

Aberrant Methylation of *HLTF* Gene in Human Esophageal Cancer

Liang Wang^{1,3*}, Xiaojing Zhang^{1*}, Peng Yin^{1*}, Yan Gao⁵, Yuan Zhang¹, Xianling Feng¹,
Si Chen¹, Huimin Yu¹, Weiling Huang¹, Yong Huang¹, Qianhe Jian¹, Zhenfu Zhao¹,
Xinmin Fan¹ and Zhe Jin^{1,2,3,4}

¹Department of Pathology, The Shenzhen University School of Medicine, Shenzhen, Guangdong, People's Republic of China, 518 060

²Shenzhen Key Laboratory of Micromolecule Innovational Drugs, The Shenzhen University School of Medicine, Shenzhen, Guangdong, People's Republic of China, 518 060

³Shenzhen Key Laboratory of translational Medicine of Tumor, School of Medicine, Shenzhen University, Shenzhen, Guangdong, People's Republic of China, 518 060

⁴Laboratory of Chemical Genomics, School of Chemical Biology and Biotechnology, Peking University Shenzhen Graduate School, Shenzhen, Guangdong, P.R. of China, 518 055

⁵Nanshan Hospital, Guangdong Medical College, Shenzhen, Guangdong, P.R. of China, 518 052

KEYWORDS Alcohol. EAC. ESCC. *HLTF*. Hypermethylation

ABSTRACT The aim of this study was to investigate whether and at which neoplastic stage promoter hypermethylation of Helicase-like Transcription Factor (*HLTF*) is involved in human esophageal carcinogenesis. The researchers examined *HLTF* promoter hypermethylation using real-time quantitative methylation-specific PCR in 229 primary human esophageal tissues of contrasting histological types. Both *HLTF* mean normalized methylation value (NMV) and hypermethylation frequency were significantly higher in dysplastic Barrett's esophagus (Dy0.0303 and 10.0%), and esophageal adenocarcinomas (EAC, 0.0079 and 10.4%) than in normal esophagus (NE, 0.0006 and 0.0%; $p < 0.05$ and $p < 0.05$, respectively). Incremental increases in the frequency of *HLTF* hypermethylation were observed during progression from NE (0.0%) to Barrett's esophagus (BE, 3.3%), D (10.0%), and EAC (10.4%). Meanwhile, *HLTF* mean NMV was significantly higher in esophageal squamous cell carcinoma (ESCC, 0.0102) than in NE ($p < 0.05$). Also, *HLTF* was hypermethylated in 7.7% ESCCs. Furthermore, mean NMV of *HLTF* was significantly higher in current alcohol drinking EAC patients (0.0194) than in non-current ones (0.0066, $p < 0.05$). *HLTF* hypermethylation is an uncommon event in human esophageal cancer, but occurs early in a subset of EAC, and is related to the alcohol drinking status of EAC patients.

INTRODUCTION

Esophageal cancer ranks as the 8th most-frequent and 6th most-fatal cancer type worldwide, with an estimated 480,000 new cases diagnosed and 400,000 deaths globally in 2008 (Bray

et al. 2013; Jemal et al. 2011). There are two major histologic types of esophageal cancer: esophageal adenocarcinoma (EAC), which is more prevalent in Western countries, with a rapid increase in incidence; and esophageal squamous cell carcinoma (ESCC), which occurs at high frequencies in many developing countries, especially in Asia, including China (Zhang et al. 2015). Since both types of esophageal cancer exhibit highly aggressive behavior, with rapid progression to death (Enzinger et al. 2003; Kleinberg et al. 2007), it is essential to gain a better understanding of the molecular events underlying these tumors in order to make further improvements in survival. Thus, it is important to discover molecular events, novel early detection biomarkers, and potential targets for chemoprevention or therapy.

Helicase-like Transcription Factor (*HLTF*), a member of the SWI/SNF2 family which mainly act as chromatin remodelers (Debaube et al. 2008), is a DNA helicase protein involved in the maintenance of genomic stability and the regu-

*Address for correspondence:

Zhe Jin
Department of Pathology,
The Shenzhen University School of Medicine
Rm 703, 3688 Nanshai Ave, Nanshan District,
Shenzhen, Guangdong, People's Republic of China
518060
Telephone: 086-0755-86671904
Fax: 086-0755-86671906
E-mail: zhejin1995@yahoo.com

Liang Wang
Department of Pathology,
The Shenzhen University School of Medicine
Rm 703, 3688 Nanshai Ave, Nanshan District,
Shenzhen, Guangdong, People's Republic of China
518060
Telephone: 086-0755-86671902
Fax: 086-0755-86671906
E-mail: liangwang@szu.edu.cn

lation of gene expression (Ding et al. 1996). Consequently, it has been reported that downregulation of *HLTF* protein in HCT116 cells induces a small decrease in DNA damage-induced proliferating cell nuclear antigen(PCNA), ubiquitination and causes increased genome instability (Motegi et al. 2008). Some investigations have also indicated that downregulation of *HLTF* gene expression may constitute a common molecular event which contributes to the initiation and/or progression of several cancers, including those arising in the digestive tract. For example, Craig et al. observed that *HLTF* protein was undetectable in four out of seven colon cancers tested, whereas the levels of SMC1 and GAPDH were relatively constant by Western blotting (MacKay et al. 2009). Other two groups of researchers also showed the loss of *HLTF* mRNA expression in some colon cancer cell lines (Hibi et al. 2003; Moinova et al. 2002). *HLTF* levels were also very low in leukaemia cell line and at moderately low levels in non-small lung tumor cell lines (MacKay et al. 2009). Therefore, these results suggest that *HLTF* possesses tumour-suppressive capabilities.

Aberrant methylation of promoter CpG islands upstream of tumor suppressor genes is now well-established as a major mechanism of gene inactivation in human tumorigenesis (Wang et al. 2009), including ESCC and EAC, where it plays an important role in pathogenesis (Agarwal et al. 2012; Clement et al. 2006; Jin et al. 2009; Mori et al. 2006; Tischoff et al. 2007). However, some of these methylation events appear to represent useful prognostic markers, as they precede and thus predict the progression of Barrett’s esophagus (BE) to EAC. Studies have

shown that CpG island methylation was one of the main mechanisms that lead to the silence of *HLTF* in numerous cancers, especially in colon, stomach, lung and cervical adenocarcinoma (Castro et al. 2010; Hibi et al. 2003; Kang et al. 2006; Leung et al. 2003; Moinova et al. 2002; Wallner et al. 2006). However, the methylation profiles of *HLTF* remain uncharacterized in human esophageal cancer.

The researcher’s goal was to determine the methylation profiles of *HLTF* in human esophageal cancer (EAC and ESCC), premalignant lesions [Barrett’s esophagus (BE), and BE with dysplasia(D)], and normal esophagus(NE) by real-time quantitative methylation-specific PCR (qMSP).

MATERIAL AND METHODS

Tissue Samples

In the current study, 67 NE, 60 BE, 40 D, 67 EACs, and 26 ESCCs were examined. All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland and Baltimore Veterans Affairs Medical Centers, where all esophagogastroduodenoscopies were performed. Biopsies were taken using a standardized biopsy protocol, as previously described (Jin et al. 2009). Research tissues were obtained from grossly apparent Barrett’s epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots obtained at endoscopy. All biopsy specimens were stored in liquid nitrogen before DNA extraction. Clinicopathologic characteristics are summarized in Table 1.

Table 1: Clinicopathologic characteristics and methylation status of *HLTF* in different human esophageal tissues

	Number of samples	Age (year) Mean	NMV		Methylation status (cut off 0.02)			
			Mean	p*	Frequency	UM	M	P
<i>Histology</i>								
Normal esophagus	67	64.4	0.0006		0.0%	67	0	
Barrett’s metaplasia	60	63.7	0.0024	>0.05 [§]	3.3%	58	2	>0.05 ^{§,†}
Dysplasia in Barrett’s esophagus	40	65.3	0.0303	<0.05 [§]	10.0%	36	4	<0.05 ^{§,†}
EAC	67	65.1	0.0079	<0.05 [§]	10.4%	60	7	< 0.01 ^{§,‡}
ESCC	26	62.5	0.0102	<0.05 [§]	7.7%	24	2	> 0.05 ^{§,†}
<i>Barrett’s Segment</i>								
Short-segment (<3cm)	14	62.3	0.0056		7.1%	13	1	
Long-segment (>=3cm)	16	62.8	0.0002	> 0.05 ^{**}	0.0%	16	0	> 0.05 ^{**} ,†

EAC: esophageal adenocarcinoma; ESCC: esophageal squamous cell carcinoma; UM, unmethylated; M, methylated; NMV: normalized methylation value; *, Student’s t test; †, Fisher’s exact test; ‡, Chi-square for independence test; §, comparisons made to normal esophagus; **, comparison made to short-segment.

DNA Extraction

Genomic DNA was extracted from biopsies using a DNeasy Tissue Kit (*Qiagen, Valencia, CA*). DNAs were stored at -80°C before analysis.

Bisulfite Treatment and Real-time Quantitative Methylation-specific PCR

DNA was treated with bisulfite to convert unmethylated cytosines to uracils prior to qMSP, as described previously (Jin et al. 2009). Promoter methylation levels of *HLTF* were determined with the ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA), using primers and probes as described previously (Mori et al. 2006). A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (CHEMICON, Temecula, CA). The normalized methylation value (NMV) was defined as follows: $\text{NMV} = (\text{HLTF-S}/\text{HLTF-FM})/(\text{ACTB-S}/\text{ACTB-FM})$, where *HLTF-S* and *HLTF-FM* represent the methylation levels of *HLTF* in sample and universal methylated DNAs,

respectively, while *ACTB-S* and *ACTB-FM* correspond to β -*Actin* in sample and universal methylated DNAs, respectively.

Data Analysis and Statistics

Receiver-operator characteristic (ROC) curve analysis (Hanley et al. 1982) was performed using NMVs for the 67 EAC, 26 ESCC and 67 NE by Analyse-it software (Version 1.71, Analyse-it Software, Leeds, UK). Using this approach, the area under the ROC curve (AUROC) yielded optimal sensitivity and specificity to distinguish normal from malignant esophageal tissues. Also, corresponding NMV thresholds were calculated for *HLTF*. The cut-off value determined from this ROC curve was applied to determine the frequency of *HLTF* methylation in each tissue type included in the present study. For all other tests, Statistica (version 6.1; StatSoft, Inc., Tulsa, OK) was used. Differences with $p < 0.05$ were deemed significant.

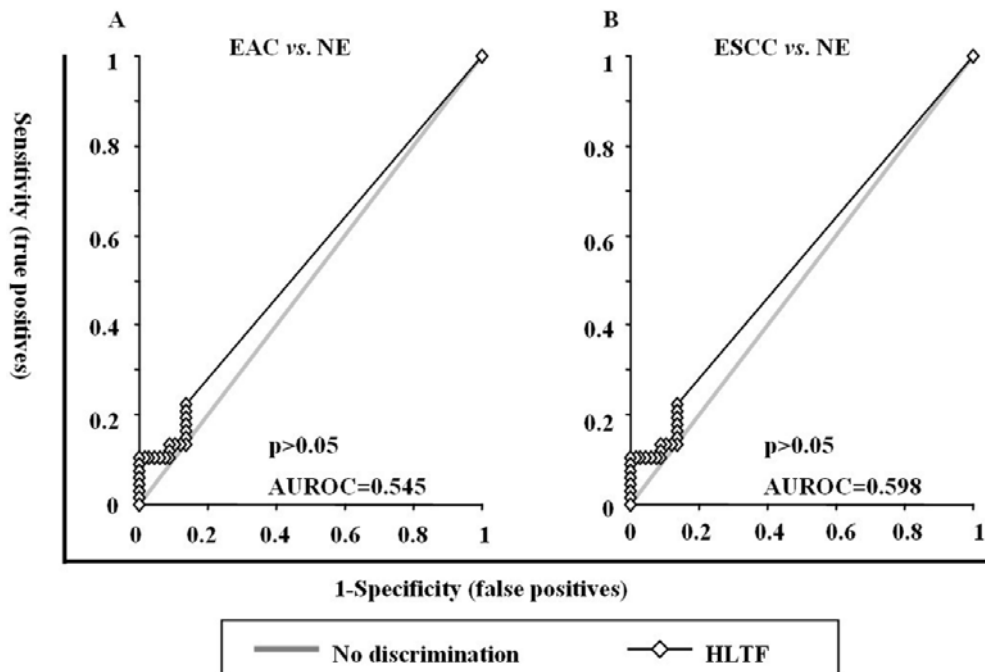


Fig. 1. Receiver-operator characteristic (ROC) curve analysis of normalized methylation value (NMV) ROC curve analysis of *HLTF* NMVs of esophageal adenocarcinoma (EAC) vs. normal esophagus (NE) (A), esophageal squamous cell carcinoma (ESCC) vs. NE (B)

RESULTS

Promoter hypermethylation of *HLTF* was analyzed in 67 NE, 60 BE, 40 D, 67 EAC, and 26 ESCC. All qMSP assays were performed in duplicate format, and data showed reproducible and concordant results. *HLTF* promoter hypermethylation didn't show significant discriminative ROC curve profiles and area under the ROC curves, not distinguishing EAC and ESCC from NE (Fig. 1).

The cutoff NMV for *HLTF* (0.02) was chosen from ROC curve in order to maximize sensitivity and specificity. Mean NMV and frequency of *HLTF* hypermethylation for each tissue type are shown in Tables 1 and 2. NMVs of *HLTF* were significantly higher in D(0.0303), EAC(0.0079) and ESCC(0.0102) than in NE (0.0006, all $P < 0.05$, Student's t test). The frequency of *HLTF* hypermethylation was significantly higher in D(10.0%) and EAC (10.4%) than in NE (0%; $p < 0.05$ Fisher's exact test and $p < 0.01$, Chi-square for independence test, respectively). Also, it increased early during EAC neoplastic progression, from 0.0% in NE to 3.3% in BE from patients with Barrett's metaplasia alone, 10.0% in D, and 10.4% in EAC. *HLTF* mean NMV was significantly higher in ESCC (0.0102) than in NE ($p < 0.05$, Student's t test), and frequency of *HLTF* hypermethylation was not significantly higher in ESCC (7.7%) than in NE (0.0%, $p > 0.05$, Fisher's exact test).

Interestingly, mean NMV of *HLTF* was significantly higher in current alcohol drinking EAC patients (0.0194) than in non-current ones (0.0066, $p < 0.05$, Mann-Whitney U test). The frequency of *HLTF* hypermethylation was clearly higher in current alcohol drinking EAC (20.0%) patients than in non-current ones (9.7%, $p > 0.05$, Fisher's exact test, Table 2). No significant associations were observed between *HLTF* promoter hypermethylation and patient age (data not shown), survival (data not shown), length of BE segment (Table1), tumor stage or lymph node metastasis, and smoking (Table 2).

DISCUSSION

In the current study, the researchers systematically investigated hypermethylation of the *HLTF* gene promoter in primary human esophageal lesions of differing histological types and neoplastic stages. Both *HLTF* mean NMV and hypermethylation frequency were significantly higher in D and EAC than in NE (Table 1). The frequency of *HLTF* hypermethylation was 0.0% in NE but increased slightly at the very early preneoplastic stage of BE (3.3%), while being maintained in D (10.0%) and EAC (10.4%, Table 1). In addition, *HLTF* mean NMV was significantly higher in current alcohol drinking EAC patients than in non-current ones (including never and former alcohol drink, Table 2). Though *HLTF* mean NMV was significantly higher in

Table 2: Relationship of clinicopathologic characteristics and *HLTF* hypermethylation in esophageal adenocarcinoma patients

	Number of samples	Age (year) Mean	NMV		Methylation status (cut off 0.02)			
			Mean	p^*	Frequency	Um	M	P
<i>UICC Stage</i>								
I	7	63	0.0000	$> 0.05^*$	0.0%	7		$> 0.05^\ddagger$
II	15	65.2	0.0060		13.3%	13	2	
III	25	64.6	0.0122		12.0%	22	3	
IV	7	66.3	0.0121		14.3%	6	1	
<i>Lymph Node Metastasis</i>								
Negative	25	64.9	0.0070	$> 0.05^{**}$	12.0%	22	3	$> 0.05^\ddagger$
Positive	25	64.6	0.0122		12.0%	22	3	
<i>Smoking Status</i>								
Non-current	30	66.5	0.0069	$> 0.05^{**}$	6.7%	28	2	$> 0.05^\ddagger$
Current	13	60.8	0.0145		23.1%	10	3	
<i>Alcohol Drinking Status</i>								
Non-current	31	64.5	0.0066	$< 0.05^{**}$	9.7%	28	3	$> 0.05^\ddagger$
Current	10	65.7	0.0194		20.0%	8	2	

NMV: normalized methylation value; UM, unmethylated; M, methylated; *, Kruskal-Wallis test; **, Mann-Whitney U test; †, Fisher's exact test.

ESCC than in NE, the frequency of *HLTF* hypermethylation was not significantly higher in ESCC than in NE. Thus, these results imply that *HLTF* hypermethylation is an uncommon event in human esophageal cancer, but occurs early in a subset of EAC. In addition, it is related to alcohol drinking status of EAC patients, suggesting that the aberrant methylation of *HLTF* may be involved in the pathogenesis of a subset of EAC.

Mutations, including changes in nucleic acid sequences, and chromosomal rearrangements or aneuploidy are central to carcinogenesis (Schmitt et al. 2012; Szyberg et al. 2015). Furthermore, *HLTF* display a double-stranded DNA translocase activity, which promotes the resolution of stalled replication forks at DNA damage lesions (Blastyak et al. 2010; Lin et al. 2011; Sommers et al. 2015) and possesses a chromatin remodeling activity, which leads to the displacement of DNA-bound proteins on stalled replication forks and facilitates DNA-damage repair (Achar et al. 2011). These findings demonstrate that *HLTF* may play an important role in an error-free post-replicative repair pathway and therefore possesses tumour-suppressive capabilities. However, to the best of our knowledge, the precise roles of *HLTF* in physiology and pathophysiology remain unclear elucidated especially in human esophageal cancer. Only two studies, till date, reported the methylation pattern of *HLTF* gene in esophageal cancer. Using traditional methylation-specific PCR (MSP), Hibi et al. (2003) examined the methylation status of *HLTF* in digestive tract cancer cell lines as well as in colorectal and esophageal cancer specimens. They found that 2 out of 4 colorectal and none out of 4 esophageal cancer cell lines exhibited abnormal promoter methylation of *HLTF* gene. In primary digestive tract cancers, 25 out of 76 colorectal and 1 out of 40 (2.5%) esophageal cancers showed *HLTF* hypermethylation (Hibi et al. 2003). Similarly, Fukuoka et al. reported that aberrant methylation of *HLTF* was detected 1 out of 35 (3.0%) ESCC specimens by traditional MSP (Fukuoka et al. 2006). However, the results of the aforementioned studies suggest that *HLTF* is not a common target for methylation and epigenetic gene silencing in esophageal cancers including ESCC. In the current study, abnormal methylation of *HLTF* was found in the patients of BE, D, EAC and ESCC by qMSP, suggesting that *HLTF* methylation occurs in human esophageal cancers, especially in a sub-

set of EAC. These inconsistent results are probably due to the difference of methylation detect methods, sample size, and the genetic background or environmental factors, like lifestyles, in modulating susceptibility for esophageal cancers.

It has been well established that lifestyle factors, especially alcohol consumption and tobacco smoking, are risk factors for upper digestive tract cancer (Dal Maso et al. 2002). Moreover, two different International Agency for Research on Cancer (IARC) working groups concluded independently that alcohol was related to esophageal cancer (Baan et al. 2007; Secretan et al. 2009). However, the carcinogenic mode of action of alcohol is not well understood. Recent studies of alcohol-dependent patients have reported alterations in the methylation levels of specific genes. For instance, Bönsch et al. (2005) observed a significant increase of the *alpha synuclein* promoter DNA methylation in patients with alcoholism which was significantly associated with their elevated homocysteine levels. Bleich et al. (2006) observed a significant increase in the *HERP* promoter DNA methylation in alcoholic patients with alcohol dependence when compared with healthy controls, which was significantly associated with their elevated homocysteine levels. Our data revealed that *HLTF* hypermethylation is related to alcohol drinking status in EAC patients suggesting that alcohol may act as a selective agent, promoting growth of biopsies with *HLTF* promoter methylation, and that the effect is independent of the effect of tobacco.

CONCLUSION

The current findings established that hypermethylation of the *HLTF* promoter is an uncommon event in human esophageal cancer, but occurs early in a subset of EAC, and is related to the alcohol drinking status of EAC patients.

ACKNOWLEDGEMENTS

This study was supported by the National Nature Science Foundation of China grant No. 81172282, the Shenzhen Peacock Plan KQCX20130621101141669, the Planned Science and Technology Project of Shenzhen No. GJHS20120621142654087, the Key Laboratory Project of Shenzhen No. ZDSY20130329 10 1130 496, Natural Science Foundation of SZU grants

201108 and T201202 to Z Jin; The Science and Technology Bureau of Shenzhen City grant JCYJ20150525092940973 to L Wang; National Nature Science Foundation of China grant 81302151 to XJ Zhang.

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Paper received for publication on July 2015
Paper Accepted for publication on March 2016