

# Suppression of MAL gene expression is associated with colorectal cancer metastasis

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Received July 24, 2014; Accepted April 8, 2015

DOI: 10.3892/ol.2015.3355

**Abstract.** Mal, T-cell differentiation protein (MAL) is a candidate tumor suppressor gene that functions in membrane trafficking processes in polarized epithelial cells. The aim of the present study was to determine its clinical significance in colorectal cancer (CRC). The RNA and protein expression levels of MAL in 30 colorectal specimens were detected by semi-quantitative polymerase chain reaction and immunohistochemistry analysis. Statistical analysis was performed using SPSS software. The RNA level of MAL was significantly downregulated in the CRC tissues compared with the adjacent healthy tissue ( $P < 0.05$ ). MAL was only positively expressed in 20% of the CRC tissues, but in 66.7% of the adjacent tissues, as determined by immunohistochemistry analysis. The expression of the MAL RNA transcript exhibited a positive correlation with protein expression. The expression levels of MAL were significantly associated with different tumor-node-metastasis stages and lymph node metastasis ( $P < 0.05$ ), but not with age, gender, tumor site, differentiation status and pathological type ( $P > 0.05$ ). Suppression of MAL expression was significantly correlated with metastasis in CRC. The present study indicated that MAL may function as an anti-metastasis factor and represent a potential biomarker for malignant colorectal tumors.

## Introduction

Colorectal cancer (CRC) is the third most common malignant cancer and the second leading cause of mortality worldwide (1). In China, the incidence rate of CRC is increasing rapidly, particular in urban areas, such as Shanghai, which was formerly considered a low-risk area (2,3). Metastasis is

the main cause of mortality in CRC patients (4). The first sites of metastatic CRC are the regional lymph nodes and the liver. Investigations into the molecular mechanisms involved in CRC metastasis are of major importance in order to develop novel strategies for targeted therapies (5).

The mal, T-cell differentiation protein (MAL) gene encodes the T-lymphocyte maturation-associated protein and functions in T-cell differentiation (6). Recently, downregulation of MAL has been associated with a variety of human epithelial malignancies. For example, Mimori *et al* (7) initially found that MAL was highly expressed in normal esophageal epithelia, but silenced in esophageal tumors. Later, inactivation of MAL was shown to be a common event in breast, head and neck, and gastric cancer (8-11). However, the functional role and mechanistic action of MAL in CRC remains largely unknown. The present study aimed to determine the clinical significance of MAL in colorectal cancer by measuring its expression level in CRC tissues.

## Materials and methods

**Tissue samples and cell line.** The present study enrolled a total of 30 CRC patients who underwent surgery between 2010 and 2011 at Shanghai First People's Hospital (Shanghai, China). The patients did not receive any pre-operative cancer treatment prior to resection. Of the 30 patients, 18 were male and 22 were female, and the age of the patients ranged between 45 and 87 years (median, 69 years). The clinicopathological data of the CRC patients are shown in Table I.

**Ethical approval.** All clinical tissues were collected from the CRC patients after obtaining informed consent according to an established protocol approved by the Ethics Committee of Shanghai First People's Hospital.

**RNA isolation and semi-quantitative polymerase chain reaction (PCR).** Total RNA was extracted from CRC patient samples using the SQ DNA/RNA/Protein Tissue kit (#R8042-01; Sangon Biotech Co., Ltd., Shanghai, China) and used for cDNA synthesis with the Takara RNA PCR kit (AMV) Ver.3.0 (#RR019; Takara Biotechnology Co., Ltd., Dalian, China). MAL and  $\beta$ -actin were detected, and their primers were as follows: MAL forward,

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**Key words:** colorectal cancer, MAL, metastasis

Table I. Clinical characteristics of the colorectal cancer patients.

Case	Gender	Age, years	Grade	Specimen site	TNM	Stage	Type
1	F	67	G1	Right	T4aN1aM0	IIIB	2
2	M	66	G1	Left	T4aN0M0	IIB	1
3	F	73	G3	Left	T4aN1bM0	IIIB	1
4	M	72	G2	Left	T4aN1bM0	IIIB	1
5	M	77	G2	Right	T4aN0M0	IIIB	1
6	M	62	G2	Left	T4aN0M0	IIB	1
7	M	69	G2	Right	T4aN0M0	IIB	1
8	M	62	G2	Left	T4aN1aM0	IIIB	1
9	F	77	G2	Rectum	T4aN0M0	IIB	1
10	M	86	G2	Rectum	T2N0M0	I	1
11	F	64	G2	Rectum	T4aN1bM0	IIIB	1
12	F	77	G2	Left	T2N0M0	I	1
13	F	75	G1	Rectum	T1N0M0	I	1
14	M	69	G2	Right	T4aN0M0	IIB	1
15	F	73	G3	Right	T4aN2bM0	IIIC	1
16	M	69	G1	Rectum	T2N1aM0	IIIA	2
17	M	53	G2	Rectum	T4aN0M0	IIB	1
18	M	70	G1	Left	T4aN0M0	IIB	1
19	F	55	G2	Right	T4aN1bM0	IIIB	1
20	M	75	G2	Left	T2N0M0	I	1
21	M	45	G2	Rectum	T4aN0M0	IIB	2
22	M	87	G2	Right	T4aN0M0	IIB	1
23	F	78	G2	Right	T2N0M0	I	1
24	F	51	G2	Right	T4aN0M0	IIB	1
25	M	78	G1	Rectum	T4aN0M0	IIB	1
26	F	61	G3	Left	T4aN1bM0	IIIB	1
27	M	63	G2	Rectum	T2N2bM0	IIIB	1
28	F	59	G2	Right	T4aN1M0	IIIB	1
29	M	75	G2	Rectum	T4aN0M0	IIB	1
30	M	72	G2	Rectum	T4aN1M0	IIIB	1

TNM, tumor-node-metastasis. The staging system used was based on the American Joint Committee on Cancer TNM system (12).

5'-TGGGTGATGTTTCGTGTCTGTG-3'; and reverse, 5'-TCAAGTTCTACTGCGGCTTTATG-3'; and  $\beta$ -actin forward, 5'-CTGGGACGACATGGAGAAAA-3' and reverse, 5'-AAGGAAGGCTGGAAGAGTGC-3'.

**Immunohistochemical staining.** Immunohistochemical staining was performed to detect the expression of MAL in the CRC and matched non-cancer tissues. The primary antibody against MAL was obtained from Santa Cruz Biotechnology Inc. (#sc-66978; Dallas, TX, USA). The intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate; or 3, strong. The extent of staining was based on the percentage of positive tumor cells: 0, negative; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The final score of each sample was assessed by the sum of the results of the intensity and extent of staining. Therefore, each case was considered negative if the final score was 0-1 (-) or 2-3 ( $\pm$ ), and positive if the final score was 4-5 (+) or 6-7 (++), respectively. These scores were determined independently by two senior pathologists.

**Statistical analysis.** Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA) and values are expressed as the mean  $\pm$  standard deviation. The  $\chi^2$ -test was used to evaluate the differences in staining of MAL according to the patient and tumor characteristics. The differences between groups were analyzed by using Student's t-test. Spearman's rank test was used for analysis of correlations between MAL RNA and protein levels.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**RNA expression level of MAL in CRC and adjacent tissues.** By performing a semi-quantitative PCR assay, MAL RNA expression was detected in the 30 CRC tissues and the adjacent non-cancer tissues. The expression of  $\beta$ -actin was considered as the endogenous control. A total of 9 CRC specimens showed expression of MAL, with a positive rate of 30% (9/30), and 23 adjacent tissues showed expression of MAL, with a positive

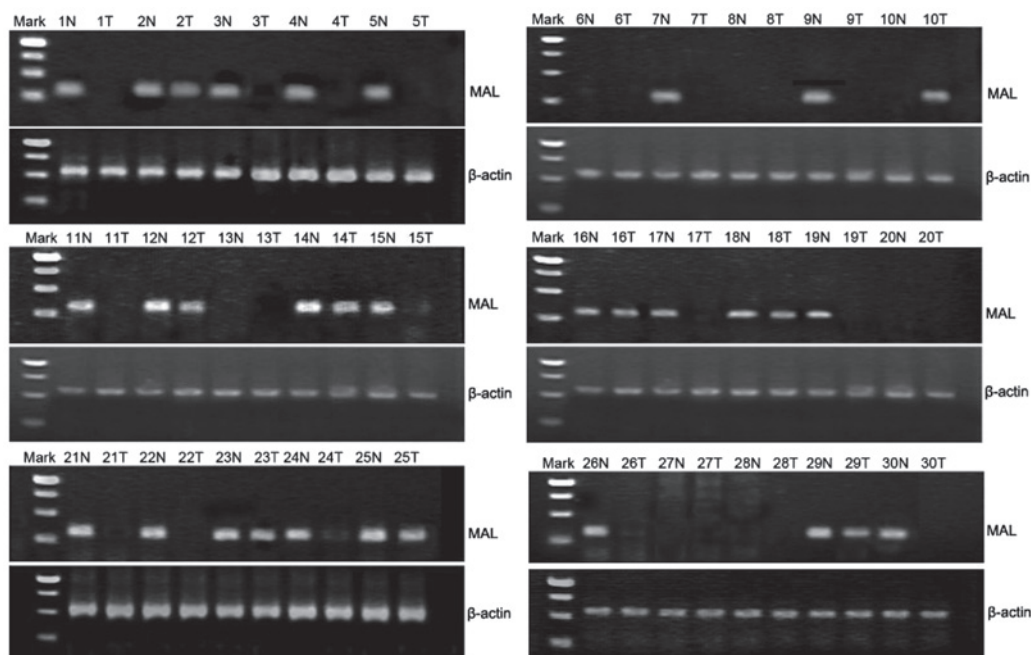


Figure 1. RNA expression of MAL in 30 CRC tissues, as determined by semi-quantitative polymerase chain reaction.  $\beta$ -actin level was considered as an endogenous control. CRC, colorectal cancer; N, adjacent tissue; T, CRC tissue. MAL, mal, T-cell differentiation protein.

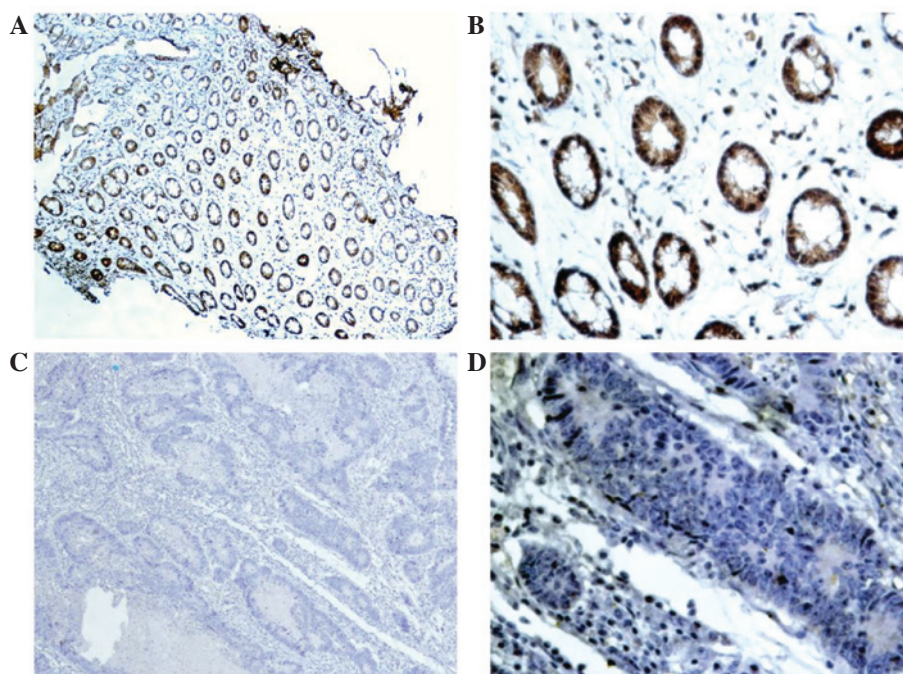


Figure 2. Protein expression of mal, T-cell differentiation protein in CRC tissues, as determined by immunohistochemical analysis. Positive adjacent tissue at (A) x100 and (B) x400 magnification; and negative CRC tissue at (C) x100 and (D) x400 magnification. CRC, colorectal cancer.

rate of 76.7% (23/30) (Fig. 1). The RNA level of MAL was significantly downregulated in the CRC tissues compared with the adjacent tissues ( $\chi^2=13.125$ ;  $P=0.001$ ).

**Protein expression level of MAL in CRC and adjacent tissues.** By performing immunohistochemical staining, MAL protein was detected in the nucleus and cytoplasm of the CRC tumor cells (Fig. 2). Immunohistochemical staining results showed that 20% (6/30) of the CRC specimens expressed MAL, while

66.7% (20/30) of the adjacent tissues expressed MAL (Table II). The protein level of MAL was significantly downregulated in the CRC tissues compared with the adjacent tissues ( $\chi^2=13.303$ ;  $P=0.001$ ). According to Spearman's rank correlation test, the RNA level of MAL was significantly positively correlated with the protein level of MAL ( $r_s=0.818$ ;  $P=0.0001$ ).

**Correlation between MAL and pathological staging, metastasis status and clinical characteristics of the CRC patients.** On the

Table II. Correlation between MAL protein level and metastatic status in CRC patients.

TNM stage	Cases, n	MAL expression		$\chi^2$	P-value
		(+), n	(-), n		
CRC with metastases	12	0	12	5.000	0.031
CRC without metastases	18	6	12		

TNM, tumor-node-metastasis; MAL, mal, T-cell differentiation protein; CRC, colorectal cancer.

Table III. Correlation between MAL protein level and different TNM stage in the colorectal cancer patients.

TNM stage	Cases, n	MAL expression		$\chi^2$	P-value
		(+), n	(-), n		
I,II	17	6	11	5.735	0.021
III	13	0	13		

TNM, tumor-node-metastasis; MAL, mal, T-cell differentiation protein.

Table IV. Correlation between MAL protein level and pathological factors in the colorectal cancer patients.

Pathological factors	Cases, n	MAL expression		$\chi^2$	P-value
		(+), n	(-), n		
Tumor location	30	6	24	1.818	0.403
Right colon	10	2	8		
Left colon	9	3	6		
Rectum	11	1	10		
Histological type				0.833	N/A
Adenocarcinoma	27	6	21		
Mucinous adenocarcinoma	3	0	3		
Differentiation status				1.429	0.490
Highly-differentiated	6	2	4		
Moderately-differentiated	21	4	17		
Poorly-differentiated	3	0	3		

MAL, mal, T-cell differentiation protein; N/A, non-applicable.

Table V. Correlation between MAL protein level and clinical characteristics in the colorectal cancer patients.

Clinical characteristics	Cases, n	MAL expression		$\chi^2$	P-value
		(+), n	(-), n		
Age, years	30	6	24	1.500	0.553
<60	5	0	5		
≥60	25	6	19		
Gender				0.139	N/A
Male	18	4	14		
Female	12	2	10		

MAL, mal, T-cell differentiation protein; N/A, non-applicable.



basis of the overall evaluation of the immunohistochemical staining score, the present study investigated whether MAL protein expression level was correlated with pathological staging, metastasis status and the clinical characteristics of the CRC patients. As shown in Table III, statistical analysis indicated that the level of MAL expression was significantly lower in CRC tumor-node-metastasis (TNM) stage III than in TNM stages I and II ( $\chi^2=5.735$ ;  $P=0.021$ ). According to the analysis of lymph node metastasis, the expression level of MAL was significantly lower in the cases of CRCs with lymph node metastasis compared with those without lymph node metastasis ( $\chi^2=5.000$ ;  $P=0.031$ ; Table II). However, the expression of MAL showed no significant correlation with the other pathological factors of the CRC patients, including tumor location, histological type and differentiation status (Table IV), and the clinical characteristics of the CRC patients, including age and gender (Table V).

## Discussion

The formation of polarity in epithelial cells depends on the rigorous maintenance of the regulation of transport and sorting process, which guarantees precise delivery of biosynthetic cargo to varying areas of the plasma membrane (13). The polarized transport of lipids and proteins to the plasma membrane is vital for the functions of the epithelial cells. MAL has been found to be a pivotal component of the machinery for the direct transport route. Loss of MAL leads to the loss of the polarized phenotype that frequently accompanies the neoplastic transformation process (14). Recent studies have suggested that downregulation of MAL has been associated with a variety of human epithelial malignancies, including esophageal (7), breast (8), ovarian (9) and cervical (15) cancers. For example, Mimori *et al* showed that overexpression of MAL in esophageal tumors exhibited decreased cellular motility, a G<sub>1</sub>/S transition block and increased levels of apoptosis via the Fas signaling pathway (7). Overmeer *et al* (9) further demonstrated the repression of MAL tumor suppressor activity by promoter methylation during cervical carcinogenesis, providing a predictive biomarker for underlying high-grade lesions in cervical cancer. Beder *et al* (10) further identified potential genetic and epigenetic mechanisms associated with the downregulation of MAL in head and neck squamous cell carcinomas, including loss of heterozygosity, mutation and hypermethylation. Buffart *et al* (11) indicated that MAL promoter hypermethylation can be used as a novel prognostic marker in gastric cancer.

For the first time, the present study evaluated the expression of MAL in CRC. RNA and protein expression levels of MAL were analyzed in 30 CRC patients and found to be significantly downregulated in the CRC tissues compared with the adjacent tissue, which was consistent with the previous studies in other epithelial malignancies. To determine the clinical significance of MAL in CRC, the correlation between its expression level and pathological staging, metastasis status and the clinical characteristics of the CRC patients was inves-

tigated. The results showed that the expression levels of MAL were significantly associated with different TNM stages and lymph node metastasis, but not with age, gender, tumor site, differentiation status or pathological type. These results indicate that MAL has a putative anti-metastasis function in CRC, and that detection of MAL expression level may be a potential biomarker for malignant CRC.

## Acknowledgements

This study was supported by the Hunan Social Development Support Program (grant number, 2012SK3190) and the Hunan Graduate student research innovation project (grant number, CX2012B090).

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