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Original Article

High expression of lncRNA MALAT1 suggests a biomarker of poor prognosis in colorectal cancer

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Abstract: Objective: This study sought to investigate the role of the long noncoding RNA MALAT1 in the prognosis of stage II/III colorectal cancer (CRC) patients. Methods: The expression of MALAT1 was evaluated in cancer tissues from 146 stage II/III CRC patients undergoing radical resection and 23 paired normal colonic mucosa samples using quantitative real-time reverse transcriptase PCR. Differences in the expression of MALAT1 between 23 CRC and paired normal colonic mucosa samples were analysed with the Wilcoxon test. Relationships between the expression level of MALAT1, patient clinicopathological parameters and disease-free survival (DFS) and overall survival (OS) were analysed using the univariate Kaplan-Meier method and the multivariate COX regression model. Results: The MALAT1 levels in cancerous tissues were 2.26 times higher than those measured in noncancerous tissues, and this difference was statistically significant ($P = 0.0004$). Based on their expression level of MALAT1, the patients were divided into a high MALAT1 expression group (n = 73) and a low expression group (n = 73). Patients with tumours harbouring higher expression of MALAT1 showed a significantly worse prognosis with a hazard ratio (HR) of 2.863 (95% CI, 1.659 to 4.943; $P < 0.001$) for DFS and 3.968 (95% CI, 1.665 to 9.456; $P = 0.002$) for OS. Furthermore, patients with perineural invasion demonstrated significantly worse DFS (HR = 3.459, 95% CI 2.008 to 5.957; $P < 0.001$) and OS (HR = 3.750, 95% CI 1.743 to 8.069; $P = 0.001$) than those without perineural invasion. Multivariate analyses indicated that MALAT1 expression and perineural invasion were two independent prognostic risk factors for patients with CRC. Conclusion: The expression of MALAT1 is upregulated in CRC tissues, and a higher expression level of MALAT1 might serve as a negative prognostic marker in stage II/III CRC patients.

Keywords: Colorectal cancer, locally advanced stage, prognosis, long noncoding RNA, MALAT1

Introduction

Noncoding RNAs (ncRNAs) are found throughout the genome, although their functions are only partially understood. Long non-coding RNAs (lncRNAs, > 200 nt in length) were initially thought to be spurious transcriptional noise but are emerging as new regulators in the cancer paradigm [1, 2]. In fact, emerging evidence indicates that lncRNAs may have complex and extensive functions in the development and progression of cancer [3-9]. Metastasis associated lung adenocarcinoma transcript 1 (*MALAT1*) is a highly abundant nucleus-restricted lncRNA that localises to nuclear speckles. Recent studies have established that *MALAT1* is involved in cancer metastasis and recurrence and is upregulated in several solid tumours, including lung cancer, sarcomas of the uterus and hepatocellular carcinomas [10-13]. Moreover, *MALAT1* was shown to enhance cellular proliferation and tumour formation, whereas depletion of *MALAT1* in tumour cells led to reduced tumourigenicity [13, 14]. *MALAT1* has also been found to regulate the activity of the E2F1 transcription factor, which is a crucial determinant of cell cycle progression and tumourigenesis [15-18]. These studies show that the upregulated expression of *MALAT1* may play a role in promoting tumourigenesis and suggest that tumours expressing a high level of *MALAT1* should be expected to display

Table 1. Distribution of the included CRC patients according to clinicopathological variables

Variables	Number (%)
Age (years)	
Median (range)	57 (27-81)
< 65	108 (74.0)
≥ 65	38 (26.0)
Sex	
Male	89 (61.0)
Female	57 (39.0)
Tumour site	
Rectum	95 (65.1)
Colon	51 (34.9)
Tumour histology	
Adenocarcinoma	140 (95.9)
Mucinous adenocarcinoma	6 (4.1)
Tumour differentiation	
Well	14 (9.6)
Moderate	99 (67.8)
Poor	33 (22.6)
T stage	
T2	10 (6.8)
T3	40 (27.4)
T4	96 (65.8)
N stage	
N0	55 (37.7)
N1	51 (34.9)
N2	40 (27.4)
TNM stage	
Stage II	55 (37.7)
Stage III	91 (62.3)
Lymph vascular invasion	
Absence	104 (71.2)
Presence	42 (28.8)
Perineural invasion	
Absence	114 (78.1)
Presence	32 (21.9)
Adjuvant chemotherapy*	
No	15 (10.3)
Yes	113 (89.7)

*All patients with stage III disease underwent adjuvant chemotherapy.

a more aggressive behaviour and have a poorer prognosis. However, the role of MALAT1 in colorectal cancer (CRC) is not well studied. In this study, we investigated the correlations between the expression of MALAT1 and the clinicopathological features and survival outcomes of stage II and III CRC patients.

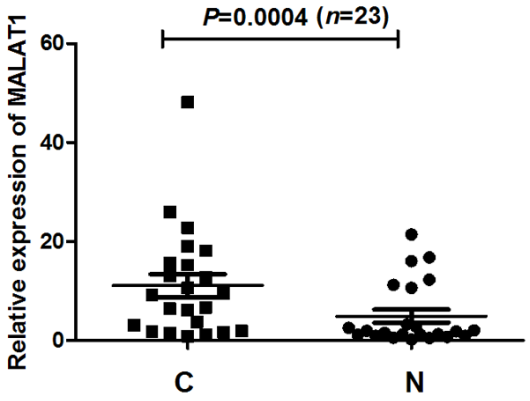

Figure 1. MALAT1 expression levels assessed by quantitative real-time PCR in cancerous (C) and paired noncancerous tissues (N) from 23 CRC samples. The MALAT1 levels were normalised to GAPDH; the MALAT1 levels in T were significantly higher than those in N (P = 0.0004).

Materials and methods

Clinical samples

A total of 146 fresh cancer tissue samples were obtained from stage II and III CRC patients who underwent radical surgical resection without preoperative chemotherapy or radiotherapy at Fudan University Shanghai Cancer Hospital in China between November 2007 and December 2009. All specimens were immediately frozen in tubes containing RNAlater preservation liquid after removal and stored at -80°C until RNA extraction. The tumour specimens were pathologically confirmed to be adenocarcinoma, mucinous adenocarcinoma or signet carcinoma and staged according to the 7th version of the American Joint Committee on Cancer (AJCC) cancer staging system.

The detailed clinicopathological characteristics of the recruited patients are summarised in **Table 1**. The follow-up data were obtained by reviewing the out-patient charts and contacting the patients by telephone or mail. This study was approved by the Research Ethics Committee of Fudan University Shanghai Cancer Center in China.

RNA preparation, reverse transcription and quantitative real-time PCR

Total RNA was extracted from cancerous and noncancerous tissue specimens using the Trizol reagent (Invitrogen, Carlsbad, CA). The

Table 2. Association between MALAT1 expression and clinicopathological variables of the studied CRC patients

Variables	Low MALAT1 expression (n = 73) n (%)	High MALAT1 expression (n = 73) n (%)	*P*
Age (years)			
< 65	57 (78.1)	51 (69.9)	0.258
≥ 65	16 (21.9)	22 (30.1)	
Sex			
Male	36 (49.3)	53 (72.6)	0.004
Female	37 (50.7)	20 (27.4)	
Tumour site			
Rectum	48 (65.8)	47 (64.4)	0.862
Colon	25 (34.2)	26 (35.6)	
Tumour histology			
Adenocarcinoma	61 (83.6)	63 (86.3)	0.644
Mucinous adenocarcinoma	12 (16.4)	10 (13.7)	
Tumour differentiation			
Well/moderate	57 (78.1)	56 (76.7)	0.843
Poor	16 (21.9)	17 (23.3)	
T stage			
T2/3	25 (34.2)	25 (34.2)	1.000
T4	48 (65.8)	48 (65.8)	
N stage			
N0	24 (32.9)	31 (42.5)	0.232
N1/2	49 (67.1)	42 (57.5)	
TNM stage			
II	24 (32.9)	31 (42.5)	0.233
III	49 (67.1)	42 (57.5)	
Lymph vascular invasion			
Absence	50 (68.5)	54 (74.0)	0.465
Presence	23 (31.5)	19 (26.0)	
Perineural invasion			
Absence	54 (74.0)	60 (82.2)	0.230
Presence	19 (26.0)	13 (17.8)	

RNA was reverse transcribed into cDNA using the Promega GoScript Reverse Transcription System (Promega, USA). MALAT1 levels were quantified using the LightCycler 480 Probes Master kit (Roche Applied Science) according to the manufacturer's protocol with the following specific *MALAT1* primers: forward, 5'-CAGTGGGGAACTCTGACTCG-3' and reverse, 5'-GTGCCTGGTGCTCTCTTACC-3'. The levels of *MALAT1* were normalised to *GAPDH* (forward, 5'-GTCAACGGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3').

Statistical analysis

Comparisons of continuous data between two groups were performed using an independent *t*-test, and categorical data were analysed using the chi-square test or Fisher's exact test.

Overall survival (OS) was calculated from the date of surgery to the date of death or the last follow-up. Disease-free survival (DFS) was defined from the date of surgery to the date of local or distant recurrence or the date of the last follow-up. Patient survival rates were calculated using the Kaplan-Meier method, and statistically significant differences in survival were identified using the log-rank test. Multivariate analysis was performed to estimate the association between clinical and genetic features and DFS and OS using Cox proportional hazard models. In the multivariate Cox model, variables with $P < 0.05$ from the univariate model were included.

All statistical analyses were performed using SPSS for Windows v.17.0 (SPSS, Chicago, IL). *P* values less than 0.05 were considered statistically significant.

Results

MALAT1 expression in CRC tissue and adjacent normal tissue

The expression levels of *MALAT1* in 23 cancerous and noncancerous tissues were examined by quantitative real-time PCR. The levels of *MALAT1* in cancerous tissues were 2.26 times higher than those measured in noncancerous tissues, and this difference was statistically significant ($P = 0.0004$; **Figure 1**). These results showed that MALAT1 was upregulated in CRC tumours.

Table 3. Univariate analysis of DFS and OS for the 146 studied CRC patients

Variables	DFS	OS
	P (Log-rank)	P (Log-rank)
MALAT-1 expression		
Low	0.001	0.003
High		
Age (years)		
< 65	0.443	0.238
≥ 65		
Sex		
Male	0.977	0.937
Female		
Tumour site		
Rectum	0.468	0.680
Colon		
Tumour histology		
Adenocarcinoma	0.912	0.437
Mucinous adenocarcinoma		
Tumour differentiation		
Well/moderate	0.517	0.747
Poor		
TNM stage		
II	0.174	0.043
III		
Lymph vascular invasion		
Absence	0.361	0.144
Presence		
Perineural invasion		
Absence	< 0.001	0.002
Presence		
Adjuvant chemotherapy*		
No	0.572	0.154
Yes		

*All patients with stage III disease underwent adjuvant chemotherapy.

Relationship between MALAT1 expression and the clinicopathological features of CRC patients

We next examined the expression of MALAT1 in 146 cases of stage II and III CRC. According to the expression of *MALAT1*, these cases were divided into a high *MALAT1* expression group (n = 73) and a low expression group (n = 73), based on a *MALAT1/GAPDH* ratio of 6.15 in cancerous tissue. The expression of MALAT1 was significantly higher in male patients than in female patients ($P = 0.004$), although no associations were found between MALAT1 expression and other clinicopathological features (**Table 2**).

Univariate analysis of prognostic factors in stage II and III CRC patients

The median follow-up period for the patients studied was 56.2 months, with a range of 11 to 72.8 months. MALAT1 expression and perineural invasion were significantly correlated with DFS and OS (**Table 3**). In particular, patients with a high level of MALAT1 expression showed significantly shorter DFS ($P = 0.001$, **Figure 2**) and OS ($P = 0.003$, **Figure 3**) than patients with low MALAT1 expression, and patients with perineural invasion demonstrated significantly shorter DFS ($P < 0.001$) and OS ($P = 0.002$) than those without perineural invasion.

Multivariate analysis of prognostic factors in stage II and III CRC patients

Further multivariate COX regression analysis indicated that both MALAT1 expression and perineural invasion served as predictors of poor prognosis regarding DFS (MALAT1: HR = 2.863, 95% CI = 1.659-4.943, $P < 0.001$; perineural invasion: HR = 3.459, 95% CI = 2.008-5.957, $P < 0.001$) and OS (MALAT1: HR = 3.968, 95% CI = 1.665-9.456, $P = 0.002$; perineural invasion: HR = 3.750, 95% CI = 1.743-8.069, $P = 0.001$) in CRC patients.

Discussion

CRC is one of the most common human malignant diseases and remains the third leading cause of cancer-related death worldwide [18]. Although recent diagnostic and therapeutic advances have improved the clinical outcomes of patients with early stage CRC, a significant fraction of early stage CRC patients still develop recurrence. Moreover, there are few reliable markers available to accurately predict metastasis in early stage CRC patients, and individual adjuvant treatment therefore remains a challenge.

In recent years, lncRNAs have been increasingly reported to be involved in human disease [20, 21]. MALAT1 is a lncRNA of more than 8,000 nt derived from chromosome 11q13 [10] and was originally found to be overexpressed in

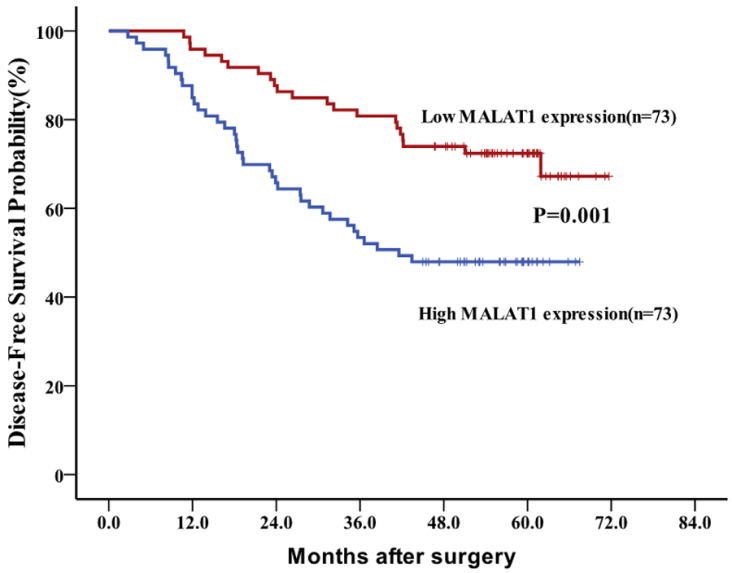

Figure 2. Kaplan-Meier curves of MALAT1 expression for stage II/III CRC patients in relation to DFS (5-year DFS 67% vs. 48%, *P* = 0.001).

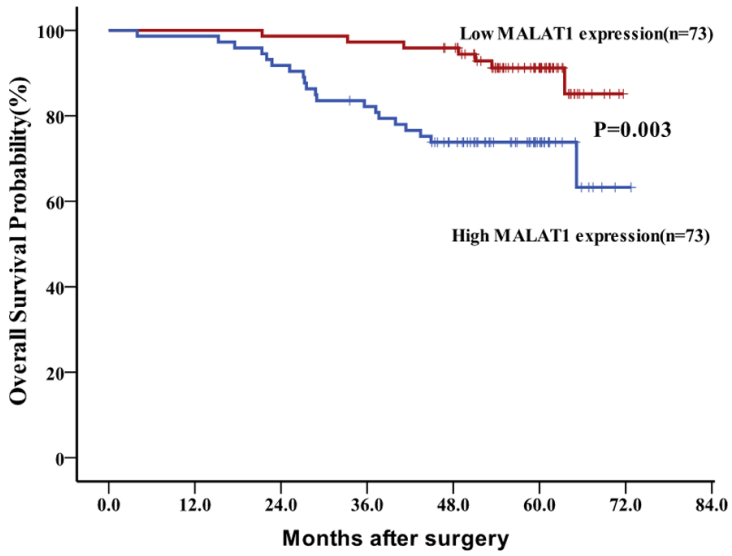

Figure 3. Kaplan-Meier curves of MALAT1 expression for stage II/III CRC patients in relation to OS (5-year OS 85% vs. 67%, *P* = 0.003).

patients with a high risk for non-small cell lung cancer (NSCLC) metastasis [10]. In addition to NSCLC, MALAT1 has been linked to several other human tumour types [11, 12, 22-25], and elevated expression of MALAT1 has been reported in liver [12, 22], breast [23, 24], and uterine cancer [11]. MALAT1 is thought to regulate tumour cell viability, invasiveness and migration, and although the roles of MALAT1 in these processes are best understood in lung cancer, they have also been observed in liver and cervical cancer [17, 26-28]. In addition, mutations in the human MALAT1 gene were recently discovered in breast cancer and CRC [15, 29]. In CRC, these mutations are enriched in the 3'-end of MALAT1, and the over-expression of MALAT1 was shown to enhance the invasive behaviour of the SW480 colon cancer cell line [15]. However, the association between MALAT1 and tumour recurrence and whether MALAT1 is upregulated in CRC was previously unknown.

In our study, we found that the MALAT1 levels in cancerous tissues were significantly higher than those in noncancerous tissues, suggesting a positive role for MALAT1 in CRC tumourigenesis. In cervical cancer cells, depletion of MALAT1 led to the induction of proapoptotic genes, such as CASP3, CASP8 and BAX, and the downregulation of antiapoptotic genes, such as BCL2 and BCL2L1 [17, 30]. However, whether this regulatory role of MALAT1 in apoptosis contributes to tumourigenesis remains unknown. Ongoing studies in our lab seek to identify the biological functions of MALAT1 in normal cells, benign adenoma cells and malignant CRC cells to elucidate the impact of MALAT1 on colorectal tumourigenesis.

Our results also demonstrated that patients with high MALAT1 expression had a significantly higher risk of metastasis after radical surgery and significantly poorer OS. MALAT1 was first associated with tumour metastasis in NSCLC patients, and knockdown of MALAT1 by zinc finger nucleases (ZFNs) in A549 lung cancer cells was shown to inhibit cell migration without affecting cell proliferation [31]. Subsequent functional analyses of knockout cells revealed that MALAT1 regulates a "metastatic signature", consisting of

genes such as LAYN [31]. Similar to these findings, the expression of MALAT1 was significantly increased in bladder cancer patients who developed metastases as compared to those without metastases [32], and further analysis of the underlying mechanism showed that MALAT1 was critical for promoting epithelial-mesenchymal transition (EMT) through Wnt signalling involving ZEB1, ZEB2 and SNAI2 [32]. Recently, Xu et al. [15] reported that MALAT1 enhanced the colony formation capability and invasive behaviour of SW480 colon cancer cells. Together, these mechanisms may explain why our patients with high expression of MALAT1 demonstrated a strong tendency for metastasis after radical surgery. Further studies, however, are required to evaluate the biological mechanism by which MALAT1 promotes CRC metastasis.

Multivariate COX regression analysis indicated that both MALAT1 expression and perineural invasion were independent predictors of DFS and OS. However, tumour stage was not identified as an independent predictor, which is a traditional prognostic factor of CRC. It can be noted that all the stage III patients underwent adjuvant chemotherapy while only 73% (40/55) of stage II patients received adjuvant chemotherapy. Adjuvant chemotherapy has been proved to increase the survival of stage III CRC patients. Therefore, the different prevalence of adjuvant chemotherapy between stage II and stage III patients might reduce the survival discrepancy between these two groups.

The identification of MALAT1 as a possible biomarker for the metastasis of CRC suggests that MALAT1 may be a promising target for intervention. In fact, the finding that MALAT is involved in lung cancer led to the successful use of antisense oligonucleotides (ASOs) to target MALAT1 in a pulmonary metastatic model in vivo, resulting in the inhibition of metastasis in a nude mouse model of lung cancer [28]. However, further research is necessary to evaluate whether MALAT1 could serve as a specific target for the treatment of CRC.

In conclusion, elevated expression of MALAT1 may be involved in the progression of CRC and may therefore be considered a prognostic factor for stage II/III CRC patients. The identification of this new biomarker offers an important glimpse into the possible molecular mechanisms underlying the recurrence and metastasis of CRC and should help to identify therapeutic targets for more effective targeted treatment.

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Disclosure of conflict of interest

None.

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