

# Plasma long non-coding RNA MALAT1 is associated with distant metastasis in patients with epithelial ovarian cancer

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**Abstract.** Human metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a newly identified metastasis-associated long non-coding RNA. In a previous study, it was identified that plasma levels of MALAT1 were significantly increased in gastric cancer patients with metastasis compared with gastric cancer patients without metastasis and healthy control individuals. However, it is unclear whether plasma levels of MALAT1 may act as a biomarker for evaluating the development of metastasis in epithelial ovarian cancer (EOC). In the present study, groups that consisted of 47 patients with EOC and metastasis (EOC/DM), 47 patients with EOC without metastasis (EOC/NDM), and 47 healthy control (HC) individuals were established. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the level of plasma MALAT1 in these groups. The results showed that levels of plasma MALAT1 were significantly increased in the EOC/DM group compared with the EOC/NDM and HC groups ( $P < 0.001$ ). Receiver operating characteristic (ROC) analysis indicated that plasma MALAT1 yielded an area under the curve (AUC) of 0.820 [95% confidence interval (CI), 0.734-0.905;  $P < 0.001$ ], distinguishing between EOC/DM and EOC/NDM. ROC analysis also yielded an AUC of 0.884 (95% CI, 0.820-0.949;  $P < 0.001$ ), with 89.4% sensitivity and 72.3% specificity for distinguishing between EOC/DM and HC. Furthermore, multivariate analysis indicated that

overexpression of MALAT1, differentiation (poor), tumor-node-metastasis stage (IV), lymph node metastasis (N3), peritoneal invasion (present) and higher serum carbohydrate antigen 125 levels were independent predictors of survival (hazard ratio, 3.322;  $P = 0.028$ ) in patients with EOC. Kaplan-Meier analysis revealed that patients with increased MALAT1 expression had a poorer disease-free survival time. In conclusion, the levels of plasma MALAT1 may act as a valuable biomarker for the diagnosis of metastasis.

## Introduction

Although the incidence of ovarian cancer ranked eighth and the mortality ranked seventh in females worldwide in 2008 (1), ovarian cancer remains one of the most prevalent, deadly and costly gynecological cancers (2). Epithelial ovarian cancer (EOC) accounts for almost 90% of all ovarian tumors (3-5) and is characterized by advanced-stage presentation, multiple organ metastases, peritoneal dissemination and refractory ascites when diagnosed (6). Although progress in early detection technology (transvaginal ultrasound, CA125 examination, computed tomography and BRCA gene mutation detection) and intervention management (surgical management for borderline ovarian tumors, ovariectomy for high-risk women) has decreased the mortality rate in multiple fields, advanced-stage diseases, particularly those that have metastasized, continue to frequently progress to recurrent disease and have a poor prognosis, with an average disease progression-free survival time of 18 months and a 5-year overall survival rate of <30% (7-9).

At present, the diagnosis of metastasis depends on detection by ultrasound and computed tomography scanning. As a supplemental diagnostic marker, the level of carbohydrate antigen 125 (CA125) is also considered (10). However, these measures have limited utility due to their inadequate sensitivity and specificity (11); the vast majority of metastases continue to be found during surgery due to false negative tests (12). Therefore, the development of novel biomarkers for early detection of EOC with metastasis (EOC/DM) is imperative and urgent.

There are a large number of non-protein-coding RNAs in the human genome, which do not encode proteins and were once considered as 'noise' in genome transcription (13,14). Previous studies have found that these non-protein coding RNAs regulate cell differentiation, growth and metabolism,

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*Abbreviations:* lncRNAs, long non-coding RNAs; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; EOC, epithelial ovarian cancer; EOC/DM, epithelial ovarian cancer with metastasis; EOC/NDM, epithelial ovarian cancer without metastasis; HC, healthy controls; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CA125, carbohydrate antigen 125

*Key words:* long non-coding RNA, MALAT1, epithelial ovarian cancer, distant metastasis

in addition to numerous other biological processes, at the transcriptional, post-transcriptional or translational levels, and at the DNA, RNA or protein levels (15-18). Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is one of the few well-studied long non-coding RNAs (lncRNAs), which presents high expression and enhanced activity in numerous tumor tissues and cells (19-21), playing a critical role in tumor invasion and metastasis (22). Studies have verified that MALAT1 is closely associated with metastasis in non-small cell lung cancer (NSCLC) (23,24) and several other human solid tumors, including colorectal cancer, osteosarcoma, bladder cancer and gallbladder cancer (25-28). At present, there is a lack of research concerning plasma MALAT1 expression in patients with metastatic EOC.

## Materials and methods

**Patients and samples.** A case-control study was designed to identify whether MALAT1 could be used as a substitute marker for EOC/DM. All patients were enrolled from the Xianyang Central Hospital (Xianyang, Shaanxi, China) between January 2010 and November 2014. The present study was approved by the Clinical Research Ethics Committee of Xianyang Central Hospital and all individuals provided written informed consent. Plasma preparation and RNA isolation were performed on the clinical samples obtained.

The present cohort totaled 141 female participants, consisting of 47 patients with EOC/DM, 47 patients with EOC without metastasis (EOC/NDM) and 47 healthy control (HC) individuals. The clinical stage of EOCs was conducted according to the tumor-node-metastasis (TNM) (29) and Federation of Gynecology and Obstetrics (30) staging systems. The inclusion criteria for EOC/DM were: Liver, lung or other distant organ metastasis identified by imaging and confirmed pathologically. The 47 patients with EOC/NDM had no pathological evidence of distant organ metastasis; HC individuals were also recruited as patients who underwent routine medical examination and had no evidence of tumor disease in the same period. The HC individuals were matched to the patients based on age and gender. The baseline features of the patients and HC individuals are summarized in Table I.

**Plasma preparation.** All plasma samples were collected prior to any therapeutic procedure, such as surgery, chemotherapy or radiotherapy. Plasma sample preparation and total RNA isolation were performed as previously described (31). In brief, 5 ml venous blood was collected in an EDTA gel tube, and the separation procedure was performed within 2 h of sample collection. Plasma samples were centrifuged at 1,000 x g for 10 min, followed by 13,000 x g for a further 10 min at 4°C. The supernatant plasma was then separated, split into 250  $\mu$ l aliquots, and frozen at -80°C until use.

**RNA preparation, reverse transcription (RT) and RT-quantitative polymerase chain reaction (qPCR).** Total RNA was extracted from plasma using TRIzol LS reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's protocol with minor modifications (when precipitating RNA, RNase-free glycogen was not added as a carrier to the aqueous phase). RNA was removed from

genomic DNA and reverse transcribed to cDNA using a PrimeScript<sup>®</sup> RT reagent Kit with gDNA Eraser (catalog no., DRR047; Takara Bio, Inc., Otsu, Shiga, Japan). Subsequently, RT-qPCR was performed using SYBR<sup>®</sup> Premix Ex Taq II (Perfect Real Time; catalog no., DRR081; Takara Bio, Inc.) on ABI7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). To examine the expression of MALAT1 in EOC, RT-qPCR amplification was performed using the following specific primers: MALAT1 sense, 5'-CTTCCCTAGGGG ATTTCAGG-3' and antisense, 5'-GCCACAGGAACAAG TCCTA-3' (32).  $\beta$ -actin was used as the internal reference, and the primers were as follows:  $\beta$ -actin sense, 5'-CTTAGT TGC GTTACACCC TTTCTTG-3' and antisense, 5'-CTGTCA CCTTACCCGTTCCAGTTT-3'. Amplification was performed under the following conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. Melting curve analysis was performed as follows: 95°C for 0 sec (at 20°C/sec from 72-95°C); and 65°C for 15 sec to 95°C for 0 sec (at 0.1°C/sec). Experiments were performed in triplicate in the same reaction. The results of RT-qPCR experiments were calculated using the  $2^{-\Delta\Delta Cq}$  method (33).

**Statistical analysis.** Normalized MALAT1 levels were demonstrated as  $\Delta Cq$ , with  $\Delta Cq = Cq$  (MALAT1) -  $Cq$  ( $\beta$ -actin). The difference between groups was analyzed by the Mann-Whitney or Kruskal-Wallis tests. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were conducted to determine the diagnostic power of plasma MALAT1 in distant metastasis. COX analysis was used to assess the clinicopathological factors that were associated with survival in the EOC patients. The clinicopathological features were assessed by one-way analysis of variance, Student's t-test or  $\chi^2$  test, as appropriate. For the post-hoc test, multiple comparisons between the groups were performed using the Student-Newman-Keuls method.  $P < 0.05$  was considered to indicate a statistically significant difference. Statistical analyses were conducted using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Graphs and charts were created in GraphPad Prism (version 5.00 for Windows; GraphPad Software, Inc., La Jolla, CA, USA) and MedCalc software (version 12.3.0.0; MedCalc Software, Ostend, Belgium).

## Results

**Evaluation of clinical features in study groups.** The baseline clinicopathological features of the participants enrolled in the present study are summarized in Table I. All 94 EOC patients and 47 HC individuals did not differ in age or gender. In addition, there was no significant difference in the distribution of TNM stage, pathological differentiation, serum CA125 level, peritoneal invasion status, lymph node metastasis and metastatic sites between the EOC/DM and the EOC/NDM groups.

**MALAT1 expression was upregulated in the plasma of metastatic ovarian epithelial carcinoma patients.** The expression levels of plasma MALAT1 in 94 EOC patients and 47 HC individuals were examined by RT-qPCR. The findings demonstrated that, compared with the EOC/NDM and HC groups, the expression of plasma MALAT1 was significantly higher

Table I. Clinicopathological features of EOC patients and HC individuals.

Variables	EOC/DM, n (%)	EOC/NDM, n (%)	HC, n (%)
Total <sup>a</sup>	47 (100.0)	47 (100.0)	47 (100.0)
Age, years			
Median	58	59	59
Range	49-64	48-66	48-68
TNM stage			
III	0 (0.0)	4 (8.5)	
IV	47 (100.0)	43 (91.5)	
Pathology differentiation			
Well	5 (10.6)	7 (14.9)	
Moderate	17 (36.2)	19 (40.4)	
Poor	25 (53.2)	21 (44.7)	
Serum CA125 level			
≤35 U/ml	2 (4.3)	3 (6.4)	
>35 U/ml	45 (95.7)	44 (93.6)	
Peritoneal invasion			
Absence	27 (57.4)	30 (63.8)	
Presence	20 (42.6)	17 (36.2)	
Lymph node metastasis			
N1	14 (29.8)	17 (36.2)	
N2	20 (42.6)	17 (36.2)	
N3	13 (27.7)	13 (27.7)	
Metastasis location			
Absence	0 (0.0)	4 (8.5)	
Liver	29 (61.7)	28 (59.6)	
Lung	11 (23.4)	10 (21.3)	
Multiple organ	7 (14.9)	5 (10.6)	

<sup>a</sup>All patients and HC individuals were female. EOC, epithelial ovarian cancer; EOC/DM, EOC with metastasis; EOC/NDM, EOC without metastasis; HC, healthy control; TNM, tumor-node-metastasis; CA125, carbohydrate antigen 125.

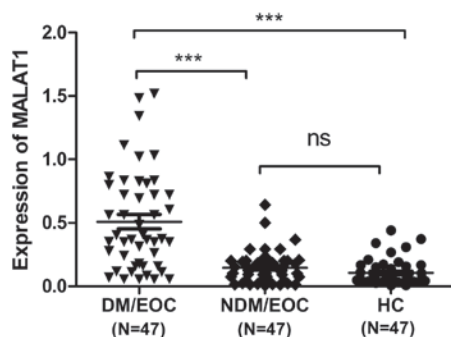


Figure 1. Plasma MALAT1 levels in EOC patients and HC individuals. The levels of plasma MALAT1 were determined by reverse transcription-quantitative polymerase chain reaction. Data are expressed as the mean  $\log_{10} 2^{-\Delta\Delta Ct}$  relative to the control  $\beta$ -actin. The horizontal lines indicate the median values. \*\*\* $P < 0.0001$ . MALAT1, metastasis-associated lung adenocarcinoma transcript 1; EOC, epithelial ovarian carcinoma; HC, healthy control; DM/EOC, EOC with metastasis; NDM/EOC, EOC without metastasis; ns, not significant.

in the EOC/DM group ( $P < 0.001$  vs. EOC/NDM and HC; Fig. 1). In addition, Youden's index (sensitivity plus specificity minus 1) was chosen as the optimal cutoff value and ROC

curves were constructed to estimate the diagnostic value of MALAT1 in discriminating distant metastasis in EOCs. ROC analyses indicated that plasma MALAT1 yielded an AUC of 0.820 [95% confidence interval (CI), 0.734-0.905;  $P < 0.001$ ], with 87.2% sensitivity and 72.3% specificity for distinguishing EOC/DM from EOC/NDM. ROC analysis also yielded an AUC of 0.884 (95% CI, 0.820-0.949;  $P < 0.001$ ), with 89.4% sensitivity and 72.3% specificity for distinguishing EOC/DM from HC. The plasma levels of MALAT1 were effectively distinguished in patients with EOC/DM from those with EOC/NDM or HC individuals (Fig. 2).

*Cox analysis and Kaplan-Meier survival analysis of prognostic factors in EOC.* Effect on the prognosis of plasma MALAT1 expression and other clinicopathological factors was also assessed. Low levels of CA125 were considered as  $\leq 35$  U/ml, while high levels of CA125 were  $> 35$  U/ml. Low levels of plasma MALAT1 were considered as a fold-change of  $< 2$ , while high levels of plasma MALAT1 were a fold-change of  $> 2$ . Univariate and multivariate Cox analysis revealed that poor differentiation, TNM stage IV, N3 lymph node metastasis, presence of peritoneal

Table II. Univariate and multivariate analysis of plasma MALAT1 and clinicopathological factors associated with survival in epithelial ovarian cancer patients.

Variable	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Differentiation (poor)	3.576	1.356-6.754	0.015	2.357	1.085-5.867	0.023
TNM stage (IV)	2.376	0.989-5.374	0.005	1.864	0.956-4.356	0.043
Metastasis location (lung)	1.137	0.376-3.202	0.082	-	-	-
Lymph node metastasis (N3)	1.271	0.352-2.328	0.043	1.479	0.561-4.546	0.037
Peritoneal invasion (presence)	2.725	0.934-6.347	0.025	2.435	0.834-4.563	0.039
Serum CA125 (>35 U/ml)	2.259	0.563-3.194	0.018	3.189	1.043-6.487	0.017
Plasma MALAT1 (high)	2.391	1.057-4.672	0.031	3.322	1.140-5.680	0.028

TNM, tumor-node-metastasis; CA125, carbohydrate antigen 125; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; CI, confidence interval.

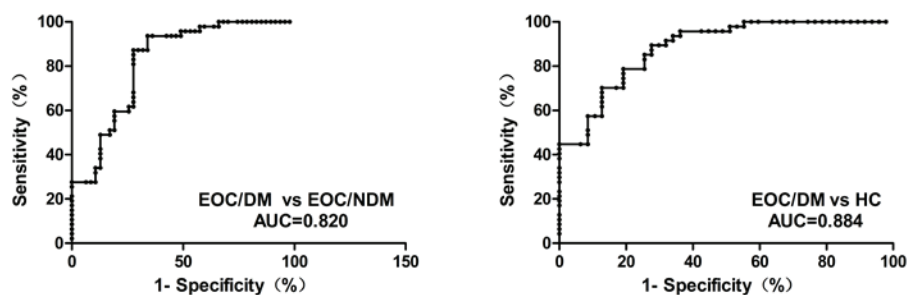


Figure 2. ROC analysis of the sensitivity and specificity of the levels of plasma MALAT1 in distinguishing DM/EOC from NDM/EOC and HC patients. The levels of plasma MALAT1 in the DM/EOC (n=47), NDM/EOC (n=47) and HC (n=47) groups were subjected to ROC analyses. The ROC-AUC for the levels of plasma MALAT1 to distinguish EOC/DM patients from EOC/NDM patients or HC individuals was 0.820 and 0.884, respectively ( $P < 0.001$ ). MALAT1, metastasis-associated lung adenocarcinoma transcript 1; EOC, epithelial ovarian cancer; EOC/DM, EOC with metastasis; EOC/NDM, EOC without metastasis; HC, healthy controls; AUC, area under the curve; ROC, receiver operating characteristic.

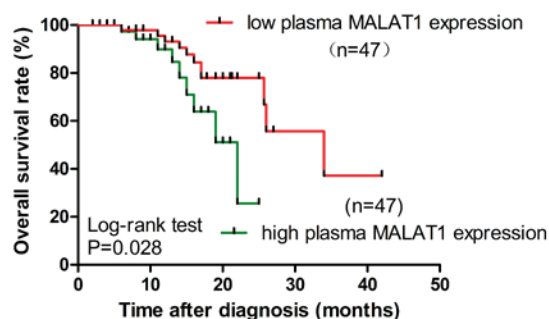


Figure 3. Kaplan-Meier survival curves for patients with EOC plotted for plasma MALAT1 levels. The P-value was calculated using the log-rank test between patients with high fold-change ( $>2$ ) and low fold-change ( $<2$ ). The OS rate of patients with EOC with low plasma MALAT1 expression was significantly higher than the OS in the patients with high plasma MALAT1 expression ( $P = 0.028$ ). EOC, epithelial ovarian cancer; OS, overall survival; MALAT1, metastasis-associated lung adenocarcinoma transcript 1.

invasion, high serum CA125 levels and high plasma MALAT1 levels were significantly associated with short life expectancy (Table II). Using the Kaplan-Meier and log-rank methods, it was found that high expression of the plasma MALAT1 was associated with a shorter overall survival (OS) of patients

with EOC (hazard ratio, 3.322; 95% CI, 1.140-5.680;  $P = 0.028$ ; Fig. 3). Upregulation of plasma MALAT1 may independently contribute to a poor prognosis in patients with EOC.

## Discussion

EOC ranks among the most common and lethal malignant diseases in gynecological cancers. Due to the location of the ovaries and the biological characteristics of insidious onset of EOC, it becomes extremely challenging to diagnose ovarian cancer at an early curable stage. In total,  $>70\%$  of patients present with advanced disease at diagnosis (34). The cure rate of women with advanced ovarian cancer is  $\leq 40\%$  as a result of challenging and inefficient surgery on metastatic disease (35). Poor prognosis of EOC is associated with the early onset of tumor metastasis. The mechanisms of EOC metastasis development are poorly understood and require additional exploration. Several studies have found that abnormal expression of lncRNAs are involved in tumor metastasis through regulating the expression of other molecules (36-39). Ji *et al* (22) identified the MALAT1 lncRNA by subtractive hybridization, establishing it as a prognostic marker for metastasis and survival in NSCLC. However, the roles of MALAT1 in the metastasis of EOC have yet to be fully elucidated.

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. Arita *et al* found that the lncRNA full-length form is not stable in plasma (40), but certain fragments in the plasma are highly stable and abundant (32,41,42). The present study assessed the relevance of plasma MALAT1 to metastasis of EOC. The results demonstrated that a specific stable MALAT1 fragment existed in plasma, which was identified by a previous study (43), thus making circulating lncRNA expression detection available.

The present results showed that the level of plasma MALAT1 was markedly increased in patients with EOC/DM compared with patients with EOC/NDM and the HC group, and could effectively identify patients in the EOC/DM group from those in the EOC/NDM and HC group, thus indicating a suitable plasma biomarker for MALAT1 in EOC metastasis development. Furthermore, through Kaplan-Meier survival analysis and Cox analysis, it was found that elevated MALAT1 expression was associated with a shorter OS time in patients with EOC, as well as poor differentiation, advanced-stage disease, multiple lymph node metastases, presence of peritoneal invasion and increased serum CA125 level. Although the mechanism remains to be elucidated, these findings indicated that MALAT1 may play an important role in the cancer metastatic process, and may be a useful novel marker for metastatic EOC. Similar to the present findings, Shen *et al* (44) found that the level of MALAT1 was significantly increased in brain metastasis compared with non-brain metastasis samples, and subsequent functional studies indicated that MALAT1 promoted lung cancer cell brain metastasis by inducing epithelial-mesenchymal transition. Several studies have revealed that there are other MALAT1-mediated molecular pathways involved in tumor metastasis, such as through binding to splicing factor, proline- and glutamine-rich (SFPQ) and releasing the oncogene polypyrimidine tract binding protein 2 (PTBP2) from the SFPQ/PTBP2 complex (25), activating the extracellular signal-regulated kinase/Mitogen-activated protein kinase or phosphoinositide 3-kinase/AKT pathway to result in distant metastasis in numerous cancer types (26,28). Overall, these mechanisms may explain why the present patients with high expression of MALAT1 expression demonstrated a strong metastasis tendency.

In summary, the high heterogeneity of metastatic EOC leads to resistance to chemotherapy drugs, poor prognosis and lack of effective targeting therapy. Therefore, identifying specific EOC metastasis-associated markers, and subsequently executing an early intervention have become the 'bottleneck' problem of EOC treatment. In the present study, it was found that plasma MALAT1 upregulation was closely associated with EOC distant metastasis and may be an independent risk factor for poor prognosis. The results of the present study may aid in understanding the molecular mechanisms of EOC with metastasis, which possess potential clinical value for metastatic EOC screening and early diagnosis, and may be a potential target for the treatment of EOC with metastasis.

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