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Expression analysis of histidine decarboxylase in esophageal squamous cell carcinoma patients

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ABSTRACT

Esophageal cancer is considered among the ten most prevalent malignancies and the sixth leading cause of cancer deaths. Histamine has role in numerous physiological/ pathological responses, including gastric acid secretion, immune response and neurotransmission as well as angiogenesis or cancer. Histidine decarboxylase (HDC) catalyzes the formation of histamine from histidine. HDC has been proposed as a novel biomarker for neuroendocrine differentiation, inflammatory pathologies and various leukemia and highly malignant types of tumors, such as small cell lung carcinoma and melanoma. HDC over-expression was reported in a number of malignancies including melanoma, small cell lung carcinoma, breast cancer and colorectal carcinoma. In the present study, we examined the mRNA expression of HDC in patients with ESCC (squamous cell carcinoma). Conducting quantitative Real-time PCR, the expression level of HDC in normal and tumor tissues of 50 consented subjects with ESCC was studied. The results of molecular analysis were correlated to the clinical parameters such as age, stage, grade of differentiation, lymph node and tumor size. Compared with normal tissues, 6 out of 50 (12%) of specimen were found to represent a HDC over expression. Statistical analysis revealed that there are no correlations between HDC expression and clinicopathological features. Considering the lack of significant statistical correlation between the level of mRNA expression and clinicopathological features, it seems that the HDC has minimal role in ESCC progression and tumorigenesis.

Keywords: Histidine decarboxylase, squamous cell carcinoma, quantitative Real-time PCR, clinicopathological features

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INTRODUCTION

The biogenic amine histamine plays a crucial role in several physiological and pathological processes, such as neurotransmission, gastric acid secretion, allergic reaction and inflammation, as either angiogenesis or cancer which are mediated by the activation of specific histamine receptors, namely H1, H2, H3 and H [1-3]. Histamine is widely distributed in different types of cells and tissues which are involved in histamine production and secretion, as well as mast cells, enterochromaffin-like cells, monocytes/macrophagesbasophils and histaminergic neurons. The level of histamine production is regulated by the activity of histidine decarboxylase, which is known the only rate limiting enzyme responsible for the formation of histamine from L-histidine[4]. Histamine can be synthesized in both tumor cells and stromal cells and overexpression of HDC is thus recognized as a specific marker for biosynthesis of histamine. HDC has been previously proposed as a novel marker for neuroendocrine differentiation, inflammatory pathologies and a number of leukemia and highly malignant types of cancer, including small cell lung carcinoma and melanoma [3]. Accounting for 90–95% of all esophageal cancer worldwide, ESCC is the major

histological type of cancer derived from the esophageal mucosa representing great prevalence in some Asian countries such as Iran. [5] Different laboratory methods have been introduced for determination of HDC expression. Among them, quantitative Real-time PCR is the most reliable and automatizable technique for quantifying HDC expression at the mRNA level. Several studies have indicated an increase in HDC expression in human cancers including melanoma, leukemia, small cell lung, neuroendocrine and gastrointestinal cancers. As a consequence, it might introduce as a novel molecular targeted therapy for ESCC [3].

Suggested to be involved in progression, tumorigenesis and various cancers, the aim of this work was to investigate the expression of HDC in ESCC patients and its associations with clinicopathological characteristics by the use of Syber Green-based quantitative real-time RT-PCR at the mRNA level. The objective of this study was to determine whether we could introduce HDC as a specific biomarker for diagnosis of ESCC.

MATERIALS AND METHODS

Tissue Samples

In the present study, a total of 50 cases with a diagnosis of esophageal squamous cell carcinoma (ESCC) were collected from Omid Oncology Hospital of Mashhad University of Medical Sciences (MUMS), Iran. Tumoral and distant tumor-free tissues were freshly obtained from patients who have never received any treatment (radiotherapy and/or chemotherapy) preoperatively. Tissue samples which were obtained during esophagectomy (surgical removal of the esophagus) were immediately stored in RNAlater solution at -70 °C until RNA extraction was performed. In this study, histopathological parameters including tumor size and location, number of lymph nodes and grade of differentiation were determined. Real-time RT-PCR method was exploited to determine the expression of HDC gene at the mRNA level.

RNA preparation and cDNA Synthesis

Total RNA was extracted from tumor and normal tissues of 50 patients with ESCC using the RNeasy Mini kit (Qiagen, Hilden, Germany), based on the manufacturer's instructions. RNA sample was re-suspended in 20µL diethylpyrocarbonate-treated water (RNase-free water) and stored at -80°C. Subsequently, RNA integrity and quality were confirmed using 1 % agarose gel electrophoresis. The quantity and purity of the extracted RNA samples were evaluated by measuring the absorbance at 260 and 280 nm and the A260/A280 ratio using a nanodrop spectrophotometer. Subsequently, RNA samples were utilized for determination of gene expression levels.

Total RNA was reverse transcribed in a final volume of 20 µl reaction mixture using random hexamers. To synthesize cDNA, 2 µg of total RNA was mixed with 1 µl of oligo $(dT)_{12-18}$ primer (0.5 mg/ml) (Fermentas, Lithuania) and was diluted with DEPC-treated water to a volume of 11 µl. The reaction mixture was incubated at 65°C for 10 min and then rapidly chilled on ice. Afterward, 4 µl of 5X buffer, 2µl of 10mM dNTP mix (GeNet Bio CO.), 1 µl of RiboLock™ RNase Inhibitor (20U/µL) and the mixture was incubated for 5 min at 37 °C. Finally, 1 µl M-MuLV reverse transcriptase (Fermentase) were added to each reaction and the mixture was then incubated for 60 min at 42°C. The reaction was stopped by incubating the mixture at 70 °C for 10 min. A negative control lacking template was included in each reverse transcription and subsequent amplification experiment. Additionally, a duplicate sample containing template but lacking reverse transcriptase was included. Thermocycling was conducted on a (Mx-3000P) thermocycler (Stratagene, La Jolla, CA). Complementary DNA was kept at -20°C for the subsequent uses.

Semi-Quantitative RT-PCR

Complementary DNA was utilized as template for RT-PCR amplification in a total volume of 20 µl. Primer sequences for the in vitro amplification of HDC gene are presented in Table 1. The reaction mixture included 2 µl 10X Buffer, 0.4 µl dNTP (10mM, Cinnagen, Iran), 1 µl of both sense and antisense primers, 0.2 µl of Taq polymerase (Parstoos, 5U/µl), 0.6 µl MgCl2 (50mM) and 2 µl of cDNA (50- 200 ng). The amounts of PCR products were determined during the exponential phase of the PCR but before the plateau phase. Amplification was conducted according to the following PCR program: after an initial incubation for 5 min at 95 °C, samples were amplified for 43 cycles of 30s at 95 °C, 20s at 62°C, and 30s at 72 °C with a final period of 5 min at 72 °C. PCR products were visualized by 1.5 % agarose gel electrophoresis stained with ethidium bromide. A control PCR reaction was carried out without reverse transcriptase to investigate possible traces of contaminating genomic DNA. As an internal control, the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also amplified.

Table 1. Primer sequences used in this study

Gene	Forward primer (5´→3‘)	Reverse primer
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT
HDC	ATGATGGAGCCTGAGGAGTACAG	CCTGAGTTGGCATGCCTGAGGTAG

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using a step one plus apparatus in a Stratagene Mx-3000P real-time thermocycler (Stratagene, La Jolla, CA). Unlike endpoint RT-PCR, real-time quantification is defined by CT (threshold cycle number) at a fixed threshold where PCR amplification is still in the exponential phase and the reaction components are not limiting gene amplification. Real-time PCR was carried out using SYBR green PCR Master Mix containing ROX as a reference dye. The following thermal cycling program was applied: 10 min at 95°C, 43 cycles of 30 s at 95°C, 20 s at 62°C, and 30 s at 72°C. The specificity of the amplification reaction was assessed based on a melting curve analysis acquired by measuring fluorescence of SYBR Green during a linear temperature transition from 65 °C to 95 °C at 0.3 °C/s. Data were normalized for GAPDH expression as a reference gene by means of comparative threshold cycle method. The PCR efficiency for GAPDH and HDC genes were measured using standard curves depicted by serial dilution of cDNA. All experiments were performed in triplicates. PCR reactions were accomplished in duplicate in a 20 µl volume, including 400 ng cDNA, 10 µl SYBR Green PCR master mix (Fermentas, Lithuania), nuclease-free water, and 2 pmol forward and reverse primers.

More than 2-fold fluorescence intensity of mRNA expression in tumor tissue compared with the corresponding normal tissue was interpreted an overexpression of each gene, while Less than 2-fold indicated underexpression and the range in between was considered as no change in expression (at the mRNA level).

Statistical Analysis

The associations between HDC expression levels and different clinicopathological factors were tested using the χ^2 -squared test. The statistical analysis was performed using SPSS 16.0 statistical package (SPSS, Chicago, IL). Differences in the various groups of patients with different levels of HDC expression were tested by means of the χ^2-squared test. All P values were two-sided and P values of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A total of 50 patients who were newly diagnosed with ESCC were enrolled in the present study. The specimens were collected before beginning any treatment, therefore the histopathological characteristics were not affected by therapeutic interventions. Clinicopathological parameters of patients are summarized in Table 2.

TABLE 2. Clinicopathological features of 50 patients

Factor	Number (%)
Grade	
WD	9 (18.4)
MD	31 (63.2)
PD	9 (18.4)
gender	
Male	27 (54)
Female	23 (46)
Tumor location	
Upper	25 (51)
Middle	23 (46.9)
Lower	1 (2.1)
Stage	
I	2 (4.2)
II	23 (47.9)
III	5 (10.4)
Lymph node metastasis	
N0	26 (56.5)
N1	20 (43.5)

WD well differentiated, MD moderately differentiated, PD poorly differentiated

Of the tumors, 9/50 (18.4%), 31/50 (63.2 %) and 9/50 (18.4%) were considered as well, moderately and poorly grades of differentiation, respectively. Male-to-female ratio was 1.17 (27:23). The size of tumor samples were as follows: < 2 cm (8/50, 16.3), between 2 to 4.5 cm (28/50, 57.2) and >5 cm (13/50, 26.5). Age distribution of 50 patients with esophageal cancer revealed that the prevalence of ESCC is more frequent in people over 50 (85.7% vs. 14.3%).The expression level of HDC gene in 50 tumor specimens was compared to their paired non-neoplastic esophageal epithelium by quantitative real-time RT-PCR. Illustrated in Fig. 1, reduction in expression of HDC

mRNA was detected in 1 of 50 of tumor specimens (2%). Of 50 patients with ESCC, 6 cases (12%) had overexpression of HDC gene, while in 86% of patients (43 of 50), no alteration was in HDC expression observed.

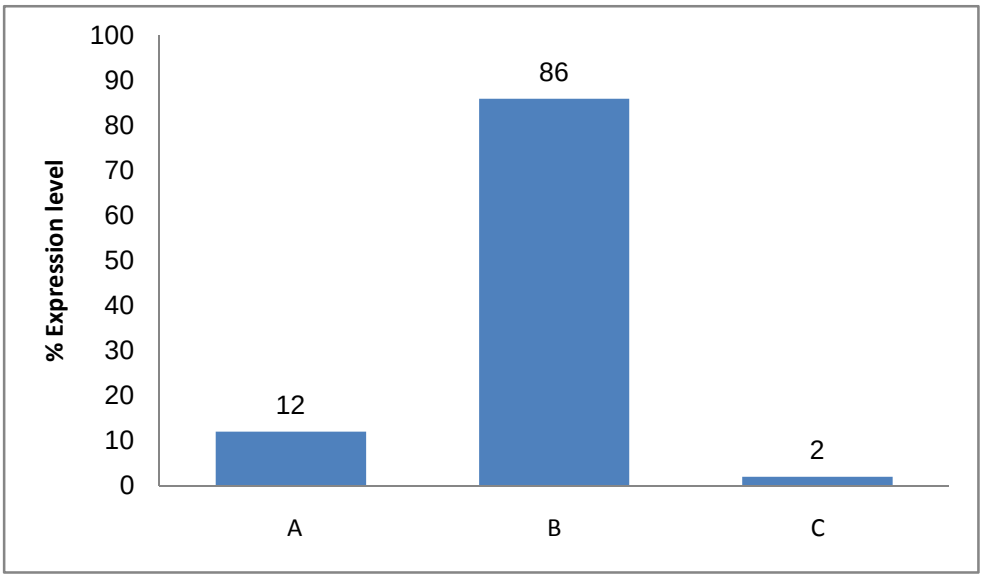

Fig. 1 The expression level of histidine decarboxylase (HDC) gene in esophageal squamous cell carcinoma (ESCC) A; Overexpression of HDC gene, B; No significant change in expression level, D; Decrease in expression of HDC gene.

To evaluate the clinicopathological consequences of HDC expression in ESCC, the correlation of different clinicopathological variables (age, grade of differentiation, stage, lymph node and size of tumor) with expression levels of the HDC gene was analyzed. To investigate the correlation between the level of HDC expression and the age of patients with ESCC, the specimens were divided in 2 groups (85.7% over 50 years and 14.3% under 50 years). Only 6 cases (11.9% of >50 vs. 14.3% <50) represented HDC overexpression, while there was no change in expression of HDC in 85.7% of patients over or under 50 years. Consequently, the expression of HDC in ESCC cancerous tissues was not significantly associated with the age of patients. No change in expression of HDC was observed in 81.5% of male and 91.3% of female specimens with ESCC. Therefore, it was suggested that there was no correlation between gender and the expression level of HDC. HDC expression did not differ in patients with regard to tumor location (middle vs. lower: 87% vs. 88%, p = 0.141). 87.5%, 82.1% and 92.3% of patients with tumor sizes of < 2 cm, 2 < < 4.5 cm and >5 cm did not show any change in HDC expression. Thus, the expression of HDC was not meaningfully related to the size of tumor. Tumor stage was not correlated with expression of HDC. Overexpression of HDC was observed in 5 of 28 specimens with lower stages of tumor (II), while 88.9% and 80% of tumors in advanced (III) and lower stages demonstrated no change in HDC expression. HDC expression was not correlated with other clinicopathological features such as the number of involved lymph nodes, grade of differentiation and node metastasis.

HDC has formerly been shown to be overexpressed in humamelanoma [6], small cell lung carcinoma [7], different types oneuroendocrine cancers [8,9] and chondrocytes of arthriticartilage[10]. Therefore, HDC has been suggested as a significant diagnostic biomarker for neuroendocrin differentiation [8], inflammatory pathologies and highly malignant tumors. The present study described the investigation of HDC expression in a total of 50 patients with ESCC tumor and its probable correlation with clinicopathological parameters. Using quantitative real-time PCR, Expression analysis of HDC in normal and tumor esophageal tissues revealed no significant change in expression of HDC gene indicated that HDC has minimal role in ESCC progression and tumorigenesis. Thus, HDC could not be considered as tumor prognostic tool in diagnosing of ESCC cancer.

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