ctDNA samples through NGS detection. Overall, 145 genes were found to have mutations in tumor tissues and ctDNA samples, including 28 deletions (DELs), 7 insertions (INSs), and 270 single nucleotide variants (SNVs). The top 3 mutant genes were TP53 (59.0%, 23/39), EGFR (33.3%, 13/39), and LRP1B (23.1%, 9/39) (Figure 1A). A total of 114 genes were found to have mutations in tissue samples, including 9 Frame_Shift_Del, 2 Frame_Shift_Ins,
In_Frame_Del, 1 In_Frame_Ins, 157 missense mutations, 16 nonsense mutations, and 1 Splice_Site (Table S1). The common mutated genes were TP53 (58.3%, 14/24), EGFR (33.3%, 8/24), LRP1B (25.0%, 6/24), KRAS (20.8%, 5/24), and ARID1A (16.7%, 4/24) (Figure 1B). A total of 78 genes were found to have mutations in ctDNA samples, including 4 Frame_Shift_Del, 2 Frame_Shift_Ins, 5 In_Frame_Del, 1 In_Frame_Ins, 85 missense mutations, 13 nonsense mutations, and 2 Splice_Site (Table S2). The top 3 mutant genes were TP53 (56%, 15/27), EGFR (33.3%, 9/27), LRP1B (20.0%, 5/27), ERBB4 (3/27), and ARID1A (13.3%, 3/27) (Figure 1C).

### The relationship between tissue DNA and ctDNA in NSCLC patients

Among the 28 patients, 11 patients underwent both tissue sample DNA testing and blood ctDNA testing. As shown in Table 2, there were 100 mutated genes detected in tissue and ctDNA samples, of which 39% were co-mutated genes (39%, 39/100). Among them, 72 mutated genes were detected in tissue samples, and 33 (45.8%, 33/72) mutated genes were ctDNA-specific. The ctDNA samples detected mutations in 67 genes, and 28 (41.8%, 28/67) mutated genes were ctDNA-specific. In addition, 39 genes were co-mutated in both samples, tissue samples accounted for 54.2% (39/72), and ctDNA samples accounted for 58.2% (39/67). The average co-mutation frequency of paired tissue DNA with ctDNA was 40.1% (0–83.3%), and the median value was 37.5%. The average accuracy of paired tissue DNA with ctDNA detection was 65.26% (0–100%), and the median value was 90.91% (Table 3).

Next, we performed GO and KEGG analysis to assess the mutated genes in tissue and ctDNA samples. This study found that for the co-enrichment of tissue and ctDNA samples, the GO terms of cellular component (CC) were cellular component, nuclear chromosome part, chromatin, nuclear chromatin, transcription factor complex, and chromosome/telomeric region (Figure 2A,2B). The GO terms of biological process (BP) were gland development, phosphatidylinositol 3-kinase signaling, inositol lipid-mediated signaling, regulation of protein kinase B signaling, phosphatidylinositols-mediated signaling, and protein kinase B signaling (Figure 2C,2D). The GO terms of molecular function (MF) were protein tyrosine kinase activity, hormone receptor binding, transmembrane receptor protein tyrosine kinase

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**Table 1** Summary of patients’ clinical information

| No. | Gender | Age (years) | Smoking | Clinical stage | Treatment | Therapeutic evaluation | Metastasis | Pathology |
|-----|--------|-------------|---------|----------------|-----------|-----------------------|------------|-----------|
| Pt.1 | Male   | 64          | Yes     | IVB            | –         | –                     | Lymph nodes, bone, kidney, pleura | Squamous carcinoma |
| Pt.2 | Female | 60          | None    | IVA            | –         | –                     | Pleura     | Adenocarcinoma |
| Pt.3 | Male   | 58          | Yes     | IVB            | –         | –                     | Lymph nodes, pleura, pelvic cavity | Adenocarcinoma |
| Pt.4 | Female | 55          | None    | IVB            | –         | –                     | Lymph nodes, bone | Adenocarcinoma |
| Pt.5 | Female | 77          | None    | IVB            | EGFR-TKI  | PR                    | Lymph nodes, bone, liver | Adenocarcinoma |
| Pt.6 | Male   | 75          | None    | IVB            | EGFR-TKI  | PR                    | Lymph nodes, bone, kidney, brain | Adenocarcinoma |
| Pt.7 | Female | 60          | None    | IVB            | Chemotherapy | SD               | Lymph nodes, bone, kidney, liver | Adenocarcinoma |
| Pt.8 | Female | 72          | None    | IVA            | EGFR-TKI  | PR                    | Lymph nodes, pleura | Adenocarcinoma |
| Pt.9 | Female | 67          | None    | IVA            | Chemotherapy, Tislelizumab | PR       | Lymph nodes, pleura | Squamous carcinoma |
| Pt.10| Male   | 69          | Yes     | IVA            | –         | –                     | Lymph nodes | Adenocarcinoma |
| Pt.11| Male   | 60          | Yes     | IVA            | –         | –                     | Nodes, pleura | Adenocarcinoma |

EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; PR, partial response; SD, stable disease.
Figure 1 Differences in mutation profiles of tissue DNA and ctDNA. (A) Oncoprint of all samples gene alterations; (B) oncoprint of tissues samples gene alterations; (C) oncoprint of ctDNA samples gene alterations. ctDNA, circulating tumor DNA.

Table 2 Differences in genetic mutations in tissue and blood samples from patients.

| Samples          | Mutation genes                                                                 | Total (n=100) |
|------------------|--------------------------------------------------------------------------------|---------------|
| Tissue           | ERCC4, FLT4, ATRX, HIST1H3C, MAP3K13, FOXL2, LAT51, MCL1, KMT2C, PTPRT, EP300,  |
|                  | CARD11, SDHA, NRAS, PTPRS, WWTr1, ERCC3, ROS1, SHOC2, MSH3, KMT2D, SDHAf2,     |
|                  | MET, PREX2, ATR, CIC, PIK3CG, PIK3CA, FGf10, SGK1, KLF4, SNCAIP, FAT1          | 33            |
| ctDNA            | ARID2, CYSLTR2, HOXB13, STAG2, FOXP1, LTK, SUZ12, KMT2B, PTPN11, FANCL, TNFRSF14, |
|                  | ELF3, PIK3CG, STAT5A, SPEN, RECQL4, PRKD1, SRC, SF3B1, TSHR, CDKN1A, NOTCH3,     |
|                  | ABL1, ALK, RASA1, AR, ERBB2, KDM6A                                               | 28            |
| Tissue and       | EPHA5, BRCA1, PRKCI, ERBB4, E2F3, KRAS, EPHA7, BRAF, IRS2, TCF3, LRP1B, TERT,  |
| ctDNA            | KDR, HGF, TPS3, ZFHX3, PTEN, ASXL1, RET, ARID1A, BCL6, STK11, NF1, RAF1, CTNNB1, |
|                  | AMER1, ETV6, EGFR, FGf12, MYC, MGA, AGO2, BCOR, KEAP1, NCO1, BRCA2, POLD1, PARP1, |
|                  | ARID1B                                                                           | 39            |

ctDNA, circulating tumor DNA.
activity, transmembrane receptor protein kinase activity, nuclear hormone receptor binding, and steroid hormone receptor binding (Figure 2E,2F). In addition, the KEGG pathway enrichment analysis showed that the mutated gene functions in tissue and ctDNA were mainly enriched in hepatocellular carcinoma, NSCLC, breast cancer, prostate cancer, endometrial cancer, and tumor-associated miRNA (Figure 2G,2H).

As showing Table 1, the follow-up treatment information was available for 5 of the 11 patients who had both tissue and ctDNA samples tested, including 3 who received EGFR-TKI therapy (all patients evaluated as partial response) and 2 who received chemotherapy (one evaluated as stable and the other as partial response). EGFR mutations were found in tissue samples and ctDNA samples from patients treated with EGFR-TKIs. In contrast, tissue samples and ctDNA samples from chemotherapy patients did not have EGFR mutations.

### Discussion

We performed NGS testing on 28 patients with lung cancer, including 24 tissue samples and 15 blood samples. In addition, 11 of the 28 patients received tissue sample testing and ctDNA sample testing. The analysis of the sequencing results revealed some crucial questions. For example, ctDNA testing found that patients with lung cancer carry tumor-related gene mutations, such as TP53, KRAS, and EGFR mutations. This detection has important clinical significance, suggesting that ctDNA mutation detection can be used as a non-invasive early detection method and can be applied to screening high-risk populations. In the process of cancer screening and early diagnosis, the combination of the quantitative level of ctDNA in circulation and the identification of ctDNA gene mutations can provide valuable information for cancer diagnosis. Existing studies indicate that gene mutations are tumor-specific and detection of mutations in local tumor tissues is often insufficient (17,18). Assessing these changes in ctDNA can provide greater diagnostic accuracy than standard protein biomarkers such as carcinoembryonic antigen (CEA) (19,20).

ctDNA from tumors can represent the entire genome pattern of tumors to a certain extent and can be used as a liquid biopsy to analyze tumor-specific genetic and epigenetic changes, including oncogene or tumor suppressor gene mutations, methylation, and gene amplification, among others. As an effective and reliable biomarker in the past few years, ctDNA has played a vital role in classifying specific patients, guiding early clinical diagnosis and treatment, and for prognostic evaluation. In addition, using ctDNA biomarkers to detect epigenetic abnormalities in specific types of cancers early, such as hypomethylation of promoters in oncogenes or tumor suppressor genes, can provide critical information for tumor biology research and clinical treatment. Several studies have shown that TP53, ITH, HCK, and TNNB1 are common in peripheral blood ctDNA in patients with liver cancer (21-24). Specific
Figure 2  Functional enrichment analysis of mutation genes in tissue samples and ctDNA samples. (A) Cellular component enrichment results of tissue samples; (B) cellular component enrichment results of ctDNA samples; (C) biological process enrichment results of tissue samples; (D) biological process enrichment results of ctDNA samples; (E) molecular function enrichment results of tissue samples; (F) molecular function enrichment results of ctDNA samples; (G) KEGG pathway enrichment results of tissue samples; (H) KEGG pathway enrichment results of ctDNA samples. The x-axis shows the ratio number of genes, and the y-axis shows the pathway terms. The −log10 value of each term is colored according to the legend. ctDNA, circulating tumor DNA; KEGG, Kyoto Encyclopedia of Genes and Genomes.
mutations suggest that peripheral blood ctDNA can be detected for the early diagnosis of cancerous hepatocytes. Gormally et al. reported that plasma ctDNA could detect KRAS gene mutations in healthy subjects as early as 2 years in advance and could detect the mutation of TP53 in healthy patients 20.8 months before cancer diagnosis (25). Although the plasma of advanced patients with early tumors or tumor micrometastasis contains only a relatively low abundance of ctDNA fragments, quantitative and qualitative analysis of trace amounts of DNA has become possible with the continuous advancement of DNA detection technology (26).

The prognosis of cancer patients is usually evaluated by combining clinical observation, tumor progression stage, histopathological changes, and biomolecular features. When tissue sections are challenging to obtain, such as non-solid tumors, or it is difficult to use preserved tissue sections for genetic analysis, the importance of using patient blood to detect ctDNA is revealed. In addition, due to tumor heterogeneity, ctDNA detection can reflect the mutation information of patients more comprehensively, while tissue DNA detection often reflects the mutation information of a single nodule. ctDNA detection can dynamically detect the prognosis of patients during treatment and support timely adjustment of patient treatment regimens. Due to the advantage of ctDNA detection. Due to the potential benefits of ctDNA detection, many studies reveal the feasibility of ctDNA detection as a tumor liquid biopsy. The study of Bettegowda et al. showed that ctDNA could be detected in more than 75% of NSCLC, breast cancer, ovarian cancer, pancreatic cancer, colorectal cancer, liver cancer, and head and neck cancer (27). Studies have shown that ctDNA can reveal a series of molecular biological characteristics related to tumors, such as RAS gene and TP53 gene mutations, APC gene hypermethylation, loss of allelic heterozygosity (LOH), and mtDNA changes (28-30). About one-third of NSCLC patients have KRAS gene mutations. Gautschi et al. studied 180 cases of lung cancer patients with ctDNA and found that KRAS mutations are associated with poor prognosis (31). Parkinson et al. analyzed the mutations of TP53 gene ctDNA in 40 patients with malignant, recurrent, high-grade serous ovarian cancer (32). They showed that after 1 course of chemotherapy, patients whose TP53 mutation allele scores decreased >60% had a more prolonged disease-relapse survival time. Morikawa et al. tested plasma PIK3CA and KRAS gene ctDNA in patients with ovarian clear cell carcinoma and showed that patients with high PIK3CA-H1047R or KRAS-G12D ctDNA had a shorter survival time (33). A series of studies have shown that the plasma ctDNA of patients with pancreatic cancer contains mutations in the KRAS gene. Mutations in the KRAS gene can monitor the development of pancreatic cancer (34,35). Dabritz et al. detected 56 cases of pancreatic ductal adenocarcinoma and 13 cases of pancreatitis with plasma ctDNA mutations in the KRAS gene (36). Among them, found KRAS mutation in 20 patients with pancreatic duct adenocarcinoma, but no KRAS mutation in patients with pancreatitis. In a study of colorectal cancer, Trojan et al. compared KRAS gene mutation and CDKN2A promoter hypermethylation in the blood ctDNA of 37 patients with colorectal cancer. They found that the KRAS gene had mutations or CDKN2A promoters. Patients with hypermethylation had a 2-year survival rate of 48%, while those without changes had a 2-year survival rate of 100% (37).

However, one limitation of ctDNA detection is that the low abundance of ctDNA fragments in blood makes its detection sensitivity challenging. Another, the results of ctDNA assays may be interfered with by clonal hematopoietic and germline mutations. Despite this, ctDNA detection has a good application prospect in the clinical diagnosis and treatment of tumors. For liquid biopsy to detect whether patients contain ctDNA and gene mutations is conducive to an early screening of patients and timely early intervention. For localized cancers, ctDNA testing can help doctors improve patient treatment plans. At the same time, during the treatment process, ctDNA detection can dynamically monitor tumor burden and curative effect. After treatment, blood ctDNA content and gene mutation can still be detected in real-time to monitor cancer recurrence and metastasis.

Conclusions

This study sequenced tissue and ctDNA samples from 28 patients with advanced NSCLC by targeted NGS. ctDNA detection was consistent with tumor tissue detection to a certain extent. In addition, patients with EGFR mutations detected in both tissue samples and ctDNA samples achieved partial remission after EGFR-TKI treatment. ctDNA detection in patients as a method of disease diagnosis and evaluation of tumor molecular status is advantageous in clinical diagnosis and treatment.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study has been approved by the relevant supervision and independent ethics committee of Guangzhou First People's Hospital (No. K-2020-051-01). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Written informed consent was obtained from all study participants.

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