

Value of exhaled hydrogen sulfide in early diagnosis of esophagogastric junction adenocarcinoma

FANG LIU¹, LAI WEI², BOSHENG ZHENG², XIN SU², JIANMEI JU², GUANGJIE LIU² and QINGYI LIU²

Departments of ¹Hospital Quality and Control, and ²Thoracic Surgery, The Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei 050001, P.R. China

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Abstract. Esophagogastric junction adenocarcinoma (EJA) has increased in recent years, and it exhibits a poor prognosis and a short survival period for patients. Hydrogen sulfide (H₂S) plays an important role in the pathogenesis of cancer and has been studied as a diagnostic factor in some tumor diseases. However, few studies have explored the diagnostic value of H₂S for EJA. In the present study, a total of 56 patients with early-stage EJA were enrolled while 57 healthy individuals were selected as the healthy control group. Clinical features were recorded, and exhaled H₂S and blood samples were collected from both groups. Exhaled H₂S and serum interleukin-8 (IL-8) expression levels were detected in both groups. The correlation between exhaled H₂S and serum IL-8 levels was analyzed using Pearson's correlation method. Receiver operating characteristic (ROC) curve was used to evaluate the diagnostic value of exhaled H₂S combined with IL-8 detection in EJA. The results showed that patients with EJA exhaled more H₂S than healthy individuals. In addition, exhaled H₂S was positively correlated with increased IL-8 expression. The ROC curve revealed that the exhaled H₂S test had an acceptable diagnostic effect and could be used to diagnose EJA. The increase in H₂S exhaled by patients with EJA indicated that H₂S may be related to the occurrence and development of EJA; however, the *in vivo* mechanism needs to be further explored. Collectively, it was determined in the present study that exhaled H₂S was significantly higher in patients with early-stage EJA than in healthy controls and combined diagnosis with patient serum IL-8 could improve diagnostic accuracy, which has potential diagnostic value for early diagnosis and screening of EJA.

Introduction

Esophagogastric junction adenocarcinoma (EJA) is a prevalent malignant tumor of the digestive tract (1). The biological behavior of EJA differs from that of its adjacent esophageal and gastric cancers, with a worse prognosis compared with that of the other two (2,3). The incidence of EJA has been alarmingly increasing worldwide in recent years. However, due to its unique biological characteristics, controversies surrounding the pathogenesis, pathology, clinical treatment and prognosis of EJA remain. Most patients with EJA are diagnosed at an advanced stage due to the lack of specific clinical symptoms and effective early diagnostic methods. By this time, the tumor may have already widely invaded and metastasized, particularly in China where gastrointestinal malignancies are highly prevalent. Consequently, most patients with EJA are diagnosed at intermediate or late stages of tumor progression with a 5-year survival rate <30% (4). Therefore, identifying convenient and effective methods to improve the rates of early diagnosis for EJA is crucial for enhancing prognosis and survival rates.

Hydrogen sulfide (H₂S) is the third endogenous gaseous signaling molecule, following carbon monoxide (5). Numerous studies have demonstrated the diverse range of biological activities of H₂S in glucose metabolism, ischemia-reperfusion injury, stress and endotoxemia (6-9). H₂S actively participates in various physiological and pathological processes within multiple systems. It exists *in vivo* as H₂S gas or sodium hydrosulfide (NaHS). The dissociated H₂S ions from NaHS combine with hydrogen ions to generate H₂S, maintaining homeostasis within the body. Mammals possess more than one pathway for producing H₂S using L-cysteine (L-Cys substrate). The primary pathways involve cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). A high concentration of NaHS can reduce cellular oxidative stress levels, subsequently activating the mitogen active protein kinase pathway while upregulating gene expression. This induction prompts intestinal epithelial cells to malignant transformation (10).

Interleukin-8 (IL-8) is a member of the chemokine family that attracts neutrophil infiltration by acting on the C-X-C chemokine receptor type 1 (11). The immunosuppressive effect of the tumor microenvironment is promoted by chemotaxis of neutrophils and myeloid suppressor cells (12). Tumor cells secrete a significant amount of IL-8 to facilitate the progression and metastasis of tumor cells (13). Known as Barrett's

Correspondence to: Dr Guangjie Liu or Dr Qingyi Liu, Department of Thoracic Surgery, The Fourth Hospital of Hebei Medical University, 12 Jiankang Road, Shijiazhuang, Hebei 050001, P.R. China

E-mail: 13932143768@163.com

E-mail: zhongmeijian_lqy@126.com

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esophagus, metaplasia of the single columnar epithelium is a precancerous lesion in the development of EJA (14). The upregulation of IL-8 expression in Barrett's esophagus tissue inflammation is related to intraepithelial neutrophil granulocytes, which may be affected by the epithelial secretion of IL-8 (15). In addition, both IL-8 mRNA and protein levels are upregulated in EJA (16). IL-8 can thus be used as an early diagnostic marker for EJA (17).

Increased H₂S concentrations have been reported in colon cancer tissues compared with those in adjacent normal tissues (18). Colorectal cancer (CRC) cells are able to synthesize more H₂S and release it into the tumor micro-environment (19). Reportedly, H₂S expression increases in multidrug-resistant CRC cells (20). Moreover, H₂S-producing *Clostridium* and *Bacillus fragilis* are significantly enriched in early-stage CRC, and H₂S produced by *Clostridium* nucleate and other microorganisms may promote tumor development by destroying host cells and DNA (21). In recent years, different diagnostic probes and combination therapy approaches, as well as tumor treatment pathways mediated by H₂S have been developed (22). It has been observed that H₂S is expressed in the tumor tissues of colorectal adenoma and bladder cancer making it a potential diagnostic marker (23,24). However, the expression level of H₂S in EJA and its diagnostic value remain unexplored. Gastrointestinal gas analysis holds promise as a diagnostic technique. Therefore, the present study aimed to assess and compare exhaled H₂S in patients with EJA with that in healthy controls. Additionally, correlations between exhaled H₂S and clinical diagnosis of EJA were analyzed to determine the clinical significance of this biomarker.

Patients and methods

Research participants. A total of 56 patients (36 males and 20 females; mean age, 56.61±10.65 years) with EJA, who underwent surgical treatment at Hebei Medical University Fourth Affiliated Hospital (Shijiazhuang, China) from January 2019 to December 2021, were included in the EJA group. All patients underwent gastroscopy and received a pathological diagnosis confirming EJA without any previous history of tumors. These patients were treated for the first time without prior radiotherapy or chemotherapy. Early-stage EJA was defined as AJCC Staging Manual 8th edition stages I and II (1). Exclusion criteria consisted of: i) Patients with other malignancies or severe cardiovascular, pulmonary, or renal diseases where diagnosis was unclear; ii) patients with a history of gastrointestinal surgery or trauma that altered anatomy and function; iii) patients who had bowel surgery or were preparing for bowel surgery, and those who were pregnant and lactating; and iv) patients on antibiotics, lactulose, acid suppressants, or drugs affecting gastrointestinal motility within the past 4 weeks. Additionally, a healthy control group consisting of 57 individuals (38 males and 19 females; mean age, 58.54±10.35 years), without any autoimmune diseases, tumors or organic lesions (Table I) was also formed. The present study adhered to the ethical standards set by the responsible committee on human experimentation, namely, the Ethics Committee of Hebei Medical University Fourth Affiliated Hospital, Shijiazhuang, China; approval no. 2018MEC108), following the principles outlined in The Declaration of Helsinki. All the patients and

Table I. Clinical characteristics of patients with EJA and the healthy control group.

Characteristics	EJA group	Healthy control group
Number of patients	56	57
Sex (male/female), n	36/20	38/19
Age, years	56.61±10.65	58.54±10.35
IL-8, pg/ml	2,200.80±641.69	243.64±70.36
Stage I	26	0
Stage II	30	0

EJA, esophagogastric junction adenocarcinoma.

their families were informed about the study details and provided written informed consent.

Exhaled H₂S determination. Based on a previously published study (23), exhaled H₂S tests were performed using Nanocoulomb breath analyzer DA6000 (Sunvou Medical Electronics Co., Ltd.). Briefly, all participants had to fast for 12 h before the test, and avoid exercise and smoking on the day of the test. Participants wrapped their lips tightly with a disposable filter. After inhaling through the filter, they held their breath for 15 sec and then forcefully exhaled. Animation software (Breathing Zone 13.0; Breathing Zone Limited) was used to coordinate the exhalation rhythm. The analyzer automatically collected the end-expiratory air. After the acquisition process was complete, the analyzer automatically analyzed the exhaled gas and displayed the results immediately. To eliminate the effects of H₂S in the environment, the participants first inhaled through the H₂S filter and then exhaled at a set flow rate, expiratory pressure and exhalation time. Calibration with 50 parts per billion (PPB) and 200 PPB H₂S/N₂ standard gas, supplied by the manufacturer prior to daily testing, was performed to ensure the accuracy of the test.

Enzyme-linked immunosorbent assay (ELISA). Patient serum was collected within 24 h of admission before treatment and immediately stored at -80°C until use. Serum IL-8 concentrations were measured using a commercial ELISA kit (cat. no. H008-1-2; Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. All trained operators were blinded to the characteristics of the patients with EJA and healthy controls. Briefly, the preparation of blank wells, standard holes and sample holes was performed. After washing, 100 µl of enzymatic secondary antibody was added, incubated at 37°C for 60 min, washed repeatedly, and then color was added to the developing solution, followed by incubation at room temperature for 20 min and the addition of termination solution. The absorbance at 450 nm per hole was measured using the developing method. The average OD values were calculated after the difference in the duplicate wells was <10%.

Statistical analysis. The experimental data are expressed as the mean ± standard deviation. SPSS 21.0 (IBM Corp.) and GraphPad Prism 8.0 (GraphPad Software; Dotmatics) were used for data

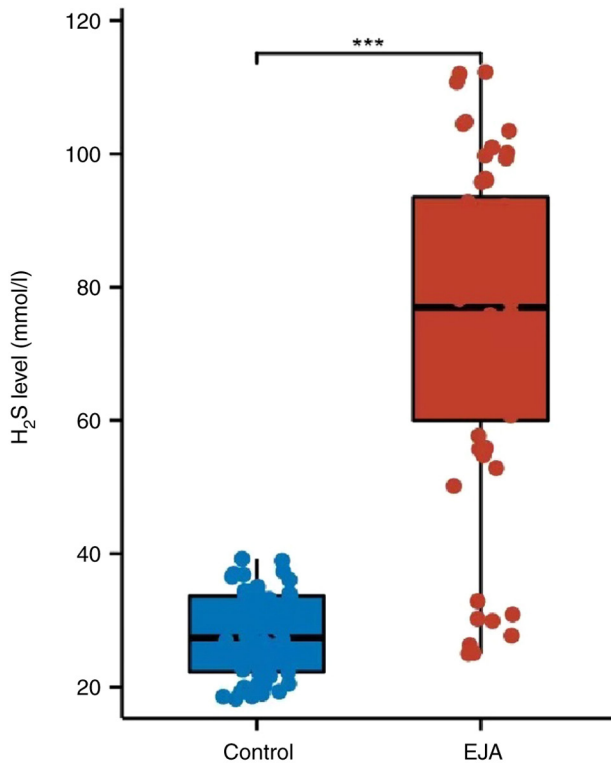


Figure 1. Exhaled H_2S in patients with EJA. *** $P < 0.05$, the EJA group vs. the healthy group. H_2S , hydrogen sulfide; EJA, esophagogastric junction adenocarcinoma.

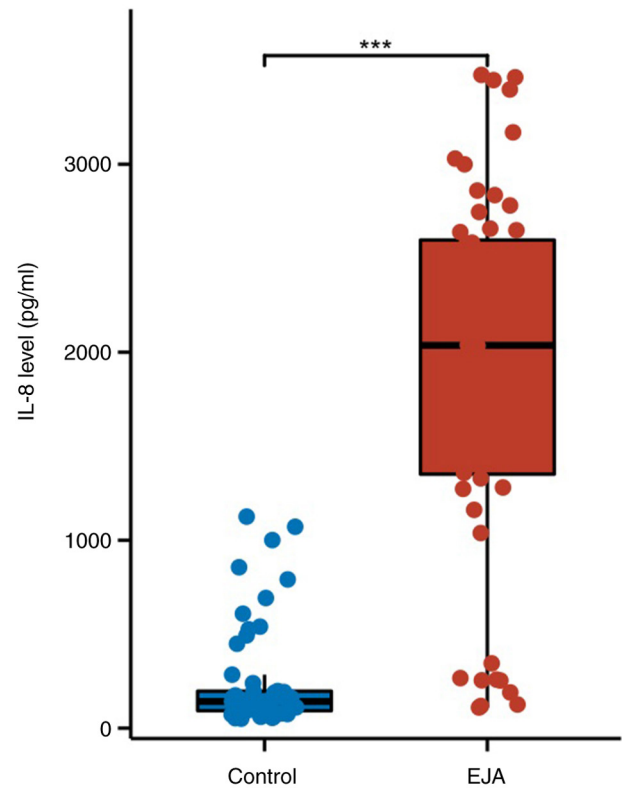


Figure 2. Increased expression of IL-8 in patients with EJA. *** $P < 0.05$, the EJA group vs. the healthy group. IL-8, interleukin-8; EJA, esophagogastric junction adenocarcinoma.

analysis. Unpaired Student's t-test was used for comparison between the two groups. Pearson correlation coefficient was used to analyze the correlation between H_2S and IL-8. The diagnostic value of H_2S or its combination with IL-8 was evaluated using receiver operating characteristic (ROC) curves, according to the area under the curve (AUC) with 95% confidence interval (CI). $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were performed three times.

Results

Exhaled H_2S is increased in patients with EJA. The exhaled indicator results showed significantly higher levels of H_2S exhaled in patients with EJA compared with those in healthy controls (Fig. 1; $P < 0.05$).

Expression levels of IL-8 in patients with EJA. The expression levels of IL-8 were determined using ELISA. The results revealed that the levels of IL-8 in patients with EJA were significantly higher than those in the control group (Fig. 2; $P < 0.05$).

Exhaled H_2S is positively correlated with IL-8 expression in patients with EJA. Pearson's correlation coefficient was used to analyze the correlation between H_2S exhaled and serum IL-8 in patients with EJA. The results showed that exhaled H_2S was positively correlated with IL-8 expression (Fig. 3; $P < 0.001$).

Evaluation of the clinical value of miR-29c and miR-146a in diagnosing EJA. To evaluate the diagnostic value of exhaled H_2S , ROC curves were generated based on the two groups.

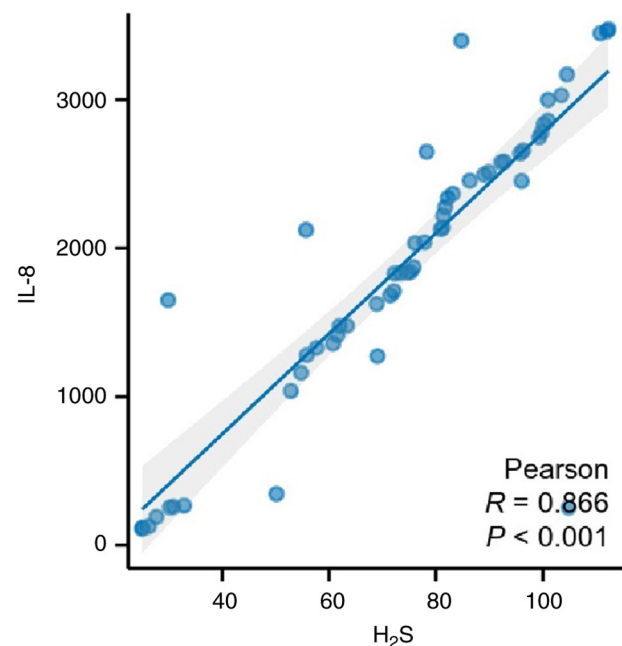


Figure 3. Correlation between the expression of exhaled H_2S and IL-8 in serum of patients with esophagogastric junction adenocarcinoma. IL-8 expression is positively correlated with exhaled H_2S ($R = 0.866$ and $P < 0.001$). H_2S , hydrogen sulfide; IL-8, interleukin-8.

The results revealed that exhaled H_2S had high diagnostic accuracy, with an AUC of 0.933 (sensitivity, 92.9%; and specificity, 85.7%) (Fig. 4A). Moreover, exhaled H_2S combined

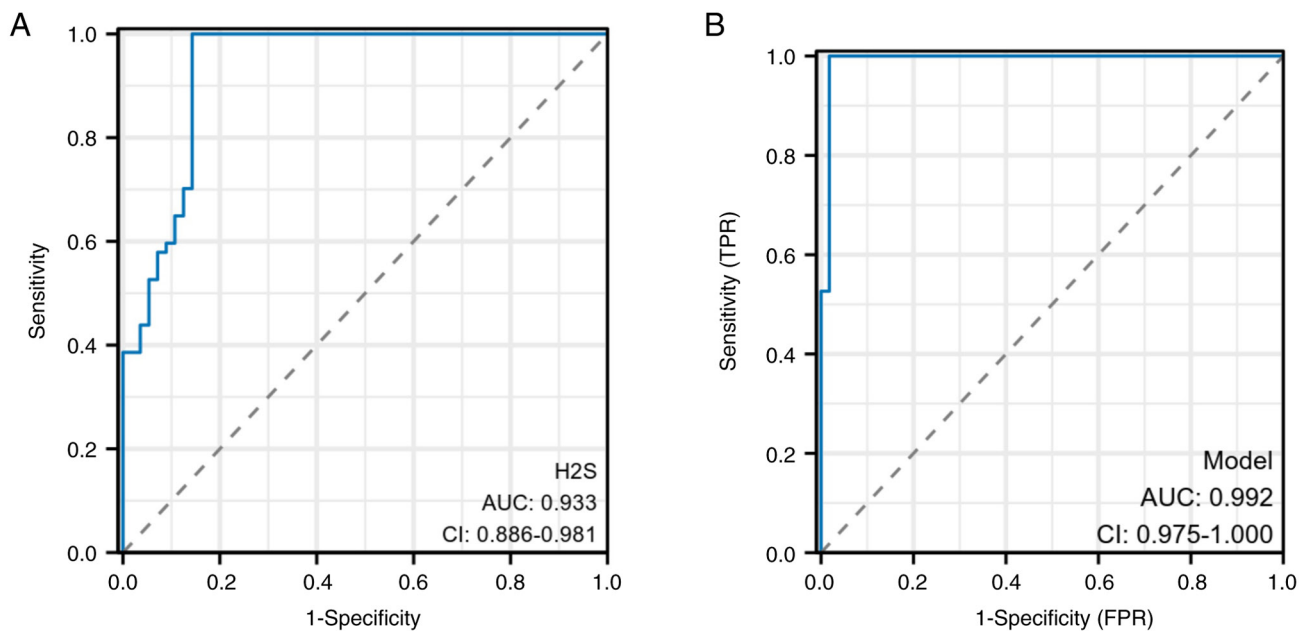


Figure 4. Evaluation of the clinical value of exhaled H₂S in the diagnosis of EJA. (A) ROC curve based on exhaled H₂S. (B) ROC curve based on exhaled H₂S + interleukin-8 expression. H₂S, hydrogen sulfide; EJA, esophagogastric junction adenocarcinoma; ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; TPR, true predictive rate; FPR, false predictive rate.

with serum IL-8 exhibited greater diagnostic value with an AUC of 0.992 (sensitivity, 99.1%; and specificity, 98.2%) (Fig. 4B), indicating that this combination led to a significant improvement in diagnostic ability.

Discussion

In the present study, a small quantity of H₂S was detected in patients with EJA, indicating endogenous origin as there was no H₂S detected in the inhaled and swallowed air of the healthy individuals. There are two pathways for H₂S synthesis in the intestinal tissue: i) Enzymatic and ii) non-enzymatic. The primary enzymatic pathway involves endogenous H₂S production by CBS and CSE enzymes and involves fermentation of sulfur-containing amino acids and sulfates by H₂S. Increased production of H₂S is a characteristic pathophysiological feature of colorectal adenoma (25). Overall there is an upward trend in the production of H₂S among patients with EJA. Additionally, it has been reported that human colon cancer cell lines (HCT-116 and SW-480) produce H₂S (26). In the present study, exhaled H₂S levels were examined in patients with EJA for the first time, revealing higher levels compared with those in healthy individuals. This may be attributed to an increase in endogenous H₂S synthesis within tumor cells, accompanied by alterations in gut microbiota metabolism, thus favoring enhanced production of H₂S.

The endogenous H₂S molecule is small and has a solubility in fat-soluble solvents that is five times higher than that in water, enabling it to freely traverse cell membranes (5). H₂S exists in the body in two forms, 1/3 is in the form of gas H₂S and 2/3 are in the form of NaHS. NaHS is not only the donor of H₂S, but also its precursor and a dynamic balance is formed between these two forms (27). Within mitochondria, glutathione catalyzes the oxidation of most H₂S into sulfate and thiosulfate. In the cytoplasm, small amounts of H₂S are

converted into less toxic methylmercaptan and dimethyl-sulfate through methylation, with these metabolites being excreted by the kidneys, intestines and lungs. Consequently, under normal physiological conditions, there is minimal accumulation of H₂S (28). Reportedly, H₂S promotes tumor proliferation, metastasis, differentiation and neovascularization while providing nutrition for tumor cell growth and facilitating tumor progression (29,30). Furthermore, in certain gastrointestinal cancers such as CRC, where increased levels of H₂S occurs due to enzymatic synthesis within colon cells or release from intestinal microorganisms followed by oxidation within colon cell mitochondria (31), upregulation of endogenous synthase may be responsible for elevated levels of H₂S in cancer cells. The high concentration of colonic cavity-derived H₂S has been suggested to contribute to CRC pathogenesis (32). However, limited research exists on the association between H₂S and EJA. The present study discovered that exhaled H₂S was significantly elevated in patients with EJA, suggesting potential involvement in EJA development. This needs to be further analyzed and confirmed in future experiments. In addition, the ROC curve in the present study showed that the AUC, sensitivity and specificity of H₂S diagnosis of EJA were 0.933, 92.9 and 85.7%, respectively, indicating that H₂S has diagnostic potential for EJA. Serum levels of IL-8 revealed significant results in the diagnosis of early-stage EJA (17). In the present study, exhaled H₂S combined with patient serum IL-8 was also used to diagnose early-stage EJA. The results demonstrated that combined detection was superior to single detection, and it significantly improved sensitivity, specificity and AUC.

The present study has some limitations. First, the patients enrolled in this study were all diagnosed with EJA and whether exhaling H₂S could be used for diagnosis in asymptomatic individuals needs to be analyzed in future studies. Second, the present study did not analyze whether there

were differences in H₂S between patients with stage I and II EJA. Therefore, whether H₂S levels change with different stages of the disease will be the topic of future research. In addition, the sample size of this study was limited and may not be representative of all patients with EJA. The high AUC values in this study may have potential overfitting. The sample size needs to be expanded to further clarify the diagnostic value of exhaled H₂S in an EJA population. Therefore, the authors of the present study anticipate to further expand the sample size in future experiments. Moreover, further *in vitro* experiments are required to confirm the underlying mechanism between H₂S and EJA. Therefore, expanding the sample size in future studies may further comprehensively identify the role played by H₂S in EJA through additional experiments.

In conclusion, the results indicated that increased levels of exhaled H₂S in patients with EJA may indicate its involvement in the occurrence and development of EJA. Exhaled H₂S holds promise as an early diagnostic indicator for EJA. Furthermore, combining H₂S and IL-8 detection serum can enhance diagnostic efficacy. These results provided broader prospect for the early diagnosis of EJA.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FL and GL made substantial contributions to the conception, design and draft of the manuscript. QL contributed in the acquisition, analysis and interpretation of the data. LW and BZ made substantial contributions to the conception of the study and its critical revision for important intellectual content. XS and JJ made substantial contributions to the conception, design and supervision of the study, and reviewed and edited the manuscript. FL and GL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy.

Ethics approval and consent to participate

The study adhered to ethical standards set by the responsible committee on human experimentation, namely the Ethics Committee of Hebei Medical University Fourth Affiliated Hospital, Shijiazhuang, China; approval no. 2018MEC108), following the principles outlined in The Declaration of Helsinki. All patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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