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Original Article

High plasma long non-coding RNA MALAT1 expression predicts a poor prognosis of cervical cancer

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Abstract: Aims: There is a great need and urgency in searching new and less invasive biomarkers to improve the detection and prognostic outcome of cervical cancer. This study aimed to investigate diagnostic and prognostic value of plasma MALAT1 expression in cervical cancer. Methods: In this research, a total of 122 cervical cancer patients and 61 age-matched healthy participants were included. Total RNA was isolated from serum using TRIzol reagent and MALAT1 expression were quantified by reverse transcription-PCR. Area under the ROC curve (AUC) was generated to assess the diagnostic values of the LncRNA MALAT1. Kaplan-Meier method to analyze the correlation between MALAT1 expression and survival. Hazard risks were calculated by Cox proportional hazards model. Results: Plasma MALAT1 expression was significantly increased in cervical cancer than in normal participants (P<0.001). The AUC (95% CI) of plasma MALAT1 in diagnosing cervical cancer patients was 0.788 (0.709-0.867). Cox proportional hazards model indicated that tumor size, FIGO stage and MALAT1 expression were independent prognostic factors for overall survival, with HRs of 1.97 (1.01-3.82, P=0.046), 2.46 (1.23-4.80, P=0.008), 2.46 (1.11-5.45, P=0.027), respectively. No significant interaction between MALAT1 expression and other risk factors was observed in predicting prognosis of cervical cancer. Conclusions: Plasma MALAT1 might be an ideal marker of prognosis in cervical cancer.

Keywords: Plasma MALAT1, cervical cancer, prognosis

Introduction

Cervical cancer is the second most common cancer in females worldwide, with 500,000 new patients and 300,000 deaths due to this cancer reported globally each year [1]. Among cervical cancer cases, 80% occur in developing countries and about 70% are identified as advanced cancer [2, 3]. Increasing evidences showed that early detection by testing for high-risk human papillomavirus (HPV) and cervical papilloma smears have reduced cervical cancer mortality. However, these methods do not detected the development of cervical cancer directly. Therefore, there was a great need and urgency in searching new and less invasive biomarkers to improve the detection and prognostic outcome of cervical cancer.

Deep sequencing recently facilitated the discovery of thousands of novel transcripts, now classified as long noncoding RNAs (lncRNAs), in many vertebrate and invertebrate species [4]. LncRNAs were broadly described as RNAs over 200 nucleotides (nt) in length, which possessed a lot of structural features of the mRNAs, containing a poly (A) tail, a 5'-cap, and a promoter structure, whereas owned no conservative open reading domain [5, 6]. Like microRNAs, lncRNA are also proving to be the key mediators of cellular differentiation, cell lineage choice, organogenesis and tissue homeostasis [4, 7]. Dysregulation of lncRNAs expression have been shown to be important in carcinogenesis and cancer metastasis and proved to as a new biomarkers in diagnosing and predicting prognosis tumor [8-10]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also called nuclear-enriched transcript 2 (NEAT2), which was found as a prognosis biomarker for the metastasis in lung cancer and even associated

with some other solid tumors [11]. It was an interesting target for anti-metastatic therapy in cancer [12]. Guo etal has declared that descending MALAT1 level reduced cell migration ability and lessened the tumor growth of cervical cancer in vivo, indicating that MALAT1 was related to the metastasis process in cervical cancer [13].

In the current study, we aimed to investigate the clinical significance of plasma MALAT1 by assessing its diagnostic values in cervical cancer screening and prognosis ability in survival of cervical cancer patients.

Materials and methods

Subjects

The research was conducted in strict accordance with the protocol approved by the Ethics Committee of Women's Hospital of Zhejiang University, and a written informed consent was obtained from each subject before their participation in the study. A total of 122 patients with cervical cancer were recruited from the hospital from Jan 2008 to Jan 2010 and 61 age-matched normal subjects from Medical Examination Center in the same period were included. Each enrolled patient has to meet the following criteria: 1) patients have no severe infection, active clinical comorbidities, or a history of any other malignancy; 2) all of the patients never received preoperative radiotherapy and/or chemotherapy before this study. All patients with cervical cancer were diagnosed as infiltrating carcinoma by pathology. The assessment was performed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer. The tumor stage and differentiation was examined by two experienced gynecological oncologists without authorship in this study.

5 mL of whole blood were collected from each participant in an EDTA gel tube. The separation procedure was performed within 2 h of sample collection. Blood samples were centrifuged at 1000 g for 10 min at 4°C to separate the blood cells. The supernatant was then centrifuged at 13000 g for 10 min at 4°C to completely remove cellular contaminants. Then plasma were aliquoted into microcentrifuge tubes, marked and stored at -80°C until use.

RNA extraction

Total RNA was isolated from 0.2 ml plasma using TRIzol reagent according to the manufacturer's protocol (Invitrogen). Briefly, 1 ml TRIzol was added to 0.2 ml of sample plasma, and then homogenated completely by a power homogenizer. 0.2 mL isopropanol was added to the mixture and incubate at room temperature for 10 minutes. Then the sample was centrifuged at 12,000× g for 10 minutes at 4°C. Wash the pellet, with 1 mL of 75% ethanol and centrifuge the tube at 7500× g for 5 minutes at 4°C. Finally, the RNA pellet was resuspended with RNase-free water. The concentration and purity of the RNA solution was measured by detecting its absorbance at 260/280 and 260/230 nm with NanoDrop 1000A spectrophotometer. (NanoDrop Technologies, Wilmington, DE). All the purified RNA samples were stored at -80°C for further processing.

Reverse transcription and quantitative real-time PCR (q-RTPCR)

Reverse transcription for total RNA was performed by the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany). The 20 μl-reverse transcription reaction contained: 1 μL Oligo $(dt)_{18}$, 1 μL Random Hexamer primer, 1 μL RNase Inhibitor, 1 μL M-MLV Rtase, 4 μL Reaction Buffer, 2 μl 10 mM dNTP Mix, 2 μl total RNA, 8 μL ddH2O (DNase-free). The reaction was performed on a MJ Research PTC-200 Peltier Thermal Cycler (Global Medical Instrumentation) at 16°C for 30 min, 42°C for 40 min, and 85°C for 5 min.

Then qRT-PCR was performed to quantify the expression level of miR-155, miR-21 and miR-10b with SYBR Green PCR Master Mix (Thermo) following the manufacturer's instructions. GAPDH was used as an intrinsic control. The 25 μl-amplifications reaction contained: 10 μL SYBR Green Mix, 2 μL MALAT1-specific forward-primer, 2 μL MALAT1-specific revere-primer, 2 μl total cDNA and 14 μl ddH2O (DNase-free). MALAT1 forward, 5'-AAAGCAAGGTCTCCCCAC-AAG-3', reverse, 5'-GGTCTGTGCTAGATCAAAA-GGCA-3', GAPDH forward, 5'-AGCCA CATCGCT-CAGACAC-3' and reverse, 5'-GCCCAATACGACC-AAATCC-3'. The RT-PCR reaction was performed at 95°C for 10 min and in 40 cycles at 95°C for 15 s and 60°C for 60 s on an ABI 7500 ther-

Table 1. The association between MALAT1 expression and clinicopathological features

	Cervical cancer	*MALAT1 expression*			Healthy
Parameters	N=121	High	Low	P	N=61
Age					
≤45	68	37	31	0.274	34
>45	54	24	30		27
Tumor size					
≤4	78	32	46	0.008	
>4	44	29	15		
FIGO stage					
Ib-IIa	83	37	46	0.081	
IIb-IIIa	39	24	15		
Differentiation					
Well/Moderate	84	25	49	<0.001	
Poor	38	26	12		

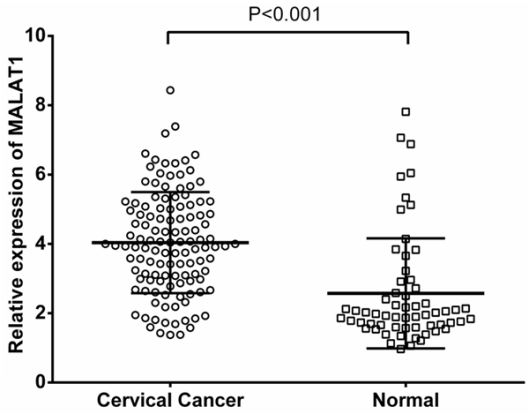

Figure 1. Relative expression levels of plasma MALAT1 in cervical cancer patients and healthy participants.

mocycler (Applied Biosystems). Relative gene expression level of each LncRNA was analyzed using the $2^{-\Delta\Delta Ct}$ method. Each blood sample was performed in duplicate wells and repeated 3 times.

Statistical analysis

All the statistical analyses were performed with R software version 2.8 (R Development Core Team 2013). P<0.05 (two-sided) was considered as statistical significance. MALAT1 expression was divided into two groups according the middle of MALAT1. Plasma MALAT1 expression between cervical cancer patients and healthy participants were compared using unpaired Mann-Whitney test, while the relationship between MALAT1 expression and clinicopathological characteristics was assessed using Pearson's χ^2 test. Area under the ROC curve (AUC) was generated to assess the diagnostic values of the LncRNA MALAT1. For survival analyzes, we used the Kaplan-Meier method to analyze the correlation between MALAT1 expression and overall actual survival, and the log-rank test to compare survival curves. The Cox proportional hazards model was used to make multivariate survival analysis for all of the significant parameters observed in the univariate analysis and transactional analysis was performed to investigate the interaction between clinicopathological characteristics, MALAT1 expression and survival.

Results

Clinical characteristics of study population

Plasma samples were acquired from 183 subjects (122 cervical cancer patients, 61 healthy participants). As presented in **Table 1**, no significant difference was observed in age between cervical cancer patients and healthy participants (57.7±7.6 vs. 56.9±6.7, P=0.469). Of 122 cervical cancer patients, 54 patients were older than 45 years, 44 patients had tumor size larger than 4 cm, 39 patients had high FIGO stage and 38 patients had poor differentiation.

MALAT1 level and cervical cancer susceptibility

To identify plasma MALAT1 expression in cervical cancer and healthy cases, we firstly quantified its expression in 122 cervical cancer cases and 61 age-matched healthy controls by qRT-PCT. Plasma relative level of MALAT1 was plotted in the form of scatter dots in **Figure 1**. As it shown, relative expression of MALAT1 in cervical cancer patients was 3.97 (2.97, 5.07), while that in healthy participants was 1.98 (1.61, 2.81). Statistically significant difference can be observed between cervical cancer patients and healthy participants (P<0.001). We further evaluated the diagnostic value of MALAT1 in distinguishing cervical cancer and normal people by ROC curves and AUC values. **Figure 2** shows

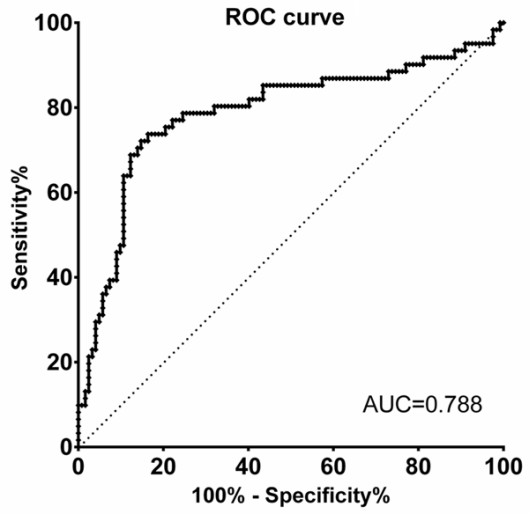

Figure 2. Receiver operating characteristic curve analysis of plasma MALAT1 in cervical cancer patients and healthy participants.

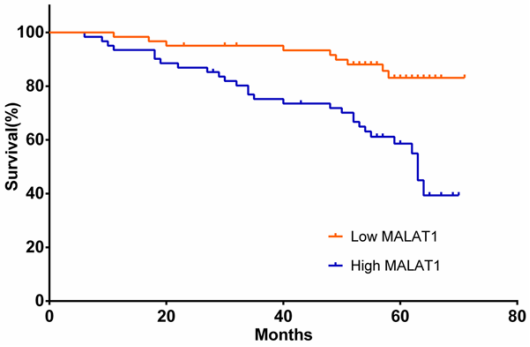

Figure 3. Kaplan-Meier survival analysis for all cervical cancer patients stratified by high plasma MALAT1 and low plasma MALAT1.

the ability of MALAT1 to diagnose cervical cancer patients. The AUC was 0.788 (0.709-0.867). We got the best cuff off value of 2.51, with sensitivity of 72.13% (59.17%-82.85%) and specificity of 85.25% (77.69%-91.02%).

To assess to association between plasma MALAT1 expression and the clinicopathological parameters, the 122 cervical cancer patients were divided into high MALAT1 expression (higher than the median level, n=61) and low MALAT1 expression (lower than the median level, n=61) groups according to the median level of MALAT1 expression (3.97). Comparisons were performed between the two groups (**Table 1**). The results showed that high MALAT1 expression was significantly correlated with tumor size (P=0.008) and differentiation (P<0.001), however age (P=0.274) and FIGO stage (P=0.081) did not show a significant association with MALAT1 expression.

High MALAT1 predicts a poor prognosis of cervical cancer

Overall survival curves in the high MALAT1 group and low MALAT1 group were given in **Figure 3**. Cervical cancer patients with high MALAT1 expression had significantly poorer overall survival (P<0.001) than that with low MALAT1 expression. As shown in **Table 2**, univariate analysis showed that HRs (95% CI, *P* value) of age (>45 years vs. ≤45 years), tumor size (>4 cm vs. ≤4 cm), FIGO stage (IIb-IIIa vs. Ib-IIa), differentiation(poor vs. well/moderate) and MALAT1 expression (high vs. low) were 1.01 (0.52-1.92, P=0.994), 2.32 (1.21-4.31, P=0.011), 2.94 (1.54-5.63, P=0.001), 2.96 (1.51-5.62, P=0.001), 3.52 (1.66-7.47, P=0.001), respectively. Then we applied multivariate analysis using the Cox proportional hazards model including these parameters and found that tumor size, FIGO stage and MALAT1 expression were independent prognostic factors for overall survival, with HRs of 1.97 (1.01-3.82, P=0.046), 2.46 (1.23-4.80, P=0.008), 2.46 (1.11-5.45, P=0.027), respectively.

Interaction between MALAT1 and other risk factors

Table 3 shows the interaction between MALAT1 and other parameters. Compared with reference, univariate analysis showed that HRs of Age & MALAT1 (>45 years & high), Tumor size & MALAT1 (>4 cm & high), FIGO stage & MALAT1 (IIb-IIIa & high), Differentiation & MALAT1 (Poor & high) were 3.67 (1.31-10.33), 6.34 (2.32-17.33), 11.64 (3.34-40.56), 8.53 (2.80-25.94), respectively (all P<0.05). However, we only observed significant interaction between Differentiation & MALAT1, with *P* value of 0.035. Then multivariate analysis were performed to calculate adjusted HRs. Compared with reference, HRs of Age & MALAT1 (>45 years & high), Tumor size & MALAT1 (>4 cm & high), FIGO stage & MALAT1 (IIb-IIIa& high), Differentiation & MALAT1 (Poor & high) were 1.95 (0.64-5.97, P=0.244), 4.74 (1.64-13.70, P=0.004), 8.83 (2.42-32.26, P=0.001), 5.82 (1.85-18.25, P=0.003). No significant interaction between MALAT1 expression and other risk factors was

Table 2. Univariate and multivariate analysis of risk factors for overall survival

	Univariate analysis		*Multivariate analysis*	
Parameters	HR (95% CI)	P	HR (95% CI)	P
Age				
≤45	Ref		Ref	
>45	1.01 (0.52-1.92)	0.994	0.98 (0.50-1.94)	0.962
Tumor size				
≤4	Ref		Ref	
>4	2.32 (1.21-4.31)	0.011	1.97 (1.01-3.82)	0.046
FIGO stage				
Ib-IIa	Ref		Ref	
IIb-IIIa	2.94 (1.54-5.63)	0.001	2.46 (1.23-4.80)	0.008
Differentiation				
Well/Moderate	Ref		Ref	
Poor	2.96 (1.51-5.62)	0.001	1.69 (0.83-3.43)	0.148
MALAT1				
Low	Ref		Ref	
High	3.52 (1.66-7.47)	0.001	2.46 (1.11-5.45)	0.027

observed, suggesting MALAT1 may be a stable prognosis factor for overall survival of cervical cancer patients.

Discussion

In this study, we confirmed that LncRNA MALAT1 expression is significantly increased in plasma of cervical cancer patients than that of normal participants. In addition, we also found high MALAT1 expression predicts a poor prognosis of cervical cancer. Furthermore, we showed that MALAT1 expression is correlated with tumor size, FIGO stage, and differentiation. More importantly, we demonstrated that there is no significant interaction between MALAT1 expression and other risk factors, suggesting MALAT1 may be a stable prognosis factor for overall survival.

In recent years, with the emergence of sequencing technique, widespread existence of lncRNAs in cervical cancer has been confirmed as well as their action mechanisms and important biological functions have gradually been elucidated. Hox transcript antisense intergenicIncRNA, named HOTAIR, has been proven to make critical effect on the most biological process of cervical cancer, via upregulating VEGF, MMP-9 and EMT-related genes and would be a potential new target in predicting recurrence and prognosis [14-16]. Additional studies have indicated that the involvement of lncRNAs in cervical cancer were through affecting on the cell proliferation and apoptosis, such as lncRNA-ANRIL, lncRNA-CCHE1, lncRNA-EBIC [17-19]. On the contrary were three downregulatedlncRNAs, namely growth arrest-specific transcript 5 (GAS5), tumor suppressor candidate 8 (TUSC8), and maternally expressed gene 3 (MEG3) [20-22].

The MALAT1 gene displays a high level of conservation throughout 33 mammalian species [23]. It was originally identified via subtractive hybridization as a prognostic parameter for patient survival of stage I lung adenocarcinoma or squamous cell carcinoma patients in 2003 anda later study identified MALAT1 as a noncoding transcript enriched in the nucleus of human primary fibroblasts or transformed lymphoblasts [23, 24]. Since its discovery a decade ago, a large amount of data have accumulated that link MALAT1 to other cancer types or diseases and provided insights into its biogenesis, interaction partners and cellular, as well as molecular functions [11]. In cervical cancer, reduction of MALAT1 in CaSki cervical cancer cells affects cervical cancer cell growth, cell cycle progression, and invasion through the regulation of gene expression, such as caspase-3, -8, Bax, Bcl-2, and BclxL [13]. Recently, a study showed that MALAT1 expression was memorably increased in tumorous tissue in comparison with adjacent tissue and was correlated with the size, FIGO stage, vessel invasion, and lymphatic diffusion acting independently as a predictive factor on prognosis in cervical cancer [25]. Due to the features of lncRNAs, such as long fragments (>200 nt), easy degradation in plasma, and extremely low concentration of total RNA in plasma, the current detection of plasma lncRNA as tumor markers becomes extremely challenging. However, some studies demonstrated that certain fragments in the plasma are highly stable and abundant [26, 27]. Chen et al. [28] indicated a specific stable MALAT1 fragment existed in plasma, which was identified by a previous study, thus making circulating lncRNA expression detection available [29].

Table 3. The interaction between plasma MALAT1 and other risk factors in predicting prognosis of cervical cancer patients

Parameters	N	Unadjusted HR (95% CI)	P	P for interaction	Adjusted HR (95% CI)	P	P for interaction
Age & MALAT1							
≤45 & low	31	Ref		0.343	Ref		0.279
≤45 & high	37	2.53 (0.69-6.98)	0.074		1.61 (0.56-4.61)	0.372	
>45 & low	30	0.70 (0.19-2.62)	0.594		0.52 (0.14-1.99)	0.340	
>45 & high	24	3.67 (1.31-10.33)	0.014		1.95 (0.64-5.97)	0.244	
Tumor size & MALAT1				0.757			
≤4 & low	46	Ref			Ref		0.912
≤4 & high	32	3.51 (1.23-9.96)	0.019		2.36 (0.79-7.06)	0.123	
>4 & low	15	2.30 (0.62-8.57)	0.215		1.84 (0.48-7.06)	0.374	
>4 & high	29	6.34 (2.32-17.33)	<0.001		4.74 (1.64-13.70)	0.004	
FIGO stage & MALAT1				0.092			
Ib-IIa & low	46				Ref		
Ib-IIa & high	37	6.34 (1.82-22.1)	0.004		4.80 (1.33-17.29)	0.017	0.131
IIb-IIIa & low	15	6.88 (1.71-29.62)	0.007		6.05 (1.50-24.45)	0.012	
IIb-IIIa & high	24	11.64 (3.34-40.56)	<0.001		8.83 (2.42-32.26)	0.001	
Differentiation & MALAT1							
Well/Moderate & low	49			0.035	Ref		0.068
Well/Moderate & high	35	5.70 (1.88-17.32)	0.002		4.74 (1.52-14.77)	0.007	
Poor & low	12	7.68 (2.05-28.76)	0.003		5.15 (1.30-20.42)	0.020	
Poor & high	26	8.53 (2.80-25.94)	<0.001		5.82 (1.85-18.25)	0.003	

Previous studies showed that MALAT1 expression was increased in tumorous tissue and was correlated with the size, FIGO stage, vessel invasion, and lymphatic diffusion. In present study, we firstly assessed the relevance of plasma MALAT1 to survival of cervical cancer and demonstrated that high plasma MALAT1 expression would also predict a poor prognosis of cervical cancer. Additionally, interaction analysis was performed between plasma MALAT1 expression and other clinical factors in predicting the prognosis of cervical cancer. Result showed no significant interaction between MALAT1 expression and other factors by multivariate analysis, indicating that plasma MALAT1 would be a stable and noninvasive biomarker for cervical cancer prognosis.

Obviously, the study on circulating LncRNA profiles offer an exciting expectation. However, some limitations and merit comments in this study should be mentioned. The population of enrolled patients and healthy participants were relatively small. Further study on a larger sample and longer follow-up is needed to confirm our results. Secondly, personal information were relatively insufficient. Lifestyle and dietary factors, which may be helpful, should be recorded. Finally, the plasma have been stored at -80°C until use. Immediate analysis should be taken after blood samples are collected for a better reflection of real condition.

In conclusion, above all, our results extend the findings of previous studies about MALAT1 in cervical cancer patients. Our data provide plasma MALAT1 was significantly higher in cervical cancer compared to normal participants, and demonstrated the ideal prognosis value to predict the survival of patients with cervical cancer.

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Disclosure of conflict of interest

None.

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