

## Original Article

# RNAi screening identifies HAT1 as a potential drug target in esophageal squamous cell carcinoma

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**Abstract:** Esophageal carcinoma (EC) is one of the most fatal carcinomas of the gastrointestinal tract. Aberrant activity of histone acetyltransferases (HATs)/deacetylases (HDACs) play a critical role in carcinogenesis through the regulation of the genes involved in cell differentiation, proliferation, and apoptosis. However, cellular functions of HATs/HDACs in esophageal cancer and its molecular mechanisms remain unclear. An RNAi screen was used in this study to identify the histone acetyltransferases (HATs) and deacetylases (HDACs) that could be critical for the survival of EC cells. We demonstrated that HAT1 (histone acetyltransferase 1) was an important determinant to regulate the proliferation of human EC Eca-109 cells. Furthermore, we showed that the knockdown of HAT1 induced a G2/M cell cycle arrest, which was associated with the disruption of cell cycle-related events, including the decrease of cyclinD1 as well as alteration in cyclinB1 expression. The expression of HAT1 was validated to be higher in the primary tumors and adjacent tissue as compared to that of the normal esophageal tissue. Furthermore, we found that HAT1 expression was directly correlated with the poor tumor differentiation of EC tissue, which suggested that HAT1 played an important role in esophageal carcinoma and that it could be a novel EC therapeutic target.

**Keywords:** Esophageal carcinoma, HAT1, G2-M, tumor differentiation, RNAi screen

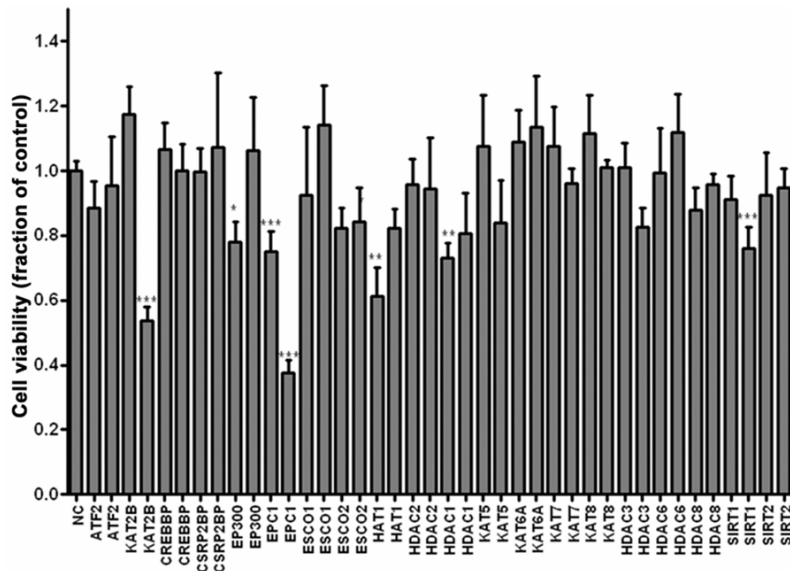
## Introduction

Esophageal carcinoma (EC), one of the most fatal carcinomas of the gastrointestinal tract, carries a poor prognosis with a 5-year survival of 15-34% [1]. Because of its poor outcomes from conventional therapies including surgery and radiochemotherapy, much efforts have been made to characterize the genes involved in EC progression and development [2, 3], which might assist in developing novel biomarkers and therapeutic targets for EC. The development of RNAi libraries and high throughput screening (HTS) technologies has made it possible to identify the key determinants of cancer cells survival [4].

The acetylation of lysine residues, located in the N-terminal tails of histone, is one of the key features associated with active gene transcription [5]. Two classes of proteins determine the acetylation status of the histones-histone acetyltransferases (HATs) and histone deacetylases (HDACs) [5]. Emerging evidence have

demonstrated that acetylation and deacetylation of histones regulate multiple cellular processes such as transcriptional activity, genome stability, and signaling pathways [6-8]. Aberrant activity of HATs/HDACs is thought to play a critical role in carcinogenesis through the regulation of genes involved in cell differentiation, proliferation and apoptosis [9, 10]. Therefore HATs/HDAC inhibitors have emerged as promising target therapy, and several classes of HAT/HDAC inhibitors have been found to have potent and specific anticancer activities in preclinical studies [11, 12]. However, the molecular mechanisms of HATs/HDACs function in EC are poorly understood and more studies should be conducted to identify novel tumor-related HATs/HDACs and clarify their roles in the initiation and progression of EC.

To identify the HATs/HDACs related to EC, we performed an RNAi screening for the genes involved in the proliferation regulation of human EC Eca-109 cells by using a lentiviral short-hairpin RNA (shRNA) library. This approach led to



**Figure 1.** RNAi screening with a lentiviral shRNA library. Eca-109 cells were plated in the 96-well plates and were infected with a library targeting 20 HATs/HDACs. Each gene contained two pools of four distinct shRNA species targeting different sequences of the target transcript. The screening was repeated in three independent plates at the same time. Negative control was infected with the empty vector lentivirus. Five days after the infection, the cell viability was assessed using tetrazolium compound (MTS) cell proliferation assay (promega, USA). Error bars represent the standard error of the mean (SEM).

the identification of histone acetyltransferase 1 (HAT1), where its knockdown remarkably inhibited the growth of Eca-109 cells. Further study showed that HAT1 silencing induced a G2/M cell cycle arrest, which was related to a decreased cyclinB1 and a decreased cyclinD1. Moreover, we observed an obviously higher HAT1 mRNA level in the EC primary tumor adjacent tissue as compared to that of the normal esophageal tissue. Furthermore, we found that a high expression of HAT1 was significantly correlated with a poor EC tumor differentiation.

## Materials and methods

### Cell cultures and reagents

Human Eca-109 cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Eca-109 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO<sub>2</sub>. A customized lentiviral shRNA library (3D-HTS, China) was used for RNAi screening, targeting 20 histone acetylase and deacetylase genes, with 8 distinct shRNA sequences per enzyme. All eight sequences targeting each enzyme were evenly divided into two pools.

### RNAi screening

Eca-109 cells (1,200 per well) were plated in 96 well plates and were infected with shRNA library 24 hr later. Infections were performed in three replicates in the primary screening and validation assays. After 120 hr post infection, the tetrazolium compound (MTS) cell proliferation assay (Promega, USA) was performed to measure the cell viability. Four distinct shRNA species targeting each gene were used to revalidate hits from the primary screening. Validation of mRNA expression was measured by quantitative PCR.

### Quantitative PCR

RNA samples were extracted with RNeasy Mini Kit (Qiagen) and were reversely transcribed to cDNA using RT reagent Kit (TaKaRa). Forward and reverse primers for HAT1 included: 5'-TGATGAAAGATGGCACTACTTCTAGT-3' and 5'-AGCCTACGGTCGCAAAGAG-3'. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. All samples were analyzed in triplicate. As for RNAi samples, gene expression was assessed relative to the expression of housekeeping gene hActb. This was further adjusted relative to the expression in shControl-infected cells. As for the clinical samples, each sample was normalized to the hActb.

### Flow cytometry

Eca-109 cells were harvested after being infected with shRNA lentivirus for 96 h, and were fixed with 70% ethanol at -20°C for at least 2 h. The fixed cell pellet was resuspended in PBS with 200 µg/ml RNase A and was incubated at 37°C for 1 h. PI was added to a final concentration of 50 µg/ml before analysis on the flow cytometer. The percentage of cell population in each phase of the cell cycle was measured using FACStar and the results were analyzed with the software CELLQUEST (Becton Dickinson and Company, New Jersey, USA).

**Table 1.** Positive genes in the RNAi screening

shRNA pool	Cell viability (% of control)	P-value	Gene symbol	Entrez gene name
CIITA-2	46.0 ± 7.8	< 0.001	CIITA	class II, major histocompatibility complex, transactivator
EPC1-1	74.8 ± 3.5	< 0.001	EPC1	enhancer of polycomb homolog 1
EPC1-2	37.4 ± 2.6	< 0.001	EPC1	enhancer of polycomb homolog 1
ESCO2-1	81.8 ± 9.0	< 0.001	ESCO2	establishment of cohesion 1 homolog 2
ESCO2-2	83.3 ± 7.3	0.028	ESCO2	establishment of cohesion 1 homolog 2
HAT1-1	60.5 ± 10.4	< 0.001	HAT1	histone acetyltransferase 1
HAT1-2	83.3 ± 2.2	0.009	HAT1	histone acetyltransferase 1
HDAC3-2	82.6 ± 5.6	0.007	HDAC3	histone deacetylase 3
SIRT1-2	75.7 ± 2.0	< 0.001	SIRT1	sirtuin 1

### Western blotting

Whole-cell lysates were resuspended in 1 × SDS loading buffer (1 mmol·L<sup>-1</sup> Na<sub>3</sub>VO<sub>4</sub>, 10 mmol·L<sup>-1</sup> NaF, and 1 mmol·L<sup>-1</sup> PMSF) containing protease inhibitors. Lysates (20 µg per lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), HAT1 (Santa Cruz, sc-390562), AKT (Santa Cruz, sc-8312), p-AKT (Santa Cruz, SC-7985-R), ERK (Santa Cruz, SC-7985-R), p-ERK (Santa Cruz, SC-7985-R), cyclinB1 (Santa Cruz, SC-7985-R), and cyclinD1 (Santa Cruz, SC-7985-R) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and were visualized by chemiluminescence detection system (Millipore, WBKLS0500).

### Patients and clinical tissue samples for qPCR and IHC

Primary tumor, adjacent tissue, and normal esophageal tissue were obtained from the Department of Thoracic Surgery, Zhongshan Hospital, Shanghai, China. The malignant samples used for immunohistochemistry were from the patients who underwent the surgery therapy at the same institute. The study was reviewed and approved by the Institutional Ethics Committee at Zhongshan Hospital, Shanghai, China. All the patients signed informed consents to give permission for the use of their tumor tissues.

### Immunohistochemistry

The tissue sections were cut for immunohistochemistry, blocked with milk for 10 min, and incubated with the primary monoclonal anti-

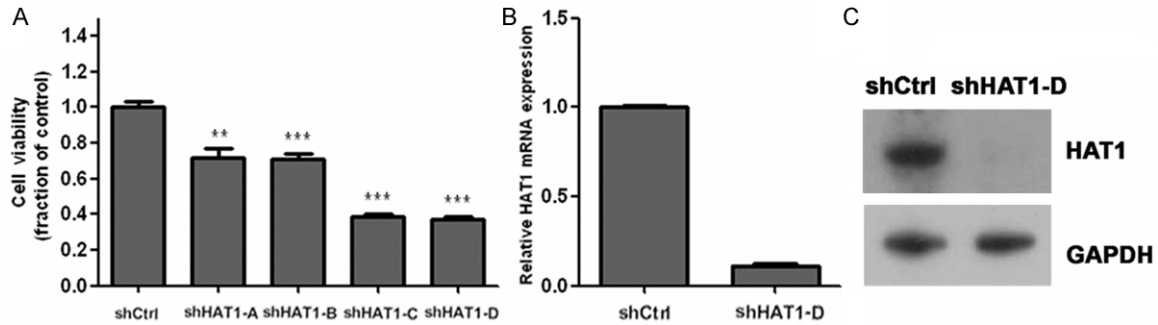
body for 2 h at 60°C. For HAT1 staining, sections were incubated with anti-HAT1 monoclonal antibody (1:2000 dilution; Gene, USA) for 24 min at 25°C. They were then incubated with HRP-DAB reagent (Roche) for 30 min. Hematoxylin was used as the nuclear counterstain and the sections were examined with a microscope (Nikon Eclipse 50i, Nikon Inc.).

The HAT1 positive Cells had brown granules in their nucleus. HAT1 expression was semi-quantitatively determined by two pathologists, as previously described [13]. Tissue sections were scored based on the immunostaining intensity of the nuclear HAT1 in the normal and tumorous esophageal epithelial cells, using the score 0, 1, 2, 3 to represent no staining, mild, moderate, and intense, respectively. Sections that scored 2 or 3 were evaluated as positive samples.

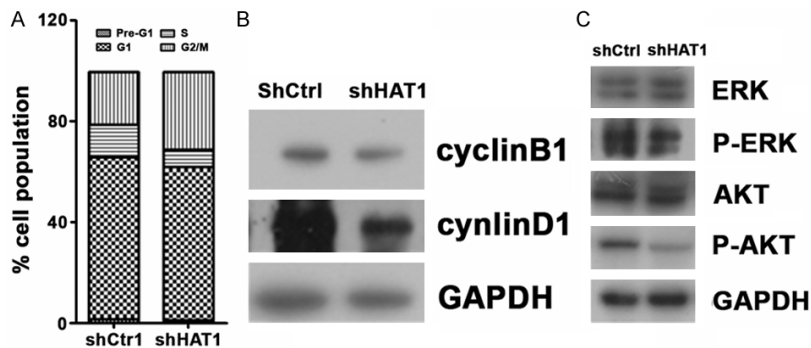
### Statistical analysis

Data were expressed as the mean±standard deviation. Statistical analysis was performed with SPSS version 19.0. One way analysis of variance was employed for the comparisons of the means among groups. A value of *P* < 0.05 was considered to be statistically significant. The relation between the HAT1 immunostaining score and its mRNA level was examined using quantitative RT-PCR and was analyzed using the Fisher's exact test. The relation between HAT1 overexpression and its clinicopathological characteristics was analyzed using the chi-square test or the Fisher's exact test. The overall survival curves were calculated using the Kaplan-Meier method and were compared using a log-rank test.

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**Figure 2.** Validation of HAT1 effect on cell viability. The positive hit HAT-1 shRNA pool from screening was revalidated with four distinct shRNA species (numbered as A, B, C, and D). Eca-109 cells were infected with four individual shRNA for five days and the cell viability was measured. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  as compared to shCtrl (Student's t test). Error bars represent the standard errors of the mean (SEM). shHAT1-D caused the most potent cell viability inhibition. Following infection of Eca-109 cells with shHAT1-D, HAT1 mRNA levels were quantified by qPCR as well as the protein levels were quantified by western-blot.



**Figure 3.** HAT1 knockdown induced G2/M cell cycle arrest. Eca-109 Cells were infected with shHAT1-D or shCtrl for five days. Cell cycle profiles were assessed by propidium iodide (PI) staining using flow cytometric analysis. Histogram represented the percentage of the cells in each phase of the cell cycle. CyclinB1 and cyclinD1 were measured by Western blot assay after the infection of the Eca-109 cells with shHAT1-D for five days. ERK 1/2 phosphorylation, AKT phosphorylation at Ser473, total ERK1/2, and total AKT were determined by Western blot assay after the infection of the Eca-109 cells with shHAT1-D for five days.

## Results

### Identification of HATs/HDACs involved in Eca-109 cell proliferation through RNAi screening

An RNAi screening was adopted to identify the HATs/HDACs regulate of cell survival as previously described. A total of six genes were identified, where their knockdown significantly decreased the cell viability by  $\geq 20\%$  as compared to the negative control (Figure 1, Table 1). Among these genes, HDAC1 has been demonstrated to play a critical role in tumorigenesis and has since become a potent target for cancer therapy [14]. Other genes, such as KAT2B and EPC1, have barely been studied in the context of cancer, except that KAT2B was

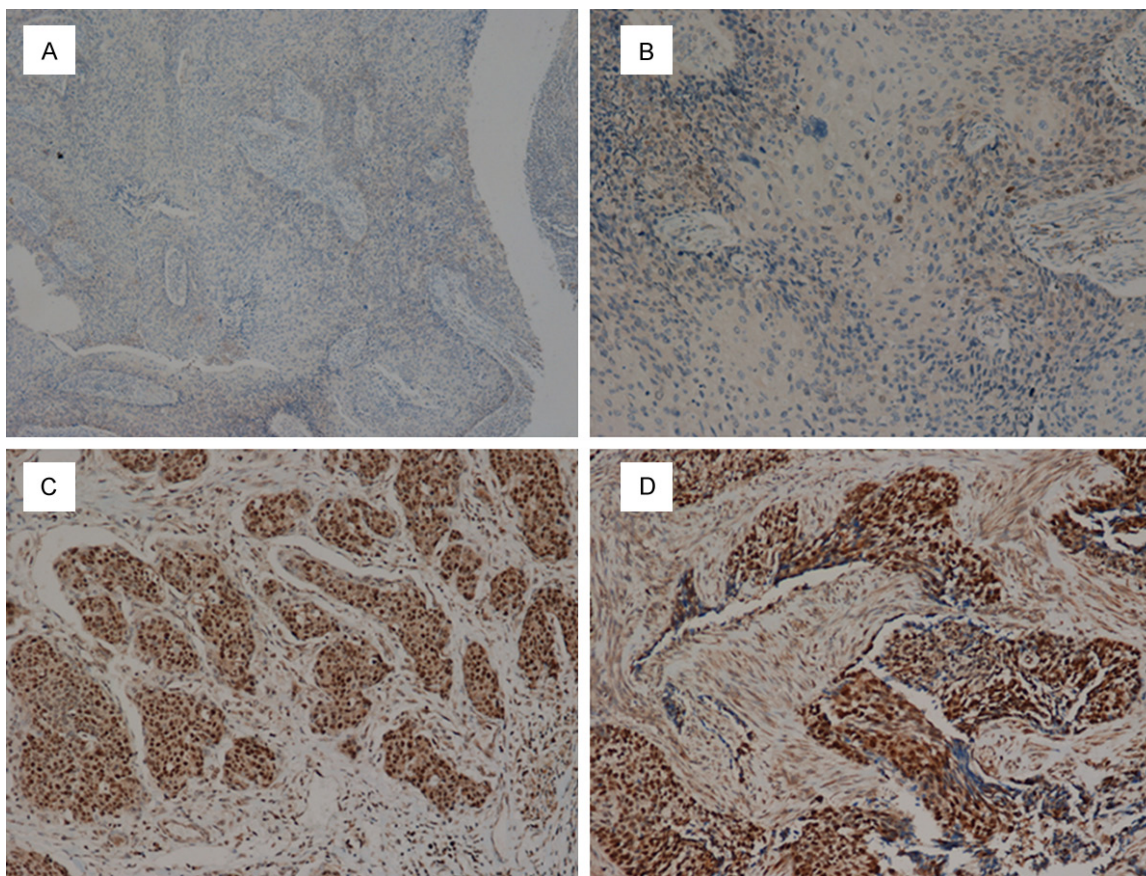
reported to acetylate a novel anticancer gene Brahma (BRM) [15]. EPC1 has been characterized as a significantly mutated gene in the pancreatic cancer [16]. Another novel distinguished gene, HAT1, was the first histone acetyltransferase identified. A number of studies have begun to link HAT1 to cancer. In many cases of diffuse large B-cell lymphoma (DLBCL) the expression of HAT1 was higher as compared to the reactive lymphoid hyperplasia, and was correlated with a poor survival [17]. In both primary and metastatic human colorectal cancer samples HAT1 mRNA and protein levels were elevated as compared to those of the normal colorectal mucosa [13]. However, the function of HAT1 in either esophageal cancer development or target therapy is not well known. Therefore, we further validated the influence of HAT1 inhibition on cell survival and elucidated the molecular mechanism through which HAT1 regulates Eca-109 cell survival.

In the primary screen, inhibition of HAT1 significantly decreased the Eca-109 cell viability by 39% ( $P = 0.009$ ). To validate the specificity of

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**Figure 4.** Representative photographs of negative HAT1 expression in well-differentiated (A&B) and positive HAT1 expression in poor-differentiated (C&D) esophageal squamous cell carcinoma(ESCC). (A) HAT1 negative tissue with a lower power view (20 ×) with less nuclear staining in the well-differentiated squamous cell carcinoma. (B) HAT1 negative tissue with a medium power view (100 ×) with less positive staining in well-differentiated squamous cell carcinoma. (C) HAT1 positive tissue with a lower power view (50 ×) with diffuse nuclear staining in poorly differentiated carcinoma. (D) HAT1 positive tissue with medium power view (100 ×) with the cell nuclear strongly immunoreactive for HAT1.

the effects observed, HAT1 was reassayed using each of the four different shRNA species that comprised the positive shRNA pool. We observed two shRNA species that caused a significant reduction in the viability of Eca-109 by  $\geq 60\%$  (**Figure 2A**). This observation indicated that the phenotype caused by HAT1 knockdown was unlikely to be the result of an off-target effect [18]. Therefore, shHAT1-D was selected for further study based on its antiproliferative effects, and the target silencing efficacy of shHAT1-D was confirmed by quantitative PCR and western-blot (**Figure 2B, 2C**).

#### *Knockdown of HAT1 induced a G2/M cell cycle arrest*

To further elucidate the mechanism of the inhibition of Eca-109 cells by the HAT1 silencing,

cell cycle profiles were monitored by flow cytometric analysis of DNA content. The results suggested that HAT1 knockdown induced a G2/M cell cycle arrest. The cells with HAT1 knockdown showed an increase in the percentage of cells in the G2/M phase from 20.8% to 30.5% as compared to the negative control. Therefore, the growth inhibitory effect of HAT1 knockdown might be due to the G2/M cell cycle arrest.

To investigate the underlying molecular mechanism of the cell cycle arrest, we examined the expression of cell cycle-related molecules by western blot analysis. Also, we found that cyclinB1 expression was decreased as well as cyclinD1 expression was reduced following HAT1 inhibition. Furthermore, phosphorylation of ERK and AKT was markedly inhibited while

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**Table 2.** Expression profile of HAT1 in 167 EC samples

Clinico-pathological parameters	No (n)	HAT1 IHC		$\chi^2$	<i>p</i>
		Positive (%)	Negative (%)		
<b>Age</b>					
≥ 65	61	38 (62.3)	23 (37.3)	0	0.997
< 65	106	66 (62.3)	40 (37.3)		
<b>Gender</b>					
Male	122	80 (65.6)	44 (34.4)	1.029	0.31
Female	43	24 (55.8)	19 (44.2)		
<b>Differentiation</b>					
High	11	2 (18.2)	9 (81.8)	18.303	< 0.0001
Median	120	71 (59.2)	49 (41.8)		
Low	36	31 (86.1)	5 (13.9)		
<b>T staging</b>					
T1	12	8 (66.7)	4 (33.3)	2.658	0.447
T2	40	22 (55.0)	18 (45.0)		
T3	108	68 (63.0)	40 (37.0)		
T4	7	6 (85.7)	1 (14.3)		
<b>Lymph node metastasis</b>					
0	99	62 (62.6)	37 (37.4)	0.396	0.82
< 3	36	21 (58.3)	15 (41.7)		
≥ 3	32	21 (65.6)	11 (34.4)		
<b>TNM staging</b>					
I (IA+IB)	39	25 (64.1)	14 (35.9)	0.765	0.858
II (IIA+IIB)	63	38 (60.3)	25 (39.7)		
III (IIIA+IIIB+IIIC)	64	40 (62.5)	24 (37.5)		
Iva	1	1 (100)	0 (0)		
Total	167	104 (62.3)	63 (37.7)		

the total AKT and ERK levels remained unaltered (**Figure 3B, 3C**). CyclinB1 activity is essential for the progression from G2 into M phase. On the other hand, cyclinD1 as a critical factor for initiation of cell cycle and proliferation was downregulated, which might have resulted from the down-regulation of the AKT and ERK pathways [19, 20].

### *HAT1 was highly expressed in clinical EC samples*

To determine the potential clinical relevance of the observed shHAT1-mediated inhibitory effect, we measured HAT1 mRNA levels in the primary tumor, adjacent tissue, and normal esophageal tissue from 10 EC patients. Compared with normal tissue, HAT1 expression was obviously higher in the primary tumor ( $P = 0.013$ ) and the adjacent normal tissue ( $P = 0.0342$ ). In the primary tumor, the expression was higher as compared to the adjacent tissue,

but this difference did not reach a statistical significance. Strikingly, the two primary tumor samples showed extremely high expression levels of HAT1 (**Figure 4**).

*High expression of HAT1 correlated with poor tumor differentiation in EC*

We carried out an immunohistochemical analysis in 167 EC samples for which the clinical features and survival time were available. As shown in **Table 2**, HAT1 expression was detected in 104 of the 167 (62%) EC patients. As shown in **Figure 4**, a high expression of HAT1 was significantly associated with a poor tumor differentiation ( $P < 0.0001$ ) (**Figure 4**), but had no statistically significant correlation with survival ( $P = 0.766$ ) (**Figure 6**), or other clinicopathological

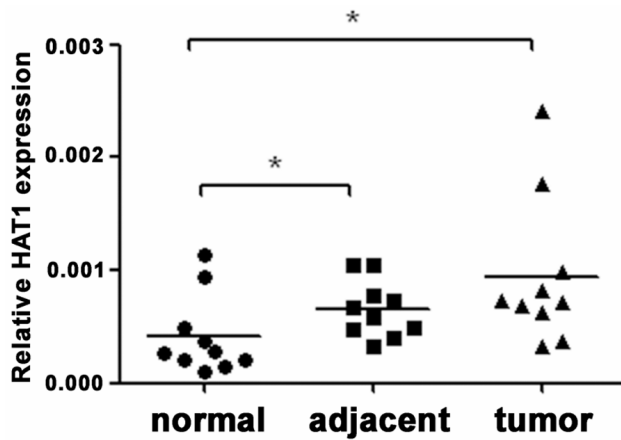
parameters, such as gender ( $P = 0.31$ ), age ( $P = 0.997$ ), T staging ( $P = 0.447$ ), lymph node metastasis ( $P = 0.82$ ), or TNM staging ( $P = 0.858$ ).

### **Discussion**

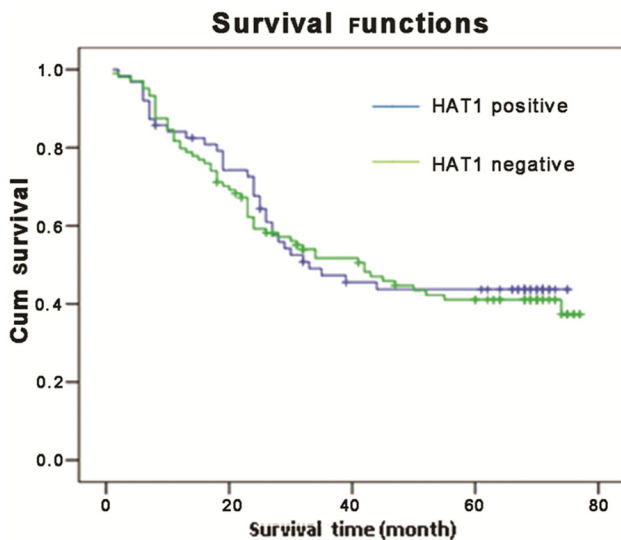
Lack of predictive biomarkers and effective therapeutic targets for esophageal carcinoma is the major limiting factor in the successful treatment of EC. Therefore, identification of the novel EC related genes would be of significant clinical benefit. In this study, we demonstrated, for the first time, the significant role of HAT1 in the proliferation of the EC cells through an unbiased cell proliferation RNAi screen on HATs/HDACs in human EC Eca-109 cells.

RNAi is an RNA-dependent gene-silencing process that is controlled by the RNA-induced silencing complex and is initiated by short double-stranded RNA molecules [21]. Elbashiret

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**Figure 5.** The mRNA expression of HAT1 in tissue samples from 10 cases of ESCC patients. The mRNA expression of HAT1 was measured by qPCR in 10 primary tumors, its adjacent tissues, and normal esophageal tissues. Each sample was normalized to the housekeeping gene GAPDH.



**Figure 6.** Comparison of the survival distribution between ESCC patients with positive and negative HAT1 expression. There was no significant difference in the overall survival between patients with positive and negative expression of HAT1, HR [95% CI] = 1.061 [0.615-1.431], ( $P = 0.766$ ).

and his colleagues discovered that RNAi could be used in mammalian cells to suppress certain gene expressions [22]. Using this powerful tool, the high throughput loss-of-function screens began to become feasible in mammalian cells. Furthermore, loss-of-function screens became popular as the genetic event mimicked the intended effect of the cancer drug reduced gene product activity [23]. The “Off-target” effects, which is the nonspecific suppression of

mRNAs by siRNA or shRNAs, might take place in RNAi. However, only a partial complementarity between the siRNA/shRNA and the mRNA might cause this suppression [24]. Having several independent siRNAs/shRNAs targeting different regions in a given mRNA that are able to produce the same phenotype might minimize the chances for an “off-target” effect [18]. So, we used eight different shRNAs for each HAT/HDAC gene for RNAi to find the target genes and choose the shRNA, which highly suppressed the expression of the target gene.

HAT1 is an evolutionarily conserved type B histone acetyl transferase. It is believed to participate in several cellular processes, such as acetylation of newly synthesized histone H4 [25], DNA repair-linked chromatin assembly [26, 27], telomeric silencing [28]. Previous researches showed that the expression level of HAT1 was increased in the stem cells as well as tumor cells [13, 17, 28, 29], while the underlying mechanism remained unclear. In the present study, we found that HAT1 knockdown induced a G2/M cell cycle arrest in the EC cells, which eventually led to growth inhibition.

The process of cell cycle depends on a precise and rigorous regulation. There are two critical restrictive sites in the cell cycle, including: G1/S phase and G2M phase [30]. CyclinD1 plays a leading role at G1 phase and in promoting cell proliferation. In our case, HAT1 silencing markedly inhibited ERK and AKT phosphorylation. We also observed a decrease of cyclinD1 as a result of AKT and ERK pathways’ inhibition, which was in line with the cell growth inhibition. Also, we found that cyclinB1 expression was decreased following HAT1 silencing. Progression of cells from G2 phase to mitosis is tightly regulated by cyclinB1, and several researches showed down-regulation of cyclinB1 might cause G2/M arrest [31-33].

AS a representative of type B histones, HAT1 is supposed to be completely localized in the cytoplasm [34]. However, a number of evidences have shown that this enzyme is actually both cytoplasmic and nuclear [35-38]. A vast majority of HAT1 is nuclear in oocytes, and in fully



differentiated cells Hat1 is found in both the cytoplasm and nucleus [39]. We also found through IHC that HAT1 was mainly expressed in the nuclear rather than plasma of the EC cells.

The expression of HAT1 was validated to be higher in the primary tumor and tumor adjacent tissue as compared to the normal esophageal tissue in our studies through quantitative PCR. A number of studies have also linked high expression of HAT1 to cancer. Pogribny et al. studied the changes in a variety of epigenetic markers during the progression of the liver tumors that resulted from a methyl-deficient diet in a rat model [40]. They found a spectrum of the histone post-translational modification changes during tumor progression, where Hat1 substantially increased in the liver tumors. Seiden-Long et al. found that HAT1 mRNA and its protein levels were elevated in the primary and metastatic human colon cancer tissues [13]. In addition, immunohistochemical staining of normal, primary, and metastatic colon cancer tissue has shown that Hat1 was primarily nuclear in normal cells, but that there was a large increase in the cytoplasmic pool of Hat1 in the tumor tissues. Hence, the subcellular distribution of Hat1 might be an important factor in its activity in mammalian cells. While still at a preliminary stage, different degrees of evidence is beginning to suggest that Hat1 might play a clinically relevant role in human cells. Researchers have found that HAT1 played a role in the chromatin assembly that occurs in conjunction with the recombinant DNA repair [41, 42]. The higher level of HAT1 seen in stem cells [28] and tumor cells [13, 40] is consistent with the proposed role for HAT1 in the process of chromatin assembly. It might reflect the obvious need of proliferating cells to maintain a high chromatin assembly capacity for the ongoing DNA replication.

Min et al. found that the degree of HAT1 expression was higher in cases of diffuse large B-cell lymphoma (DLBCL), peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) or extranodal NK/T-cell lymphoma, nasal type (NKTCL) as compared to that of the reactive lymphoid hyperplasia and HAT1 might be indicators for a poor prognosis in cases of diffuse large B-cell lymphoma as cooperating factors [17]. We found that HAT1 expression was directly correlated to the EC tissue tumor differentia-

tion. Furthermore, an increased HAT1 expression was correlated to poor differentiation, but it was not correlated with survival (**Figure 5**). The exact role of Hat1 in the EC cells is still not clear and needs more studies.

Our study unraveled, for the first time, a critical role for HAT1 in Eca-109 cell proliferation. Our data also showed that the expression of HAT1 was higher in the primary tumor as compared with that of the normal esophageal tissue. Furthermore, high expression of HAT1 was correlated with a poor tumor differentiation in EC. The exact role of HAT1 in EC still needs more investigations.

#### Disclosure of conflict of interest

None.

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