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RESEARCH

Clinicopathological Sex- Related Relevance of Musashi1 mRNA Expression in Esophageal Squamous Cell Carcinoma Patients

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Abstract The cancer stem cell theory is considered as the spotlight of cancer biology, in which a subpopulation of tumor cells show unlimited proliferative and self renewal capacities. Post-transcriptional regulation is involved in different cellular functions such as cell differentiation and proliferation which results in cellular diversity. Musashi1 (Msi1) is one of the most important RNA-binding proteins (RBPs) which are involved in translational inhibition. Although, Msi1 targets are largely unknown, p21WAF-1, a cell cycle regulator, and Numb, inhibitor of notch signaling pathway, are well-known factors which are suppressed by the Msi1 in normal and cancer stem cells. Msi1 expression in tumor tissues from 53 ESCC patients was compared to normal tissues using real-time polymerase chain reaction (PCR). Msi1 was significantely overexpressed in 41.5 % of tumor samples and we observed a significant correlation between Msi1 expression and sex, in which the males had shown a higher level of Msi1 expression in comparison with the females (2.00 Vs 0.78 fold changes, $p=0.05$). In this study, we assessed whether Msi1 is expressed in ESCC samples suggesting this protein as a novel cancer stem cell marker which requires further studies.

Keywords Esophageal squamous cell carcinoma · Musashi1 (Msi1) · Expressional analysis, Real-time PCR

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Introduction

Esophageal squamous cell carcinoma (ESCC) is the second leading cause of cancer related deaths in north-east of Iran [1]. Despite all of the hypotheses that have been suggested for the tumorigenesis of ESCC, the cancer stem cell theory is considered as the spotlight of ESCC biology, in which there is a subpopulation of tumor cells with an unlimited proliferative and self renewal capacities [2, 3]. Post-transcriptional regulation is involved in different cellular functions such as cell differentiation and proliferation resulting in cellular diversity. Expressional study of cancer stem cell factors involved in post transcriptional regulations, and is one of the main ways to explore the probable role of cancer stem cells in tumorigenesis. Protein synthesis regulation is an important post transcriptionalprocess in cell development and growth. It has been demonstrated that, translation is dominant regulation process in comparison with the transcription, in which only 40 % of variation in protein levels is under the regulation of transcription [4]. Therefore,

every deregulation of translation can results in cell death or malignancy. Specific synthesis of involved proteins in tumorigenesis that are affected by translation initiation influences cancer progression and treatment. Translational initiation is governed by the initiation complex comprised of eIF4E, eIF4F, eIF4G, eIF4A and eIF4B in 5′ untranslated region (UTR) and poly-A binding proteins (PABP) located in 3′UTR [5, 6]. eIF4G acts as a mRNA activator via binding with the PABP, it also involved in AUG start codon scanning [7].

Musashi1 (Msi1) is one of the most important RNA-binding proteins (RBPs) involved in protein expression through translational regulation and exerts translational inhibition by a specific sequence located in the 3' UTR of mRNA, leading to decline of the related protein levels [8, 9]. Indeed, Msi1 acts as an antagonist of the eukaryotic translation initiation factor 4G (eIF4G) to bind with PABP, resulting in prohibition of the 80S ribosome assembly [10]. Although, Msi1 targets are largely unknown, p21WAF-1 and Numb as a cell cycle regulator and Notch signaling pathway inhibitor, respectively, are some of well-known factors which are suppressed by the Msi1 in vertebrates [8, 11]. It has been demonstrated that Numb acts as a suppressor of Notch [12] and Sonic Hedgehog signaling pathways [13]. Numb suppresses the Notch pathway via the ubiquitination process of notch intracellular domain (NICD), its ubiquitination inhibits the NICD nuclear translocation [14, 15]. Furthermore, it targets Dickkopf3, a Wnt pathway inhibitor [16, 17]. Generally, it is accepted that Msi1 overexpression results in cell proliferation and apoptosis by translational inhibition of Numb, p21WAF and Dickkopf3, which are well known factors in cancer stem cell biology and cell cycle regulation. Msi1 targets the p21, as a negative regulator of wnt pathway and cell cycle regulator resulting the increased proliferation through the β-catenin nuclear accumulation, Wnt activation and cell cycle regulation [8, 17, 18]. It is also demonstrated that Msi1 activates the Wnt pathway through the Frat1, which increases the Lef-mediated transcription via its interactions with glycogen synthase kinase 3β and Dishevelled [19]. In the recent years, expression of the Msi1 have been reported in different cancers and cell lines such as glioma, hepatoma, endometrial carcinoma and colorectal adenoma [20–23]. To explore the involvement of Msi1 in the ESCC tumorigenesis, we assessed its mRNA expression in ESCC samples and analyzed its probable correlation with different clinicopathological features of patients. Since introducing the new molecular markers involved in tumor progression is a key aim to design novel effective therapeutic modalities, in this study, we assessed whether Msi1 is involved in ESCC tumorigenesis, probably introducing a novel cancer stem cell marker for ESCC development and progression.

Materials and methods

Tissue samples

Fresh tumor and normal margin of fifty-three ESCC samples were obtained from patients who had undergone esophagectomy before receiving any chemo- or radio-therapeutic treatments at Omid and Imam-Reza Hospitals of Mashhad University of Medical Sciences (MUMS). After the surgery and before resected tumors were transferred to the RNA later solution (Qiagen, Hilden, Germany), all the fresh tissues were microscopically examined by the pathologist to ensure the originality of samples. Finally, the samples were stored at - 20 ° C prior mRNA extraction. The patients filled and confirmed the informed consent forms which were approved by the MUMS ethic committee.

RNA extraction, cDNA synthesis and quantitative RT-PCR

The cDNA synthesis from the total mRNA was performed using oligo dT procedure in first-strand synthesis kit (Fermentas, Lithuania) following the RNA extraction from the normal and tumoral specimens with the RNeasy Mini kit (Qiagene, Hilden, Germany). Comparative relative RT-PCR was performed in triplicate reactions using primers presented in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a normalizer gene regarding the comparative threshold cycle system based on SYBR Green method (GENETBIO, Korea) in Stratagene Mx-3000P (Stratagene, La Jolla, CA). Thermal profile was applied as initial denaturation step (10 min in 94 °C), followed by 45 cycles of 30 s in 94 °C, 40 s in 62 °C and finally 72 °C for 30 s. mRNA expression was compared in tumor with normal tissues and fluorescent intensity was measured as described before [24].

Statistical analysis

All the correlations were assessed using the SPSS 16.0 (SPSS, Chicago, IL), in which correlations between continuous variables such as tumor size, age and number of metastatic lymph nodes and the level of Msi1 mRNA expression were assessed by the Pearson's or Spearman

Table 1 Primer sequences used for comparative real-time qRT-PCR

	Forward	Reverse
Msi1	TGAGCAGTTTGGGAAGGTG	TCACACACTTTCTCCACGATG
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT

tests, regarding the results. Moreover, correlational studies between clinicopathological features (lymph node metastasis, tumor depth of invasion, tumor location, grade and stage) and the level of mRNA expression were performed via the *t*-test and ANOVA. A p value of <0.05 was considered to be significant.

Results

Study population

In this study 53 histologically confirmed ESCC patients were enrolled including 28 male (52.8 %) and 25 female (47.2 %). Age range was between 30 and 84 years with a total mean age of (61.85±11.42) years. All samples were obtained from the patients prior to any radio- or chemo-therapy. All tumor tissues were confirmed to contain at least 70 % tumor cells based on hemotoxylin and eosin staining. Despite one sample with whole esophagous involvement, the other tumors were almost distributed equally between lower and middle esophagous, 25 (47.2 %) and 27(50.9 %), respectively. The size of tumor samples ranged between 0.50 and 12.00 cm with a mean size of (4.08± 1.93) cm. In the case of clinicopathological features (as summarized in Table 2); fifty-one cases were in stages of II/III (96.2 %), nine (17 %) tumors were poorly differentiated, 34 (64.2 %) and 10(18.9 %) had shown moderately and well differentiation, respectively. Most of tumor tissues were in T3 (41/53, 77.4 %) and (25/53, 47.2 %) of samples had shown lymph node metastasis.

Msi1 overexpression in ESCC patients

We assessed the Msi1 mRNA expression in 53 patients using comparative relative real time PCR, comparing the expression in tumor tissues with the paired normal samples. Twenty two out of 53 samples (41.5 %) showed overexpression in Msi1 mRNA level. Totally, the fold changes were scattered between −3.8 and 8.8 folds (Mean ± SD, 1.42±2.3). The mean and standard deviation of fold changes for the overexpressed and normal/underexpressed tumors were (Mean ± SD, 3.56±1.63) and (Mean ± SD, -0.08±1.28), respectively. The expressional level of Msi1 in all tumors is illustrated by fold changes in Fig. 1. Moreover, expression levels in N/underexpressed and overexpressed groups were compared together schematically in Fig. 2.

Clinicopathological features and Msi1 mRNA expression

To assess the probable roles of Msi1 mRNA overexpression in progression and emerging of ESCC, we performed a correlational study between the level of mRNA expression and clinicopathological features of the patients (Table 2).

Table 2 Correlations between Msi1 gene expression and patient clinicopathological characteristics

	Total	Msi1 overexpression	p value
Patients	53	22	
Mean age (mean ± SD)	61.8± 11.4 years	58.5±14.6	
Size (mean ± SD)	4.1±1.9 cm	4.2±2.3	
Sex			**0.050**
Male	28(52.8 %)	12(54.5 %)	
Female	25(47.2 %)	10(45.5 %)	
Location			0.914
Lower	25(47.2 %)	9(41.0 %)	
Middle	27(50.9 %)	12(54.5 %)	
Middle and lower	1(1.9 %)	1(4.5 %)	
Grade			0.618
P.D	9(17.0 %)	5(22.7 %)	
M.D	34(64.2 %)	15(68.2 %)	
W.D	10(18.9 %)	2(9.1 %)	
Lymph node			0.885
Yes	25(47.2 %)	11(50.0 %)	
No	28(52.8 %)	11(50.0 %)	
Stage			0.869
I	2(3.8 %)	–	
II	31(58.5 %)	13(59.1 %)	
III	20(37.7 %)	9(40.9 %)	
Depth of Tumor invasion (T)			0.395
T1	3(5.7 %)	1(4.5 %)	
T2	8(15.1 %)	2(9.1 %)	
T3	41(77.4 %)	19(86.4 %)	
T4	1(1.9 %)	–	

The bold number mentions that there is a significant correlation between the level of Msi1 mRNA expression and sex

Although, there was not any significant correlation between clinicopathological features and Msi1 expression, in the case of tumor differentiation, most of tumors with Msi1 overexpression were moderately differentiated (15, 68.2 %). All the Msi1 overexpressed tumors were in stages II and III with 13(59.1 %) and 9(40.9 %), respectively. Half of the cases with Msi1 overexpression had lymph node metastasis. Although there was not any significant correlation between Msi1 expression and tumor depth of invasion, most of overexpressed cases (19, 86.4 %) were in T3, which emphasizes the noticeable role of this factor in metastasis. In the case of tumor location, most of tumors with Msi1 overexpression were located in the middle esophagous (12, 54.5 %) while the others were located in lower part of esophagus.

Interestingly, we observed one Msi1 overexpressed sample which was spread in the whole esophagous. Normal/ underexpressed tumors were almost distributed equally in

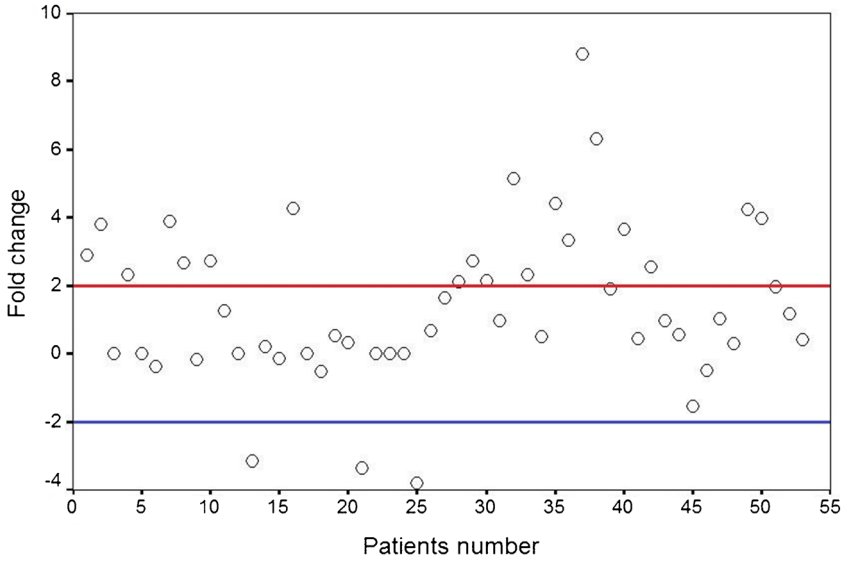

Fig. 1 Scatter plot of Msi1 expression in ESCC patients. The Y axis indicates relative gene expression, and the X axis represents the patients. Level of mRNA expression between −2 to +2 folds are considered as normal, whereas more than +2 and lower than −2 are defined as overexpression and underexpression, respectively

the middle and lower esophagous with 15 (48.4 %) and 16 (51.6 %) cases, respectively. There was a significant correlation between sex and Msi1 mRNA expression ($p=0.05$), in which the males showed a higher levels of Msi1 expression. The mean of fold changes were 2.00 and 0.78 in males and females, respectively. To assess more details about the probable significant correlation between the Msi1 expression and tumor stage we categorized the stages of tumors in two groups of stages I/II and III/IV. Similarly, we categorized the tumor depth of invasion in T1/T2 and T3/T4. As it has mentioned in the Table 3, we have observed a significant correlation among sex, tumor stage and level of mRNA expression in which, 66.7 % of tumors (8 out of 12) belonging to the males were in stages of I/II ($p=0.050$). Eleven out of 17 cases (64.71 %) with Msi1 over expression had not any lymph node metastasis, where seven cases (63.6 %, $p=0.044$) were male. Furthermore, it has also showed that there is a significant correlation among sex, tumor depth of invasion (T) and mRNA expression, in which 14 out of 23 cases (61 %) with n/under expressed tissues and T3/4 tumor depth of invasion were in females ($p=0.035$). Nineteen of 22 (89.4 %) tumor tissues with Msi1 overexpression were in T3. Interestingly, there was a significant correlation between tumor depth of invasion and sex, in which in females, 22 out of 25 (88.0 %) cases were in T3 ($p=0.050$).

Discussion

RNA-binding proteins are among the most important involved factors in cell fate, differentiation and self-renewal maintenance [25]. They play important roles in gene

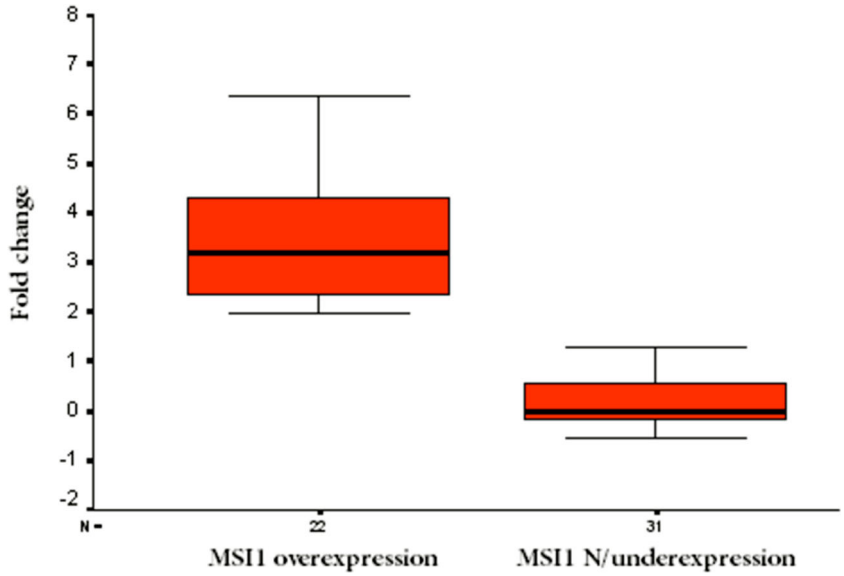

Fig. 2 Box plot illustration of Msi1 relative quantitative mRNA expression in ESCC patients. The Y axis shows the mRNA expression fold change, and the X axis represents patient groups considering the level of Msi1 mRNA expression. This plot represents the lowest, lower quartile, median, upper quartile and highest observations of fold changes in patients either with normal/underexpressed or overexpressed Msi1

Table 3 Significant correlations among the Sex, Msi1 expression and clinicopathological features

	Msi1 overexpression		Msi1 N/underexpression		*p* value
	Male	Female	Male	Female	
Lymph node					0.044
Yes	5(41.7 %)	6(60 %)	6(37.5 %)	8(53.3 %)	
No	7(58.3 %)	4(40 %)	10(62.5 %)	7(46.7 %)	
Stage					0.050
I	–	–	2(12.5 %)	–	
II	8(66.7 %)	5(50 %)	10(62.5 %)	8(53.3 %)	
III	4(33.3 %)	5(50 %)	4(25 %)	7(46.7 %)	
Depth of Tumor invasion (T)					0.035
T1	–	1(10 %)	2(15.5 %)	–	
T2	2(16.7 %)	–	5(31.3 %)	1(6.7 %)	
T3	10(83.3 %)	9(90 %)	9(56.3 %)	13(86.7 %)	
T4	–	–	–	1(6.7 %)	

expression via various processes, including post transcriptional modifications, RNA nuclear export, maintenance and translation. Therefore, it is clearly expectable to observe abnormal expression of RNA-binding proteins in different tumor types. Msi1 is an RNA binding protein with an important role in self renewal [26], and its overexpression is commonly reported in many types of brain tumors [27, 28]. It has been shown that the Msi1 exerts its tumorigenic role via several ways. It induces cell cycle progression by inhibition of p21WAF-1 as one of the most important cyclin-dependent kinase suppressors to increase Rb phosphorylation and deactivation through enhanced cyclinA-CDK dimers in S phase. Furthermore, it activates the wnt and notch pathways through the inhibition of DKK3 and Numb, respectively [13, 17]. In addition, it activates the Wnt pathway via an autocrine process [17]. And Msi1 is probably the target gene of wnt signaling pathway because of presence of the different TCF binding sites in its 5′-upstream sequence [11]. It is also involved in the regulation of sonic hedgehog pathway which is another important regulatory pathway of self renewal maintenance [29]. Taken together, Msi1 is a spotlight in regulation of crosstalk among these signaling pathways. Besides, It has been demonstrated that, Msi1 is involved in reprogramming via the famous stem cell markers such as Oct4, Nanog and Sox2 [30–33].

Although, Msi1 was initially known as a neuronal stem cell marker, its expression was also observed in other normal tissues like the colonic and intestinal crypts, which nominated that as a gastrointestinal stem cell marker [27, 28, 34]. Previously Bobryshev et al. have reported the importance of Msi1 in Barrett's esophagous and adenocarcinoma [35]; however, this study is the first report of Msi1 expression profiling in ESCC patients.

Musashi1 expression was studied in different malignancies such as esophageal, gastric, colorectal and bladder carcinoma [35–38]. Therefore, it is important to investigate the probable involvement of Msi1 in ESCC pathogenesis. This report proved that the Msi1 expression was elevated in the ESCC samples at the level of transcription. However, there is not any report about the probable importance of Msi1 in ESCC progression and its prognostic value. Despite the noticeable percentage of Msi1 overexpression among the fifty-three ESCC patients, there was not any significant statistical correlation between the level of mRNA expression and clinicopathological features like the tumor depth of invasion, lymph node, grade and stage of tumor. But we have observed a significant correlation between Msi1 expression and sex for the 1st time, in which the males had higher mean fold changes in comparison with the females (more than 2.5 folds). Moreover, these data demonstrated that although there was not any significant correlation between Msi1 overexpression and tumor metastasis, considerably most of overexpressed tumors were in T3/T4, which relies to the probable role of Msi1 overexpression in tumor progression and metastasis. Finally, as can be clearly seen in Fig. 1, there was one patient with fold change of 8.80 which was significantly higher than the mean fold of 3.56 in overexpressed tumors. This sample was from a male patient with an invasive tumor to the adventitia (T3) and poorly differentiated in tumor stage of II.

On the whole, we observed a significant overexpression of Msi1 mRNA in 41.5 % of tumor tissues in comparison to normal esophagous epithelia, which was associated with sex significantly. In spite of the lack of a significant correlation between Msi1 expression and ESCC clinicopathology, and due to its high expression in tumor samples compared to normals, it can be introduced as a valuable novel cancer stem cell marker in ESCC. The overexpression of Msi1 in ESCC may shed light in cancer stem cell-based diagnosis and targeted therapy applications of ESCC. Further studies should be performed to clarify the detailed roles of Msi1 in the

biology of ESCC. Regarding the significant correlation between Msi1 overexpression and sex in ESCC patients, in which the males had shown higher rates of expression, it will be probable to introduce this marker as a sex dependent stem cell marker. Translational defects not only can be tumorigenic through the increase in the level of proteins which are involved in cell proliferation and growth, but also can exert such tumorigenic role via decrease in the level of protein synthesis in the case of tumor suppressors [7]. Therefore, assessment of negative translational regulators like the Msi1, indubitably presents novel therapeutic targets in ESCC cancer. Further studies also are required to elucidate the different aspects of Msi1 involvement in biology of ESCC.

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