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

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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 (H_2S) 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 H_2S 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 H_2S and blood samples were collected from both groups. Exhaled H_2S and serum interleukin-8 (IL-8) expression levels were detected in both groups. The correlation between exhaled H_2S 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 H_2S combined with IL-8 detection in EJA. The results showed that patients with EJA exhaled more H_2S than healthy individuals. In addition, exhaled H_2S was positively correlated with increased IL-8 expression. The ROC curve revealed that the exhaled H_2S test had an acceptable diagnostic effect and could be used to diagnose EJA. The increase in H_2S exhaled by patients with EJA indicated that H_2S 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 H_2S 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.

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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 (H_2S) is the third endogenous gaseous signaling molecule, following carbon monoxide (5). Numerous studies have demonstrated the diverse range of biological activities of H_2S in glucose metabolism, ischemia-reperfusion injury, stress and endotoxemia (6-9). H_2S actively participates in various physiological and pathological processes within multiple systems. It exists *in vivo* as H_2S gas or sodium hydrosulfide (NaHS). The dissociated H_2S ions from NaHS combine with hydrogen ions to generate H_2S, maintaining homeostasis within the body. Mammals possess more than one pathway for producing H_2S 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

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 H_2S 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 H_2S and release it into the tumor microenvironment (19). Reportedly, H_2S expression increases in multidrug-resistant CRC cells (20). Moreover, H_2S-producing *Clostridium* and *Bacillus fragilis* are significantly enriched in early-stage CRC, and H_2S 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 H_2S have been developed (22). It has been observed that H_2S 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 H_2S 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 H_2S in patients with EJA with that in healthy controls. Additionally, correlations between exhaled H_2S 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 their families were informed about the study details and provided written informed consent.

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.

Exhaled H_2S determination. Based on a previously published study (23), exhaled H_2S 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 H_2S in the environment, the participants first inhaled through the H_2S filter and then exhaled at a set flow rate, expiratory pressure and exhalation time. Calibration with 50 parts per billion (PPB) and 200 PPB H_2S/N_2 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

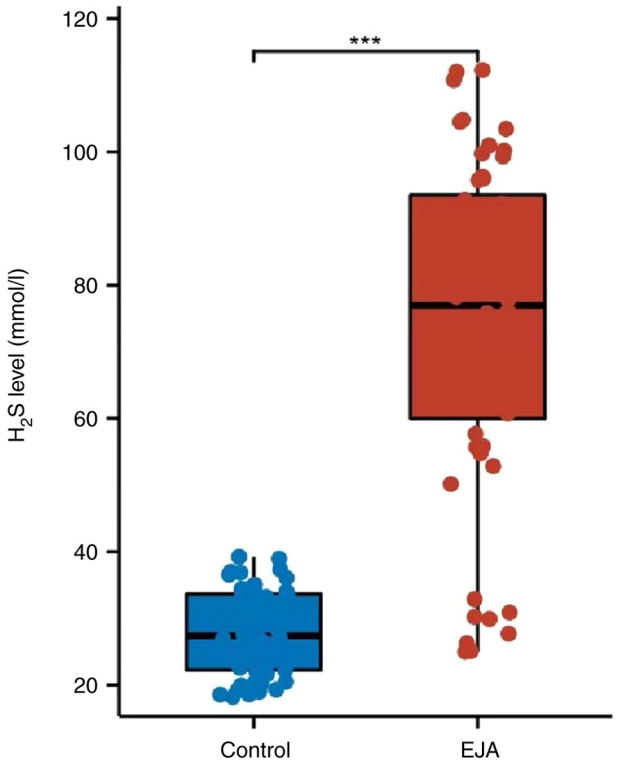

Figure 1. Exhaled H_2S in patients with EJA. ***P<0.05, the EJA group vs. the healthy group. H_2S, hydrogen sulfide; EJA, esophagogastric junction adenocarcinoma.

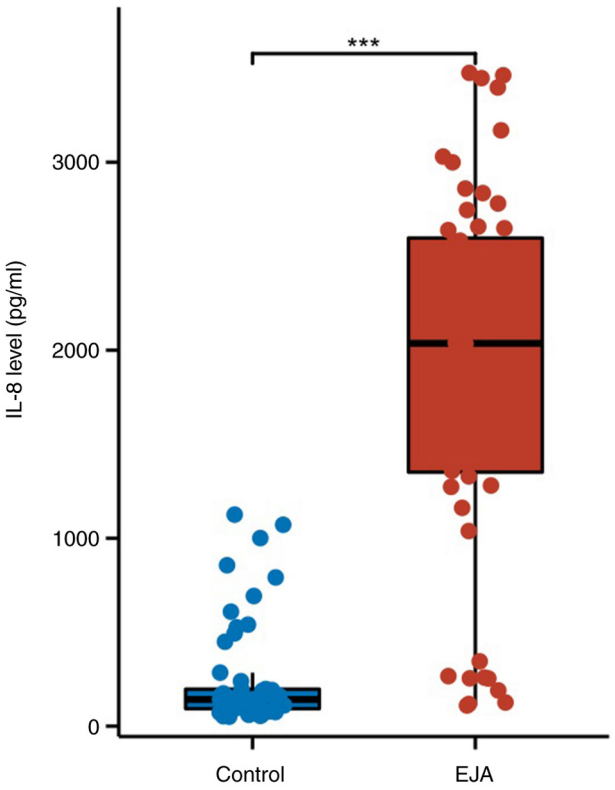

Figure 2. Increased expression of IL-8 in patients with EJA. ***P<0.05, the EJA group vs. the healthy group. IL-8, interleukin-8; EJA, esophagogastric junction adenocarcinoma.

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.

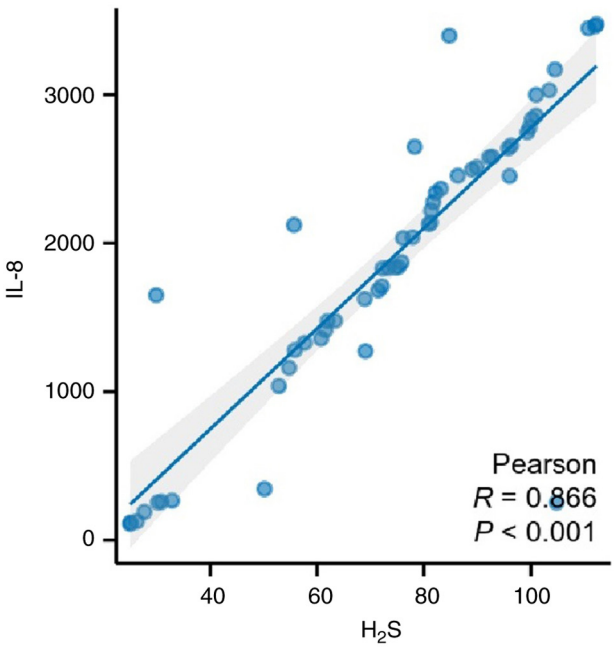

Figure 3. Correlation between the expression of exhaled H_2S and IL-8 in serum of patients with esophagogastric junction adenocarcinoma. IL-8 expression is positively correlated with exhaled H_2S (R=0.866 and P<0.001). H_2S, hydrogen sulfide; IL-8, interleukin-8.

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

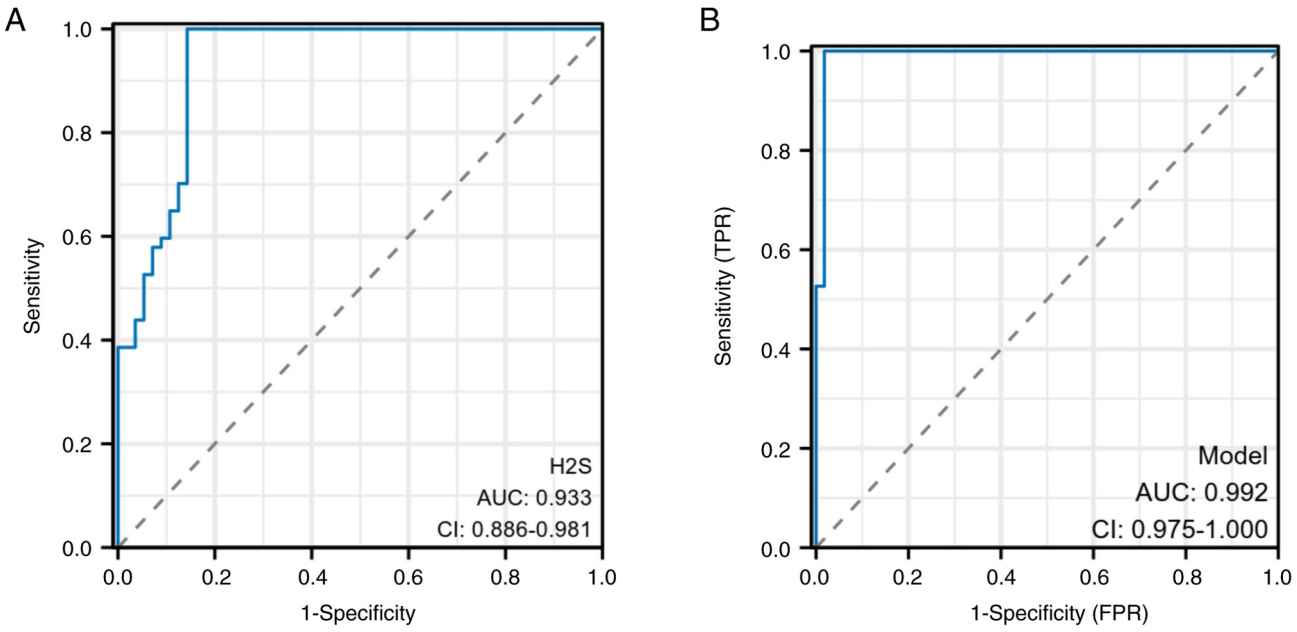

Figure 4. Evaluation of the clinical value of exhaled H_2S in the diagnosis of EJA. (A) ROC curve based on exhaled H_2S. (B) ROC curve based on exhaled H_2S + interleukin-8 expression. H_2S, hydrogen sulfide; EJA, esophagogastric junction adenocarcinoma; ROC, receiver operating characteristic; AUC, area 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 H_2S was detected in patients with EJA, indicating endogenous origin as there was no H_2S detected in the inhaled and swallowed air of the healthy individuals. There are two pathways for H_2S synthesis in the intestinal tissue: i) Enzymatic and ii) non-enzymatic. The primary enzymatic pathway involves endogenous H_2S production by CBS and CSE enzymes and involves fermentation of sulfur-containing amino acids and sulfates by H_2S. Increased production of H_2S is a characteristic pathophysiological feature of colorectal adenoma (25). Overall there is an upward trend in the production of H_2S among patients with EJA. Additionally, it has been reported that human colon cancer cell lines (HCT-116 and SW-480) produce H_2S (26). In the present study, exhaled H_2S 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 H_2S synthesis within tumor cells, accompanied by alterations in gut microbiota metabolism, thus favoring enhanced production of H_2S.

The endogenous H_2S 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). H_2S exists in the body in two forms, 1/3 is in the form of gas H_2S and 2/3 are in the form of NaHS. NaHS is not only the donor of H_2S, but also its precursor and a dynamic balance is formed between these two forms (27). Within mitochondria, glutathione catalyzes the oxidation of most H_2S into sulfate and thiosulfate. In the cytoplasm, small amounts of H_2S are converted into less toxic methylmercaptan and dimethylsulfate through methylation, with these metabolites being excreted by the kidneys, intestines and lungs. Consequently, under normal physiological conditions, there is minimal accumulation of H_2S (28). Reportedly, H_2S 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 H_2S 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 H_2S in cancer cells. The high concentration of colonic cavity-derived H_2S has been suggested to contribute to CRC pathogenesis (32). However, limited research exists on the association between H_2S and EJA. The present study discovered that exhaled H_2S 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 H_2S diagnosis of EJA were 0.933, 92.9 and 85.7%, respectively, indicating that H_2S 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 H_2S 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 H_2S 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 H_2S between patients with stage I and II EJA. Therefore, whether H_2S 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 H_2S 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 H_2S and EJA. Therefore, expanding the sample size in future studies may further comprehensively identify the role played by H_2S in EJA through additional experiments.

In conclusion, the results indicated that increased levels of exhaled H_2S in patients with EJA may indicate its involvement in the occurrence and development of EJA. Exhaled H_2S holds promise as an early diagnostic indicator for EJA. Furthermore, combining H_2S 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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