

Notch Signaling Target Genes are Directly Correlated to Esophageal Squamous Cell Carcinoma Tumorigenesis

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Abstract Notch signaling is an important cellular pathway which affects the development and function of many organs. It plays critical roles in maintaining of progenitor stem cell population as well as balancing cell proliferation, survival, differentiation and apoptosis. It has been shown that notch signaling is aberrantly activated during the carcinogenesis of a variety of human cancers. In this study we aimed to explore activation of this signaling pathway in esophageal squamous cell carcinoma (ESCC) through expressional analysis of notch signaling target genes. The mRNA expression of HEY1 and HEY2 was comparatively analyzed by real-time PCR in tumor and related margin normal tissues of 50 ESCC patients. Comparative quantitative real-time PCR indicates the overexpression of HEY1 and HEY2 in 54 and 30 % of ESCC samples, respectively. Overexpression of HEY1 was significantly associated with stage of the tumor ($p=0.048$) and tumor location ($p=0.008$). HEY2 overexpression was also significantly correlated to node metastasis of tumor cells ($p=0.043$).

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Overexpression of HEY1 and HEY2 in ESCC is correlated to different indices of poor prognosis and it is extrapolated that such overexpression is important in progression and development of ESCC tumorigenesis. To the best of our knowledge, this is the first report introducing aberrant activation of notch signaling target genes in ESCC, where it plays roles in development and progression of the malignancy and may be considered in therapeutic modalities to restrict ESCC progression.

Keywords Notch signaling pathway · Esophageal squamous cell carcinoma · Real time PCR · HEY1 · HEY2

Introduction

Notch signaling is a contact dependent cell-cell communication event in multi-cellular organisms which plays a fundamental role in embryonic development, differentiation process, proliferation and apoptosis programs as well as regulation of cell fate decision [1, 2]. Notch proteins are members of single-pass transmembrane receptors which mediate interaction between neighbor cells [3]. Their activation is happened through direct contact with transmembrane ligands of the other cells [3], and after two proteolytic cleavages, the notch intra-cellular domain (NICD) is released and translocated into the nucleus to activate transcription of related target genes [1].

The notch signaling pathway is communicated with multiple developmental activities including stem cell survival, stem cell fate decision [2, 4], and regulation of stem cell self renewal by cross-talk with other cell signaling pathways such as Wnt and Hedgehog [5, 6]. Furthermore, it has a fundamental role in controlling stem cell numbers through transcriptional activation of HEY (Hairy/enhancer of split related with YRPW motif) gene family members. It also represses tissue

specific transcription factors leading to inhibit cell differentiation [7, 8]. On the other hand, notch signaling pathway not only participates in cancer development by activating transcription factors which cause cell survival, but also contributes to progression of tumor growth through cancer stem cells which have stem cell-like characteristic [9, 10]. It has been reported that notch signaling inhibits cell differentiation in tumorigenesis process [10].

HEY genes encode the nuclear proteins belonging to the hairy and enhancer of split-related family. These basic helix-loop-helix (bHLH) transcription repressors are expressed as target genes of notch signaling pathway [11]. There are 13 members of this family in human which have important roles in embryonic stem cells maintenance [12], and can influence cell proliferation and differentiation during embryogenesis [13]. HEY1 and HEY2 are expressed in endothelial cells that suggest the role of notch pathway in angiogenesis [12].

Previously we elucidated significant overexpression of MAML1, the main transcription factor of notch signaling transcriptional complex, in ESCC and revealed its significant correlation with the metastasis of tumor cells to the lymph nodes, which may indicate the importance of this protein as a marker of metastasis in esophageal squamous cell carcinoma (ESCC) [14]. However, it is not clear that whether MAML1 acts through notch signaling pathway or not. Therefore, our aim in this study was to analyze mRNA expression of HEY1 and HEY2 as essential notch signaling target genes to clarify the probable role of notch signaling pathway activation in ESCC development and progression.

Materials and Methods

Tissue Samples

Tumoral and corresponding margin normal tissues of esophagus were collected from 50 ESCC patients after surgery at Omid Oncology Hospital of Mashhad University of Medical Sciences (MUMS), and were transferred to RNA later solution (Qiagen, Hilden, Germany) instantly. All patients were new cases without receiving any therapeutic treatment such as chemo- or radiotherapy. Histopathological characteristics of samples such as tumor size, grade of differentiation, and tumor location were determined. Furthermore, surgical stage was defined on the basis of the union international center TNM classification guidelines [15]. The study was approved by the ethic committee of MUMS and all enrolled patients formally declare their recruitment consent.

cDNA Synthesis and Quantitative RT-PCR

RNA extraction and cDNA synthesis were performed as described before [16]. Comparative real-time PCR of cDNAs

was done with peculiar primer sets (Table 1) in stratagene Mx-3000 Pro real-time thermocycler (Stratagene, La Jolla, CA) using SYBR green as master mix and ROX as a reference dye. The following thermal profile was applied: 10 min at 95 °C, 40 cycles of 15 s at 60 °C, 30 s at 57 °C, and 45 s at 72 °C. Data was normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR efficiency for GAPDH, HEY1 and HEY2 was measured using standard curves generated by serial dilutions of cDNA. All experiments were performed in duplicate. For checking the fold change of gene expression, the comparative threshold cycle method was used [17].

Statistical Analysis

The SPSS 19.0 statistical program (SPSS, Chicago, IL) was used for statistical analysis. The correlations between different histopathological characteristics and genes expression were analyzed by χ^2 test or the Fisher exact test. To compare expression levels of each gene and different categorical data we used independent sample *t* test and ANOVA. Pearson correlation was used to assess the correlation between HEY1 and HEY2 level of genes expression. A *P* value less than 0.05 was considered statistically significant.

Results

The clinicopathological features of 50 ESCC patients are presented in Table 2. Patients did not receive any treatment such as chemo- or radiotherapy before the surgery and therefore tumor tissues were not affected by preoperative modalities. The mean age (\pm SD) of the patients was 62.8 (\pm 10.78), ranged from 37 to 83 years. Male-to-female ratio was 1.08 (26:24). The size of tumor samples ranged between 2 and 12 cm (mean \pm SD: 4.1 \pm 1.8), which were resected from upper, middle or lower parts of esophagus.

Table 1 primer sequences used for real-time PCR

Gene	Sequence (5'→3')	Amplicon size (bp)
HEY1	F: ACG AGA ATG GAA ACT TGA GTT C R: AAC TCC GAT AGT CCA TAG CAA G	319
HEY2	F: ATG AGC ATA GGA TTC CGA GAG TG R: GGC AGG AGG CAC TTC TGA AG'	314
GAPDH	F: GGAAGGTGAAGGTCGGAGTCA R: GTCATTGATGGCAACAATATCCACT	101

Table 2 Clinicopathological features of the 50 ESCC patients and their correlations with HEY1 and HEY2 mRNA expression

	Number (%)	HEY1		P value	HEY2		P value
		↑	↓		↑	↓	
Tumor invasion				0.20			0.65
T1	1(2)	1	–		–	–	
T2	8(16)	2	–		2	2	
T3	40(80)	24	5		9	8	
T4	1(2)	–	1		–	–	
Node metastasis				0.97			0.043*
N0	28(56)	15	3		5	6	
N1	22(44)	10	3		7	4	
Stage				0.048*			0.70
I	1(2)	1	–		1	–	
Ia	26(52)	14	4		5	6	
Ib	1(2)	1	–		1	–	
III	22(44)	11	2		5	5	
Sex				0.43			0.49
Male	26(52)	15	2		6	7	
Female	24(48)	11	4		5	3	
Location				0.008*			0.82
Upper	–	–	–		–	–	
Middle	27(54)	13	3		7	4	
Lower	23(46)	13	3		5	5	
Grade**				0.86			0.75
P.D	10(20)	5	1		4	2	
M.D	32(64)	18	3		5	4	
W.D	8(16)	1	2		2	3	

*Statistically significant

**PD poorly differentiated, MD moderately differentiated, WD well differentiated

Overexpression of HEY1 and HEY2 in ESCC

The mRNA expression levels of HEY1 and HEY2 genes were analyzed in tumors and their margin normal esophageal epithelium of 50 patients by comparative real-time RT-PCR. The expression pattern of the genes is depicted as a scatter plot in Fig. 1. Significant overexpression of HEY1 and HEY2 was detected in 54 % (27 of 50, $P=0.012$) and 30 % (15 of 50, $P=0.021$) of tumor samples, respectively. Overexpression of at least one gene was found in 64 % (32 of 50) of the patients, and co-overexpression of the genes was also detected in 20 % (10 of 50) of tumor specimens. mRNA expression of both genes was significantly correlated to each other ($P=0.005$).

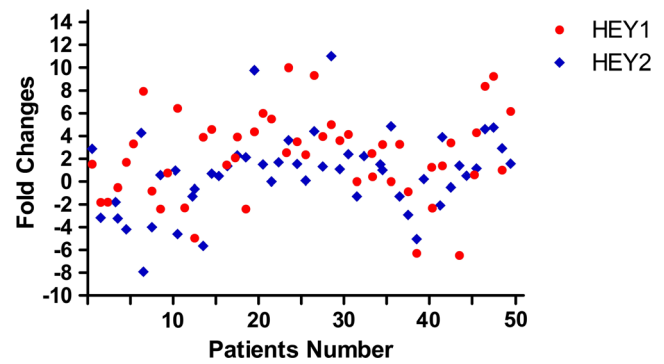


Fig. 1 Scatter plot represent descriptive analysis of relative gene expression of HEY1 and HEY2 in ESCC patients

Association of HEY1 and HEY2 with Clinicopathological Features

Having categorized the samples into HEY1 and HEY2 overexpressed groups, we found valuable significant correlations describing close relation of the genes expression with indices of tumor progression and development. Overexpression of HEY1 was significantly correlated to the advanced stages (III/IV) of tumor progression ($P=0.048$) and location of tumor ($P=0.008$). In addition, overexpression of HEY2 was significantly correlated to the metastasis of tumor cells to the lymph nodes ($p=0.043$). Furthermore, in HEY1 overexpressed tumor samples, overexpression of HEY2 was significantly associated with different clinicopathological variables including lymph node metastasis ($p=0.012$), advanced stages of tumor progression (III, $p=0.004$) and location of tumor ($p=0.004$). There were no any other significant association between overexpression of the genes and clinicopathological variables.

Discussion

Notch signaling is critical in tissue self-renewal and maintenance of many organs including but not limited to skin, blood, intestine, liver, kidney, central nervous system, bone, and muscle. In stem cell biology, the output of notch signaling activation is highly cell context-dependent, and the biological consequences of the pathway activation can vary from stem cell maintenance or expansion to promotion of stem cell differentiation [18]. Furthermore, notch signaling pathway acts as a double-edged sword in different types of cancer based on the cell context [19]. Therefore, expression analysis of downstream target genes of notch pathway is critical to indicate its significant role in different tumors progression and development.

Various studies have demonstrated the dual role of notch signaling network in a variety of cancers. In some

malignancies such as skin, uterus and hepatocellular carcinomas, notch signaling plays a tumor suppressor role, while in some others including acute T-cell lymphoblastic leukemia, breast and prostate cancers, pancreatic, cervical and lung carcinomas, and neuroblastoma, this important signaling pathway acts an oncogenic role [20]. In normal tissue of the esophageal epithelium, notch signaling is active and is required for squamous cell differentiation through a CSL-dependent transcriptional network [21]. MAML1 overexpression in ESCC is contributed in aggressiveness and metastasis of the malignancy through probable crosstalk with epithelial-mesenchymal transition (EMT) factor TWIST1 [14]. However, a question was raised upon such extrapolation that does MAML1 plays its role via activation of notch signaling pathway in tumor cells or it acts via crosstalk with other cell signaling pathways in a notch-independent manner.

To answer this question, in this study we analyzed the expression pattern of two important target genes of notch signaling pathway, HEY1 and HEY2, in ESCC patients. Our results showed that not only these genes are significantly overexpressed in ESCC and their expression is correlated to each other, but also there are significant associations between the level of HEY1 and HEY2 genes expression and MAML1 ($P < 0.05$), as well as different indices of poor prognosis including stage, lymph node metastasis, and location of tumor cells. These results may illustrate the contribution of notch signaling pathway in ESCC tumorigenesis and it is reasonable to speculate about the hypothesis of notch-dependent activity of MAML1 in aggressiveness and metastasis of ESCC. However, we cannot ignore its function in tumorigenesis through probable crosstalk with other cell signaling pathways.

HEY1 and HEY2 are two important target genes of notch signaling pathway which are belonged to a family of bHLH transcription factors. Since these genes play a critical role in the expression of downstream genes in notch signaling cascade, they can be considered as good candidates for estimation of the rate of notch signaling pathway activation in the cell. Adepoju et al. described the role of notch signaling pathway in infantile hemangiomas, a very common tumor in newborn infants. They showed significant HEY1 and HEY2 gene expression in hemangiomas stem cells and endothelial cells of hemangiomas, respectively [12]. Hulleman .G et al. have indicated that HEY1 is up-regulated in glioblastoma which can lead cell proliferation [22]. Also Tanaka et al. has showed the overexpression of HEY1 and HEY2 in osteosarcoma, which can cause maintenance of cell proliferation. In all of these cancers, notch signaling plays oncogenic role, clearly. Such studies have showed upregulation of HEY1 and HEY2 which means aberrant function of notch signaling pathway in these malignancies may be contributed to cancer progression through advanced stages.

In conclusion, this study indicated the clinicopathological relationship of HEY1 and HEY2 mRNA expression and

elucidated deregulated activation of notch signaling pathway in ESCC patients. To the best of our knowledge, this is the first report describing the oncogenic role of notch signaling target genes in ESCC. Despite to different roles of notch signaling pathway in various cancers, we showed that downstream target genes of this pathway are significantly overexpressed in association with clinicopathological features of ESCC patients that confirm oncogenic role of this pathway in esophageal carcinoma. Furthermore, we introduced HEY1 and HEY2 as new molecular markers of advanced tumors which determine the characteristics and behavior of aggressive ESCC. These genes may be new therapeutic candidates in order to block tumor cell progression and metastasis. In addition, significant correlation between HEY1 and HEY2 may suggest probable interplay between these genes to initiate a cascade resulting in tumor invasion and metastasis, although further studies are needed to clarify their exact role.

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