Research Article

HAAH Is a Potential Diagnostic Biomarker for Esophageal Carcinoma

Yihao He[1] and Yu Qiu[2#]

[1]College of Life Science, Hebei University, 180 Wusi Dong Road, Baoding, Hebei 071002, China
[2] Department of Clinical Laboratory, Beijing Gobroad Boren Hospital Co., Ltd., 2F-B Jitong East Road, Beijing 10071, China

Abstract: The human aspartyl-asparaginyl-β-hydroxylase (HAAH) is typically overexpressed in various types of cancer but remains undetectable or expressed at very low levels in most normal mature tissues. This suggests that HAAH could serve as a potential biomarker for cancer diagnosis. In this study, we examined 23 esophageal carcinoma tissues and 23 adjacent non-tumor tissues using semi-quantitative RT-PCR to detect HAAH mRNA expression. Our findings revealed that HAAH mRNA expression was detectable in all 23 esophageal cancer specimens (100%), while it was absent in 4 adjacent non-tumor tissue samples. Furthermore, HAAH was overexpressed in 17 esophageal carcinoma specimens (73.9%), compared to only 3 normal non-tumor tissue specimens (13%). These results strongly suggest that the HAAH gene is frequently overexpressed in esophageal carcinoma, providing support for its potential as a diagnostic marker for this condition.

Keywords: HAAH gene, esophageal carcinoma, semi-quantitative RT-PCR, biomarker, diagnosis.

Introduction

Esophageal carcinoma (EC) stands as one of the most formidable malignant tumors worldwide. Despite advancements in preoperative staging techniques, such as endoscopic ultrasound and positron emission tomography scanning, the majority of esophageal carcinomas are diagnosed only in their advanced stages. Consequently, this disease continues to pose a significant threat, with an overall 5-year survival rate lingering between a mere 10–20% [1].

To enhance survival rates, there's a pressing need for more refined methods to detect tumors in their early stages. A crucial avenue involves exploring and characterizing biomarkers within these tumors that could serve as reliable diagnostic indicators. Identifying specific and highly sensitive biomarkers for EC holds the potential to detect the disease before clinical symptoms emerge. Moreover, if these biomarkers are expressed on the cell surface, they could also serve as targets for precision therapy. Currently, clinics utilize various tumor markers, such as carcinoembryonic antigen (CEA), cytokeratin 19 fragment (CYFRA 21-1), and squamous cell carcinoma antigen (SCC), found in the serum of EC patients to diagnose EC [2]. However, their sensitivity levels have fallen short of providing a consistently reliable diagnostic tool. Hence, the pursuit continues for additional complementary biomarkers that can bolster diagnostic accuracy in the quest to identify EC at its earliest and most treatable stages.

HAAH is a type 2 transmembrane protein and a member of the α-ketoglutarate–dependent dioxygenase family of proteins [3]. HAAH catalyzes the hydroxylation of specific aspartyl and asparaginyl residues in epidermal growth factor (EGF)-like domains of proteins [4,5]. Previous studies have shown that the HAAH gene is abundantly expressed in a wide variety of malignant neoplasms [3]. However, its expression in esophageal carcinoma has not been examined. In this study, we analyzed HAAH mRNA expression in 23 esophageal and para-tumor tissues by RT-PCR to determine if HAAH expression levels could help diagnose EC.

Materials and Methods

Human tissue specimens

Corresponding Author: Yu Qiu
E-mail: qiuyu_qy2012@126.com

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23 esophageal carcinoma and 23 adjacent non-tumor specimens were obtained from 16 males and 7 females who did not receive any prior therapy. Their ages ranged from 43 to 77 years (average is 60 years). Fresh specimens were snapped in the RNA Later (Ambion) immediately after resection and stored at -20℃. 16 serum samples were obtained from individuals with histologically confirmed EC patients. 16 normal control serum samples were obtained from age-matched and sex-matched cohorts. All of the samples were collected with the approval of IRB at Hebei University Affiliated Hospital, Hebei, China.

Extraction of mRNA and preparation of cDNA

Total RNA was extracted from the stored esophageal tissues using TRIZOL (Invitrogen) following the manufacturer's recommendations. The first-strand cDNA was synthesized by using Oligo$(dt)_{15}$ (Tiangen) and reverse transcriptase M-MLV (Promega).

Semi-quantitative RT-PCR

RT-PCR was performed in a 25μl reaction mixture containing 1μl of cDNA template, 1μl of each primer, and 1U of Taq DNA Polymerase (Sangon), as follows: after one cycle at 95℃for 5 min, 30 cycles at 94℃ for 1 min, 61℃ for 1 min, and 72℃ for 1.5 min, followed by 72℃ extension for 10 min. The nucleotide sequences of the primers were as follows: HAAH: sense 5'-ATC TGT CTG GCA ACG CTC A-3', antisense 5'-ACA TCG AAT CTT GCA GCC T-3'. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal quantitative control for gene expression, and GAPDH primer sequences are: sense 5'-GTT GGA GGT CGG AGT CAA CGG A-3', and antisense 5'-GAG GGA TCT CGC TCC TGG AGG A-3'. The PCR products were separated by electrophoresis on 2% agarose gels. The images of gels were scanned by a gel document system and analyzed by using Quantity One software. The relative expression of HAAH was calculated by dividing the intensity of *HAAH* band with the intensity of *GAPDH* band.

Statistical Analysis

All statistical calculations were carried out by using the SPSS.16 software. The differences between the mean level of HAAH mRNA expression in both carcinoma and the adjacent non-tumor tissues were statistically analyzed using independent t-test, followed by receiver operating characteristic (ROC) curve analysis to determine cut off value with optimal diagnostic accuracy and likelihood ratios. *P* value less than 0.05 was statistically significant.

Results

HAAH expression in EC and normal tissues

Our semi-quantitative RT-PCR results showed that HAAH mRNA was expressed in all 23 esophageal cancer tissues (100%). In contrast, HAAH mRNA expression was relatively low in 19 adjacent non-tumor samples and was undetected in the remaining four adjacent non-tumor tissues (**Figure 1**). The relative HAAH gene expression values in 23 EC and 23 adjacent non-tumor tissues were 0.690±0.236 and 0.306±0.159, respectively. The independent t-test indicated that the level of HAAH mRNA expression in cancer tissues was significantly higher than that in non-tumor tissues ($P<0.01$). Multicultural Strategies, Digitalization Promotion Strategies, Brand Building Strategies, and Fan Culture and Community Engagement Strategies.

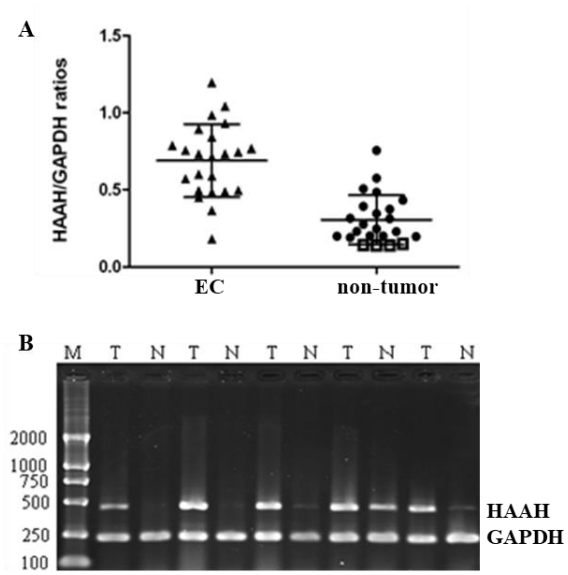

Figure 1. (A) Semi-quantitative RT-PCR analysis for the expression of HAAH gene in EC and adjacent non-tumor tissues. There is statistically significant difference between EC and the corresponding non-tumor tissues ($P<0.001$). Graphs are the mean ± SD. (B) Representative RT-PCR result showed that HAAH mRNA expression in esophageal carcinoma (T) and adjacent non-tumor tissues (N).

HAAH gene expression as a diagnostic marker

To assess the potential of the HAAH gene as a diagnostic marker, the ROC curve was generated using the data obtained in Figure 1 (**Figure 2**). The cutoff level in this assay was established to ensure optimal diagnostic accuracy and likelihood ratios, minimizing false-negative and false-positive results for HAAH. The ROC analyses determined a cutoff value of relative HAAH mRNA expression at 0.489. This value

corresponded to its overexpression in 17 out of 23 esophageal carcinomas (73.9%) and only 3 out of 23 normal tissues (13%).

Discussion

The HAAH gene, located on chromosome 8q12.1, spans an open reading frame (ORF) of 2217 bp and encodes at least 12 proteins through splicing [3]. Its conservation across bovine, human, and mouse species indicates its fundamental biological significance [6-8]. Functionally, HAAH belongs to the α-ketoglutarate–dependent dioxygenase family, known for catalyzing hydroxylation in specific aspartyl and asparaginyl residues within EGF-like domains of proteins. These domains, found in various proteins like Jagged, extracellular matrix proteins, Notch homologues, or Notch ligand homologues, contain conserved motifs forming repetitive sequences [9, 10].

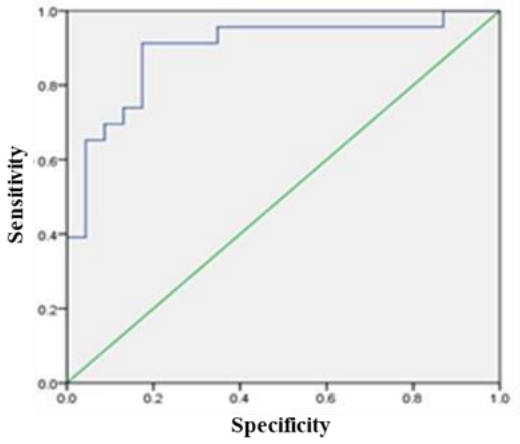

Figure. 2. ROC curve analysis for evaluating the diagnostic value of HAAH gene. The area under the curve is 0.896 and the model is significant (P<0.01).

Studies suggest that HAAH's overexpression in malignant neoplasms correlates with heightened activation of the Notch signaling pathway [10]. Abundant expression of HAAH is observed across a spectrum of malignant neoplasms and transformed cell lines, such as hepatic, biliary, breast, colorectal, and pancreatic cancers while normal mature tissues typically exhibit low to undetectable HAAH levels [3,12-14]. Moreover, *in vivo* and *in vitro* experiments Have demonstrated a direct association between elevated aspartyl beta-hydroxylase expression and malignant transformation, increased cell mobility, invasive growth, and unfavorable prognosis in malignant neoplasms [15-18]. These collective findings underscore HAAH's potential role in the pathogenesis and progression of cancer. In our study, we assessed HAAH gene expression in EC and adjacent non-tumor tissues, employing statistical analysis to evaluate its diagnostic relevance.

Our findings revealed a significantly higher mean level of HAAH mRNA expression in EC tissues compared to normal tissues (P<0.01). Through ROC curve analysis, we identified overexpression of HAAH in 17 out of 23 esophageal carcinomas (73.9%) versus only 3 out of 23 normal tissues (13%). These results strongly indicate the HAAH gene as a potential diagnostic marker EC. Previous research has linked HAAH overexpression to cellular transformation and invasive growth in malignant neoplasms. Considering the highly invasive nature of EC and the observed HAAH overexpression, we postulate that heightened HAAH gene expression may play a significant role in the pathogenesis or rapid progression of EC. This underscores the potential clinical relevance of HAAH as a marker for disease diagnosis and prognosis in EC.

The expression product of the HAAH gene, being a transmembrane protein and showing negligible or weak expression in adjacent normal tissues, holds potential as an immunotarget for antitumor agents. Previous in vitro experiments demonstrated significant inhibition of HAAH expression and motility in neuroblastoma cells, A549 lung carcinoma cells, and cholangiocarcinoma cells using antisense aspartyl beta-hydroxylase oligodeoxynucleotides, as opposed to sense or mutated antisense versions [16,19,20]. However, further exploration is needed to ascertain the impact of antisense aspartyl beta-hydroxylase oligodeoxy-nucleotides on HAAH expression and cell motility specifically in EC.

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Conflicts of interest: The authors declare that there are no conflicts of interest.

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