RESEARCH ARTICLE

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Evaluation of Long Non-Coding RNAs: *HNF1A-AS1* and *MVIH* Expressions and Their Clinical Significance in Human Gastric Cancer

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A B S T R A C T

Gastric cancer is one of the most common cancers in the world. Late diagnosis is the main cause of the high rate of treatment failure and death among patients with gastric cancer; therefore identifying the molecular basis of cancer initiation and metastasis is so critical for developing efficient methods for early diagnosis and therapy. long non-coding RNAs (lncRNAs) are the largest group of noncoding RNAs, which are involved in cancer pathogenesis. Regarding the roles of *lncRNA-HNF1A-AS1* and *lncRNA-MVIH* in cancer pathogenesis and progression, we aimed to evaluate expression profiles and clinicopathological relevance of these two genes in human gastric cancer. Real-time PCR was performed to assess relative gene expressions in 60 tumoral and non-tumoral gastric tissues. The association between gene expressions and clinicopathological characteristics were also analyzed. Expression and clinicopathological data of the *lncRNA-HNF1A-AS1* from 318 gastric cancer patients were also downloaded from the TCGA database. *HNF1A-AS1* was down-regulated in GC tissues and *MVIH* did not show any significant differential expression in GC tissues. Otherwise, the expression of *lncRNA-HNF1A-AS1* was significantly higher in TCGA tumor samples. Furthermore, *lncRNA-HNF1A-AS1* expression was associated with tumor metastasis and that of *lncRNA-MVIH* showed association with tumor grade and stage. *lncRNA-HNF1A-AS1* expression status did not show any significant correlation with GC overall survival. In conclusion, *lncRNA-HNF1A-S1* and *lncRNA-MVIH* genes may play a critical role in gastric cancer progression. Functional studies on the mechanism of action of these two lncRNAs could help to understand their role in gastric cancer progression.

Introduction

Gastric cancer is one of the most common cancers in the world and the second leading cause of cancer death in the world (Danaei *et al*., 2005). Gastric cancer is also the most common gastrointestinal cancer in Iran. Its incidence is dependent on the geographic area. The northern regions of Iran have the highest rate of incidence (Malekzadeh *et al*., 2009; Radmard, 2010).

Frequently, gastric cancer is diagnosed at an advanced stage of cancer, and with metastasis resulted in reduced 5-year survival rates of patients. Diagnosis of gastric cancer before metastasis to the muscular layer can be enhanced 5-year survival rate up to 90%; therefore this is important to understand molecular mechanisms that are involved in the gastric cancer progression and metastasis (Correa, 2013).

Long non-coding RNAs (lncRNAs) are the largest group of non-coding RNAs, which are larger than 200 nucleotides in length (Brosnan and Voinnet, 2009). lncRNAs can regulate gene expression at different levels and with different mechanisms (Wang and Chang, 2011). These molecules are engaged in various biological processes and misregulated in various pathological processes like cancer initiation and metastasis (Mathieu *et al*., 2014; Fang and Fullwood, 2016). The long non-coding RNA *HNF1A-AS1* is a lncRNA with 2.4 kb length which is located in chromosome 12q24.31. This gene is transcribed as antisense of the *HNF1A* gene and controls cell cycle by regulating E-cadherin, cyclin D1, N-cadherin, and β-catenin expressions. *HNF1A-AS1* can induce epithelial-mesenchymal transition (EMT) and metastasis by down-regulating E-cadherin through binding to DNMT1 (Liu *et al.,* 2016; Yang *et al*., 2014; Wu *et al*., 2015). *MVIH* is a lncRNA that is located in an intron of the ribosomal protein S24 gene. *MVIH* is misregulated in some cancers. It enhances metastasis by inhibition of PGK1 secretion and regulating MMP2 and MMP9 expressions (Shi *et al*., 2015; Yuan *et al*., 2012; Lei *et al*., 2016; Nie *et al*., 2014; He *et al*., 2014). Due to dysregulation of *lncRNA-HNF1A-AS1* and *lncRNA-MVIH* expressions in different cancers, in the current study, we evaluated the expression profile of these two genes in human gastric cancer specimens as well as their correlation with clinicopathological parameters. Furthermore, we analyzed gene expression and clinicopathological characteristics of the *lncRNA-HNF1A-AS1* gene in gastric cancer from the Cancer Genome Atlas (TCGA) database.

Materials and Methods

Subjects

Thirty paired gastric tissue samples (tumoral and non-tumoral) were obtained from Iran Tumoral Bank (Tehran, Iran) and examined for gene expression (Emadi-Baygi *et al*., 2015; Nikpour *et al*., 2014). The experimental design was certified by the Committee of Shahrekord University. Before participation, the patients' written informed consent was obtained by Iran Tumoral Bank. The data set from an independent cohort in the TCGA database (http://cancergenome.nih.gov) was utilized for the evaluation of the *lncRNA-HNF1A-AS1* expression and its clinicopathological relevance. The lncRNA Reads Per Kilobases per Million reads (RPKM) expression value in the TCGA database was downloaded through The Atlas of Noncoding RNAs in Cancer (TANRIC) which contains 285 gastric cancer tissues and 33 non-tumoral tissues. Clinical information about these 318 patients was also downloaded from the TCGA database (Li *et al*., 2015; Weinstein *et al*., 2013). Furthermore, TCGA survival data were retrieved from OncoLnc (Anaya, 2016).

Total RNA extraction and cDNA synthesis

Total RNA from tumoral and adjacent non-tumoral tissue specimens was isolated with TRIzol® Reagent (Invitrogen, Carlsbad, California, United States) as explained in the manufacturer's protocol. One percent agarose gel electrophoresis was used to determine RNA quality. Nanodrop instrument (Nanolytik, Düsseldorf, Germany) was used for determining RNA concentration by measuring optical density at 260 nm. For the elimination of genomic DNA, DNase treatment was carried out by using the DNase set (Fermentas Co. Lithuania). cDNA synthesis was performed using random primers and an M-MLV reverse transcriptase (Fermentas Co. Lithuania).

Gene expression analyses

The relative expression of *lncRNA-HNF1A-AS1* and *lncRNA-MVIH* genes was determined by quantitative real-time RT-PCR in comparison to *GUSB* (β-Glucuronidase) as a reference gene (Baygi *et al*., 2010). The primers for target genes (Table 1) were designed with GeneRunner software, version 4.0.9.66 Beta, and specificity of the primers was verified by the BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Sequences of primers.

Gene name	Accession number	Amplicon size (bp)	Primer sequence (5' → 3')
HNF1A-AS1	ENSG00000241388	134	GATGCTGTTCCTTCTACCTG
			CAGTGGTGCTCAAATATGG
MVIH	NCBI no.: AK094613	162	GCCAGCAGAACTACTCACTAAG
			AAAGGCTGGATGAAGAGAAC

Real-time PCR was carried out with SYBR *Premix Ex Taq* (Tli RNase H Plus) (Takara, Otsu, Shiga, Japan) and run on the Rotor-gene 6000 (Qiagen, Hilden, Germany). The conditions of the PCR for target genes included of the earliest denaturation at 95 °C for 2 minutes, 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 56 °C for *lncRNA-HNF1A-AS1* and 57°C for *MVIH* genes for 35 seconds and annealing at 60°C for *GUSB* gene for 60 seconds, then extension at 72 °C for 35 seconds for *lncRNA-HNF1A-AS1* and *MVIH* genes and 15 seconds for *GUSB* gene.

Statistical analyses

The duplicate reaction was done for each gene. The ΔCt method was used to quantify the relative levels of gene expression (Nourbakhsh *et al.*, 2018) and the result was statistically analyzed by the t-test and chi-square. The SPSS software, version 21.0 and Prism (GraphPad, San Diego, CA) was utilized for statistical analyses and variations with $p < 0.05$ were considered as statistically significant.

Results

Expression of *HNF1A-AS1* and *MVIH*

qRT-PCR was performed to determine the expression levels of *lncRNA-HNF1A-AS1* and *lncRNA-MVIH* genes in human gastric tissues. Relative expression of target genes was determined using the ΔCt method by subtracting its Ct value to that of the *GUSB* gene. The results of real-time qRT-PCR showed that *lncRNA-HNF1A-AS1* expression was significantly down-regulated in cancerous tissues compared to the adjacent non-cancerous tissues (Fig. 1a, $p=0.04$). Subsequently, we surveyed the expression levels of *lncRNA-HNF1A-AS1* in the TCGA stomach cancer (STAD) cohort and found that *lncRNA-HNF1A-AS1* was significantly overexpressed in gastric tumoral tissues compared to normal tissues (Fig.1b, $p=0.0002$). Statistical analysis of relative expression levels of the *MVIH* gene failed to show any significant difference between cancerous and non-cancerous gastric tissue samples (data not shown, $p=0.34$).

***lncRNA-HNF1A-AS1* and *lncRNA-MVIH* expressions and clinicopathological characters**

To determine the clinical significance of *MVIH* and *HNF1A-AS1*, we analyzed the relationship between their expression and clinicopathological features of the patients with GC.
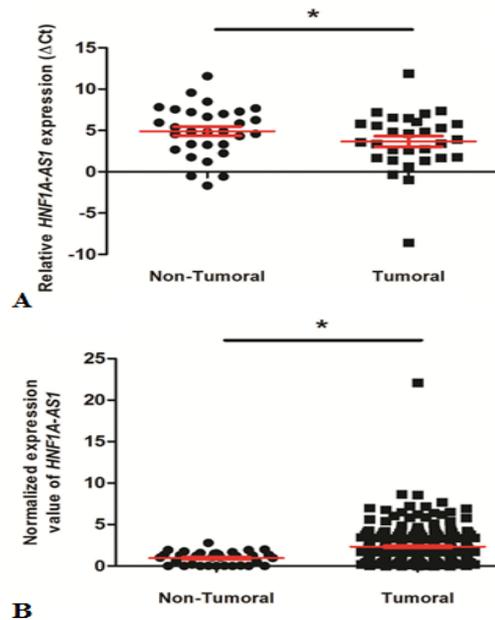
Fig. 1. Expression profile of *HNF1A-AS1* in gastric tumoral and adjacent non-tumoral samples of our study group and TCGA cohort. *HNF1A-AS1* expression was significantly lower in tumoral samples compared to adjacent non-tumoral ones both in our patients (A) In TCGA cohort; Tumoral samples showed significantly higher *HNF1A-AS1* expression levels (B); Data are expressed as means±SEM. The asterisk indicates a statistically significant value.

We categorized *MVIH* and *HNF1A-AS1* expression as low and high based on median ΔCt value and investigated the association between their expressions and clinicopathological features of patients. The result of the statistical analysis pointed out that *HNF1A-AS1* lower expression was associated with a higher metastasis level (Table 2, $p=0.03$).

Besides, analysis of *HNF1A-AS1* median gene expression data in the TCGA cohort showed a significant negative association with M classification (Table 3, $p=0.024$). Moreover, the result of the statistical analysis indicated that *MVIH* expression was associated with TNM stage ($p=0.04$) and tumor grade ($p=0.02$) (Table 4). Later, in cancerous samples (30 GC tissues), the Spearman correlation analysis showed *HNF1A-AS1* expression was positively correlated with *MVIH* expression levels ($p<0.0001$, $\rho=0.722$).

Correlation of *lncRNA-HNF1A-AS1* expression levels with overall survival in the TCGA cohort

We further assessed the prognostic significance of *lncRNA-HNF1A-AS1* by analyzing the correlation between its expression and overall survival across all cancer types in the TCGA database (according to the p-values from the univariate Cox proportional hazards model and log-rank test and visualization through a Kaplan-Meier plot). Lower expression of *lncRNA-HNF1A-AS1* was significantly associated with worse prognosis in patients with Pancreatic adenocarcinoma (PAAD) (*p*=0.019). However, there was no significant correlation between these lncRNA expression levels and the survival status of other cancer types including gastric cancer patients (Fig. 2, *p*=0.97).
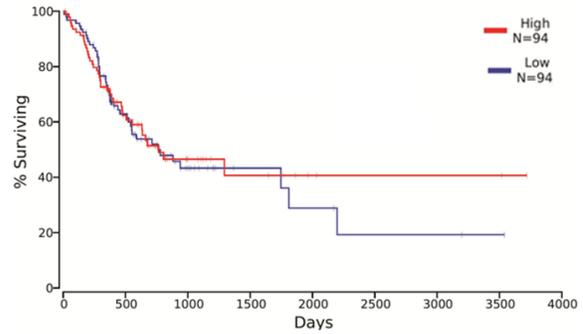
Fig. 2. Disease-free survival of gastric cancer patients based on *HNF1A-AS1* expression levels in the TCGA cohort.

Table 2. Correlation between *HNF1A-AS1* expression and clinicopathological parameters of gastric cancer patients.

	Characteristics	Numbers	Higher expression	Lower expression	*p*-value
Sex	Male	16	7	9	0.35
	Female	14	8	6	
Age (years)	≥70	16	6	10	0.136
	<70	14	9	5	
Depth of invasion	T2	1	0	1	0.5
	T3-T4	29	15	14	
N classification	N0 –NX	6	2	4	0.3
	N1	12	7	5	
	N2-N3	12	6	6	
M classification	MX	6	1	5	**0.03****
	M0	18	12	6	
	M1	12	2	4	
TNM stage	I-II	16	9	7	0.341
	III	8	4	4	
	IV	6	2	4	
Tumor grades	I	10	3	7	0.111
	II	8	4	4	
	III	12	8	4	
Tumor types	Diffuse	13	5	8	0.241
	Intestinal	17	15	7	
Perineural invasion	Yes	24	12	12	0.5
	No	6	3	3	
Lymphatic invasion	Yes	18	8	10	0.35
	No	12	7	5	
Tumor size (cm)	≥5	8	5	3	0.341
	<5	22	10	12	

** Statistically significant

Table 3. Correlation between *HNF1A-AS1* expression and clinicopathological parameters of gastric cancer patients of the TCGA cohort based on median gene expression level.

	Characteristics	Numbers (#285)	Higher expression (#142)	Lower expression (#143)	*p* value
Sex	Male	174	92	82	0.1974
	Female	111	50	61	
Age (years)	≥70	113	60	53	0.3042
	<70	167	81	86	
	NA	5	1	4	
Depth of invasion	T1	13	9	4	0.1111
	T2	72	33	39	
	T3	113	63	50	
	T4	78	35	43	
	TX	9	2	7	
N classification	N0	94	50	44	0.6692
	N1	78	34	44	
	N2	47	25	22	
	N3	53	28	25	
	NX	13	5	8	
M classification	M0	252	124	128	**0.0240****
	M1	18	6	12	
	MX	15	12	3	
TNM stage	I	40	20	20	0.0649
	II	99	53	46	
	III	101	55	46	
	IV	25	6	19	
	NA	20	8	12	
Tumor grades	G1-GX	12	6	6	0.4877
	G2	91	50	41	
	G3	182	86	96	
Tumor types	Diffuse	51	23	30	0.1687
	Intestinal	95	56	39	
	Mixed	136	62	72	
	NA	3	1	2	

** Statistically significant

Table 4. Correlation between *MVIH* expression and clinicopathological parameters of gastric cancer patients.

	Characteristics	Numbers	Higher expression	Lower expression	*p*-value
Sex	Male	16	8	8	0.5
	Female	14	7	7	
Age (years)	≥70	16	7	9	0.35
	<70	14	8	6	
Depth of invasion	T2	1	0	1	0.5
	T3-T4	29	15	14	
N classification	N0 -NX	6	1	5	0.059
	N1	12	6	6	
	N2-N3	12	8	4	
M classification	MX	6	3	3	0.074
	M0	18	11	7	
	M1	6	1	5	
TNM stage	I-II	16	8	8	**0.041****
	III	8	6	2	
	IV	6	1	5	
Tumor grades	I	10	2	8	**0.021****
	II	8	6	2	
	III	12	7	5	
Tumor types	Diffuse	13	9	4	0.069
	Intestinal	17	6	11	
Perineural invasion	Yes	24	12	12	0.5
	No	6	3	3	
Lymphatic invasion	Yes	18	9	9	0.5
	No	12	6	6	
Tumor size (cm)	≥5	8	5	3	0.34
	<5	22	10	12	

** Statistically significant

Discussion

In this study, we evaluated *lncRNA-HNF1A-AS1* and *lncRNA-MVIH* genes expression in cancerous and adjacent non-cancerous gastric tissue specimens by real-time qRT-PCR. We also analyzed the expression level of the *lncRNA-HNF1A-AS1* and its association with clinicopathological characteristics in an independent large TCGA cohort. Our results showed that the expression of *HNF1A-AS1* in cancerous tissues is lower than that in adjacent non-cancerous tissues and the difference was statistically significant. However, we found that *lncRNA-HNF1A-AS1* was significantly up-regulated in GC tissues compared with non-tumoral ones in TCGA patients. We further revealed that *HNF1A-AS1* expression inversely associated with distant metastasis in both our samples and TCGA patients. TCGA survival data analysis for this lncRNA showed that patients with pancreatic adenocarcinomas have a worse prognosis (lower overall survival time) if they are categorized in the *HNF1A-AS1* low expression group.

Recently, Dang et al reported that *HNF1A-AS1* expression is down-regulated in gastric cancer. Dang et al also reported that *HNF1A-AS1* expression is related to tumor size, venous invasion, lymphatic metastasis, perineural invasion, and invasion depth (Dang *et al.*, 2015). Our results are concordant with the mentioned report favoring the potential tumor-suppressive role of *HNF1A-AS1* in GC progression. However, Liu. et al. showed an upregulation in HNF1A-AS1 expression in gastric cancer samples in which it is associated with growth and metastasis (Liu *et al.*, 2018). The plausible differences in the measurement of expression of this gene in different cancers and different samples of certain cancer can be caused by high heterogeneity among cancers, which makes heterogeneity in the expression profile of different samples of certain cancer and even different parts of a tumor.

In 2014, Yang *et al.* reported that *HNF1A-AS1* is upregulated in esophageal adenocarcinomas (Yang *et al.*, 2014). Analysis of *HNF1A-AS1* expression in lung adenocarcinoma by Wu et al revealed that this gene is up-regulated in lung adenocarcinoma compared with corresponding non-tumoral tissues. Furthermore, they revealed that *HNF1A-AS1* expression is related to clinicopathological features of patients including tumor size, TNM stage, and lymph node metastasis (Wu *et al.*, 2015). Moreover, Liu et al analyzed *HNF1A-AS1* expression in hepatocellular carcinoma (HCC) and reported that its expression is upregulated in cancerous tissues compared to noncancerous tissues. They further reported that *HNF1A-AS1* expression is associated with TNM stage and tumor size (Liu *et al.*, 2016). Considering the expression of *HNF1A-AS1* in different cancer types shows that expression of this gene in different cancers follow a different pattern while up-regulated in some cancers (lung, HCC and esophageal) and maybe act as an oncogene, it is downregulated in some others (gastric, pancreatic) and maybe act as a tumor suppressor (Müller *et al.*, 2015). Furthermore, studies in different cancers have generally shown that HNF1A-AS1 regulates different oncogenes including BCl-2, Notch, Wnt/β-Catenin, Sox-4, and CDK-6 and tumor suppressors such as p21 and p53 via sponging different miRNAs resulting in increased growth and metastasis in various cancers (Liu *et al.*, 2019; Yang *et al.*, 2014; Wu *et al.*, 2015; Liu *et al.*, 2019; Zhang *et al.*, 2018; Fang *et al.*, 2017; Zhan *et al.*, 2017; Guo *et al.*, 2020; Wang *et al.*, 2017; Wang *et al.*, 2017). However, this difference(s) in expression pattern and plausibly function of HNF1A-AS1 could be explained by this point that lncRNAs usually are expressed tissue-specifically and act in the different pathway(s) in different tissue(s) with different expression profiles (Gibb *et al.*, 2011).

Survival data analysis in lung adenocarcinoma patients studied by Wu *et al.*, (2015) revealed that patients with higher *HNF1A-AS1* expression levels have lower survival rates. This is the only report on this gene that assessed the survival data. TCGA data analysis showed that except for pancreatic cancer, there is no statistically significant association between *HNF1A-AS1* expression levels and overall survival. Finally, we found an apparent discrepancy between our results and the TCGA results, regarding the expression of *HNF1A-AS1*. In our own data analysis, we noticed that the distribution of patients with different tumor types (intestinal vs. diffuse) was different according to *HNF1A-AS1*

expression. Patients with diffuse gastric cancer type had been categorized more in a class of higher *HNF1A-AS1* expression (although not statistically significant). This trend was also evident according to the comparison of *HNF1A-AS1* mean expression levels between intestinal and diffuse samples. The decreased expression of E-cadherin has been mainly observed in diffuse-type (Chan, 2006). We speculate that the reduction of E-cadherin expression through binding of *HNF1A-AS1* to DNMT1 may result in metastasis, so it is more plausible to find its overexpression in diffuse-type gastric cancer similar to the trend we observed in this study. Furthermore, the real-time qRT-PCR is the gold standard and the sequencing data (from TCGA) should be verified by real-time qRT-PCR (Costa *et al*., 2013).

Besides, we analyzed the expression of *MVIH* in 30 paired tissue samples of cancerous and adjacent non-cancerous gastric tissue by real-time qRT-PCR. Our results demonstrated that there was no significant difference in *MVIH* expression between tumoral and non-tumoral tissues. We also revealed that *MVIH* expression levels were associated with TNM stage and tumor grades. To the best of our knowledge, this is the first report on examining the expression of *MVIH* in gastric cancer. However, expression of this gene was analyzed by Yuan et al and Shi et al in HCC and they reported that this gene is upregulated in cancerous tissues compared with adjacent non-cancerous ones. Moreover, they showed that *MVIH*'s expression is associated with clinicopathological features such as microvascular invasion and TNM stage (Shi *et al*., 2015; Yuan *et al*., 2012). Analysis of *MVIH* expression in breast cancer tissues by Lei et al showed that *MVIH* is up-regulated in tumoral tissues compared to non-tumoral tissues (Lei *et al*., 2016). Nie et al analyzed *MVIH* expression in non-small cell lung adenocarcinoma. They reported that *MVIH* is up-regulated significantly in cancerous tissues compared with adjacent noncancerous ones. Furthermore, they reported that *MVIH* expression is related to clinicopathological features such as tumor stage, and lymph node metastasis (Nie *et al*., 2014).

Finally, the correlation between *MVIH* and *HNF1A-AS1* expressions in our cohort showed that *HNF1A-AS1* expression was positively correlated with *MVIH* expression. We hypothesize that due to the structural relationship of *HNF1A-AS1* and DNMT1 and the recognized role of DNMT1 in gene expression, it may be speculated that *HNF1A-AS1* has a role in regulating the expression of *MVIH* (Wu *et al*., 2015; Chen and Li, 2006).

In conclusion, we showed that *HNF1A-AS1* was under-expressed in gastric cancerous tissues. We further revealed that *HNF1A-AS1* expression was inversely associated with distant metastasis. Moreover, we demonstrated that *MVIH* expression levels were associated with TNM stage and tumor grades. Although our results show co-expression of the *MVIH* and *HNF1A-AS1* expressions in cancerous tissues, further studies should be conducted to clarify and shed light on the mechanism of action(s) of these lncRNAs in gastric cancer initiation and progression.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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