



## Original Research

## ORMDL1 is upregulated and associated with favorable outcomes in colorectal cancer

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## ABSTRACT

**Background:** The ORMDL1 gene is known as a crucial negative regulator of sphingolipid biogenesis. However, the ORMDL1 gene has rarely been studied in a tumor-related context. Therefore, its prognostic value and functional significance in colorectal cancer (CRC) remain to be explored.

**Methods:** TCGA CRC cohort analysis, qRT-PCR, and immunohistochemistry (IHC) were used to examine the ORMDL1 expression level. The association between ORMDL1 expression and various clinical characteristics was analyzed by chi-square tests. The overall survival (OS) of CRC patients was analyzed by Kaplan-Meier analysis. *In vitro* and *in vivo* cell-based assays were performed to explore the role of ORMDL1 in cell proliferation, invasion and migration. Transcriptional changes in cells with either ORMDL1 knockdown or overexpression were compared and analyzed.

**Results:** ORMDL1 was upregulated in CRC tissues in both the TCGA and our cohort. Interestingly, its expression was significantly lower in patients with metastasis than in patients without metastasis, and the high expression group had longer OS than the low expression group. Knockdown of ORMDL1 expression can promote proliferation, colony formation and invasion, while attenuating migration in CRC cell lines. In contrast, forced overexpression of ORMDL1 reduced cell proliferation, colony formation and invasion, while enhancing cell migration. Stable knockdown of ORMDL1 can promote cancer cell proliferation *in vivo* to some extent. Finally, Rho GTPase activity was influenced by ORMDL1, and the expression of ORMDL1 was enhanced by DTT treatment.

**Conclusion:** ORMDL1 is upregulated and may serve as a biomarker to predict favourable outcomes in colorectal cancer.

## Introduction

Colorectal cancer (CRC) is a malignant tumor with high incidence in the gastrointestinal tract; it is the third most common cancer and the second leading cause of cancer mortality overall [1,2]. Usually, the occurrence and development of CRC go through four successive stages:

initiation, promotion, progression and metastasis, and the liver is the most common metastatic organ, followed by the lungs, bones and peritoneum [3]. The 5-year survival rate of advanced CRC patients with metastasis to distant organs is very low (below 5%) [4]. Although new treatment strategies for CRC in addition to surgical resection, e. g., radiotherapy, chemotherapy, immunotherapy or targeted therapy have

**Abbreviations:** CRC, colorectal cancer; IHC, immunohistochemistry; OS, overall survival; EMT, epithelial-to-mesenchymal transition; GEF, guanine nucleotide exchange factors; GAP, gtpase-activating proteins; TCGA, the cancer genome atlas; HE, hematoxylin and eosin; RTCA, real-time cell analyzer; IF, immunofluorescence; DEG, differentially expressed gene; qRT-PCR, quantitative real time PCR; ER, endoplasmic reticulum; GWAS, genome-wide association study; MSI, microsatellite instability; SPTLC1, palmitoyl transferase long chain base subunit 1; ccRCC, clear cell renal cell carcinoma; FC, free cholesterol.

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been developed, approximately 50% of CRC patients who develop metastasis still exhibit a poor survival rate [5–7]. Some biomarkers have been found to achieve early prognosis as well as for targeted therapy and molecular stratification in CRC [8,9]. Similarly, finding more promising biomarkers for the detection and diagnosis of early metastases of CRC is a valuable and advantageous approach to improve patient survival.

Tumor cells possess many unique hallmarks, of which the most important is their ability to proliferate indefinitely, invade and metastasize. Through epithelial–mesenchymal transition (EMT) and remodeling of the cytoskeleton, certain cancer cells become motile so that they acquire the abilities to migrate and spread to other tissues [10,11]. During this process, Rho GTPases act as a molecular switch regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) to regulate tumor cell morphology, cell adhesion to extracellular matrix and cytoskeleton reorganization [12,13]. Rho GTPases also regulate cell movement by activating a variety of downstream effectors [14,15]. Given the close relationship between Rho GTPases and cancer cell migration and metastasis, targeting Rho GTPases may help prevent tumors from deteriorating [16–18]. Moreover, if drugs can block the interaction between Rho GTPase and downstream effectors in cancer specifically, side effects would be less frequent than with Rho GTPase inhibition in bulk [16].

The endoplasmic reticulum (ER) is essential for the synthesis of proteins and lipids. Studies have shown that endoplasmic reticulum stress is closely related to the occurrence of various diseases, including cancer [19]. Inducing endoplasmic reticulum stress with cytotoxic drugs can accelerate the apoptosis of cancer cells [20]. These results indicated that the endoplasmic reticulum plays an important role in tumor development and progression. The Mammalian ORM DL (or osomucoid-like) gene family (ORMDL1, ORM DL2, ORM DL3) can encode transmembrane proteins of the endoplasmic reticulum and functions crucially in sphingolipid homeostasis [21,22], which is regulated by ceramide level sensing [23,24], as well as participating in proper folding of endoplasmic reticulum proteins [25]. By a genome-wide association study, ORM DL3 was identified as a risk factor for ulcerative colitis [26]. Related to cancer, it was reported that ORM DL family proteins interact with SPTLC1 and are associated with clear cell renal cell carcinoma (ccRCC) development [27]. However, the expression of ORM DL1 in CRC and its prognostic significance are unclear, and whether it is involved in the progression of CRC is unknown.

In the current study, we initially investigated The Cancer Genome Atlas (TCGA) cohort and discovered that ORM DL1 was upregulated in CRC tissues. Analysis of clinical characteristics showed that patients with low ORM DL1 expression were more inclined to have metastasis and to have shorter OS. By performing various cell-based assays, we investigated the malignant cellular behavior of CRC cells, which was influenced by ORM DL1 expression. With the evidence presented, we concluded that ORM DL1 is a valuable and promising biomarker whose heightened expression is associated with favorable outcomes in CRC, providing candidates for molecular stratification and potential therapeutic targets in comprehensive CRC treatment regimens.

## Materials and methods

### Patients and tissue samples

The frozen paired samples used for mRNA quantification were stocked in RNAlater solution (Invitrogen, Thermo Fisher Scientific, USA), and were acquired from 41 CRC patients with primary CRC tissues and matched adjacent normal tissues who had undergone surgery between November 2012 and December 2017 at the Sixth Affiliated Hospital of Sun Yat-sen University (SYSU).

We also constructed tissue microarrays of primary CRC from 217 patients from the Sixth Affiliated Hospital of SYSU. None of these patients received chemotherapy, radiotherapy or other related treatments prior to surgical resection of the tumor. Among these patients, 122

patients had synchronous metastasis or disease relapse or metastasis within 6 months after surgery, and 95 patients had no synchronous metastasis. Our study was approved by the Institutional Ethics Committee of Sixth Affiliated Hospital, Sun Yat-sen University.

### Cell culture and transfection

All cell lines (HCT15, HCT8, SW480, SW620, LOVO, DLD1, SW48, HCT116) used in this study were obtained from ATCC. Cell lines were typically cultured at 37 °C in an incubator with 5% CO<sub>2</sub>. The SiRNAs used were as follows: siORMDL1#1: 5'-GGAGTTGGCTTGCTTCATA-3', and siORMDL1#2: 5'-CTGGCAAGTTTCTATACGA-3'. Negative scramble fragment (RiboBio Co., China) was used as a control.

### Quantitative real time PCR (qRT-PCR)

mRNA was extracted from CRC cell lines and tissues with TRIzol (Invitrogen, CA, USA). The sequences of the PCR primers were as follows: ORM DL1, forward: 5'-TTC AGT GTT CCT GTT GCT TG-3' and reverse: 5'-AGC CTT GCT TTA CCC TGG TC-3'; β-actin, forward: 5'-TTG TTA CAG GAA GTC CCT TGC C-3', and reverse 5'-TTG TTA CAG GAA GTC CCT TGC C-3'. β-Actin was used for normalization. The siRNAs were transfected into CRC cells via Lipofectamine RNAiMAX (Invitrogen) and overexpression plasmids of ORM DL1 (full-length ORM DL1 cDNA inserted onto pEZ-M98 plasmid, FulenGen, Guangzhou, China) were transfected with Lipofectin 3000 (Invitrogen).

### Western blotting

Cells were lysed and boiled for 10 min (min). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and detected with relevant antibodies against RhoA (1:1000 dilution, Cat# 2117, Cell Signaling Technology, CST), RhoB (1:1000 dilution, Cat# 63,876, CST), RhoC (1:1000 dilution, Cat# 3430, CST), E-cadherin (1:1000 dilution, Cat# 14472S, CST), α-SMA (1:1000 dilution, Cat# ab184705, Abcam), Slug (1:1000 dilution, Cat# 9585, CST), Snail (1:1000 dilution, Cat# 3879, CST), and ZEB1 (1:1000 dilution, Cat# 3396, CST), CHOP (1:1000 dilution, Cat# 381,679, ZEN-BIOSCIENCE), BiP/GRP78 (1:1000 dilution, Cat# 200,310-4F11, ZEN-BIOSCIENCE). Blotting for α-tubulin (1:20,000 dilution, Cat# 66,031-1-Ig, Proteintech) or GAPDH (1:20,000, Cat# 5174, CST) was used as a loading control.

### Hematoxylin and eosin (H&E) and immunohistochemistry assay

The tissue microarray and related clinicopathologic information were collected from the Sixth Affiliated Hospital of SYSU. IHC for ORM DL1 was carried out on CRC tissue microarray slides. The slides were first incubated at 60 °C for 4 h, deparaffinized in xylene, and then rehydrated in alcohol. After heating in citrate buffer for 21 min, we used an IHC kit (Cat# SP9000; ZSGB-Bio) to block endogenous peroxidase activity. Slides were blocked with 5% goat serum for 1 h and incubated in the anti-ORM DL1 antibody (1:100 dilution, Cat# PHA065643, Atlas Antibodies) overnight at 4 °C. The next day, after 3 washes with PBST, slides were incubated with secondary antibody for 1 h and then stained with a DAB kit (Cat# ZLT-9017; ZSGB-Bio). After the experiments, the slides were observed by microscope. The IHC scores were independently assessed by two pathologists. An IHC score of 6.3 was selected as the cutoff value to divide the high and low expression of ORM DL1.

### Cell proliferation assay

HCT116 and DLD1 cells were selected to perform this experiment. We separately seeded 5000 DLD1 cells and HCT116 cells after transfection with ORM DL1 overexpression plasmid or siORM DL1 into 96-well plates. A real-time cell analyzer (RTCA, xCELLigence system, ACEA Biosciences, USA.) and an IncuCyte Essens Bioscience incubator

(Essens Bioscience, Birmingham, UK) were used to monitor cell growth. The live cells were recorded automatically every 30 min.

#### Colony formation assay

Five hundred cells were seeded into 6-well plates and incubated for 8 days in a 37 °C incubator. Then colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

#### Wound-healing assay

After transfection, cells were seeded into 24-well plates and incubated until 100% confluence. A scratch was made in the cell layer using a 10 µl pipette tip, and then HCT116 cells were cultured in FBS-free McCoy's 5A and DLD1 in FBS-free RPMI-1640. Live cell photos were collected in an IncuCyte Essens Bioscience incubator every 30 min.

#### Invasion assays

Eight-micrometer transwell filters (Corning, NY, USA) were used to perform the invasion assay. The upper chambers were covered with Matrigel (Corning, NY, USA) and placed in a 37 °C incubator for 1 h. Then,  $8 \times 10^4$  DLD1 cells were seeded into the upper chamber with RPMI-1640, and we added 700 µl RPMI-1640 with 10% FBS to the 24-well plates. After 24 h, we wiped the upper chamber with cotton swabs and stained cells on the other side of the membrane with crystal violet for 5 min. The other HCT116 cell line was generated with the same method. However, it was cultured with McCoy's 5A medium and was planted for  $10 \times 10^4$  cells.

#### In vivo subcutaneous tumor growth assay and H&E and IHC assay

The shORMDL1 plasmid (ORMDL1 specific shRNA sequence same as siORMDL1#1 inserted onto the pLKD-CMVEGFP-2APuro-U6-shRNA plasmid, Obio Tech, Shanghai, China) or empty control plasmid was stably transfected into DLD1 cells using the lentivirus transfection method, and subjected to stable selection by puromycin. The BALB/c nude mice were injected with  $4 \times 10^6$  stable shORMDL1 DLD1 cells subcutaneously, and each group had 7 mice. Tumor sizes were measured every 3 days and then calculated as volume =  $1/2$  (length  $\times$  width<sup>2</sup>). After 15 days, mice were sacrificed and tumors were measured and then collected. All specimens were fixed with formalin and embedded in paraffin for slide preparation. Slides were stained by H&E and anti-Ki67 accordingly.

#### Immunofluorescence [28] staining

DLD1 cells layered on confocal-compatible slides were transfected with control or siORMDL1 reagent and then fixed 24 h afterwards with 4% paraformaldehyde. After incubation with anti-ORMDL1 antibody (1:100, PHA065643, Atlas Antibodies) and secondary antibody according to the product manual, all slides were observed and imaged (Leica SP8, Mannheim, Baden-Wuerttemberg, Germany).

#### RNA sequencing

HCT116 cells with transient transfection of either ORMDDL1 overexpression plasmid or knockdown (siORMDDL1 and siControl) reagent, were collected 48 h after transfection. Cell medium was collected as well for contamination detection for mycoplasma. Whole transcriptome sequencing (RNAseq) was conducted by GeneSeed Company (Guangzhou, China) with the HiSeq X10 PE150 platform. Differential genes were generally defined as the difference between the two groups more than 1.5 times and  $P < 0.05$ .

#### Activated RhoA and ATP content measurement

Activated RhoA (GTP-bound) within cells was measured by a colorimetric method according to the product manual (Cytoskeleton, BK124). ATP content within cells was measured by a luciferase method according to the protocol of ATP detection kit (Beyotime, S0026).

#### Statistical analysis

Except for other notes, statistical analyses were performed using GraphPad Prism 8.0 (Chicago, IL, USA) or SPSS 25.0 (California, USA). Associations between ORMDDL1 expression and clinical variables were analyzed by chi-square tests. Overall survival was analyzed using Kaplan-Meier analysis and the  $P$  value was calculated by the log-rank test. A Cox regression analysis model was used to evaluate univariate and multivariate survival analyses. Differences between groups were analyzed by a two-tailed Student's  $t$ -test or a Mann-Whitney  $U$  test.

## Results

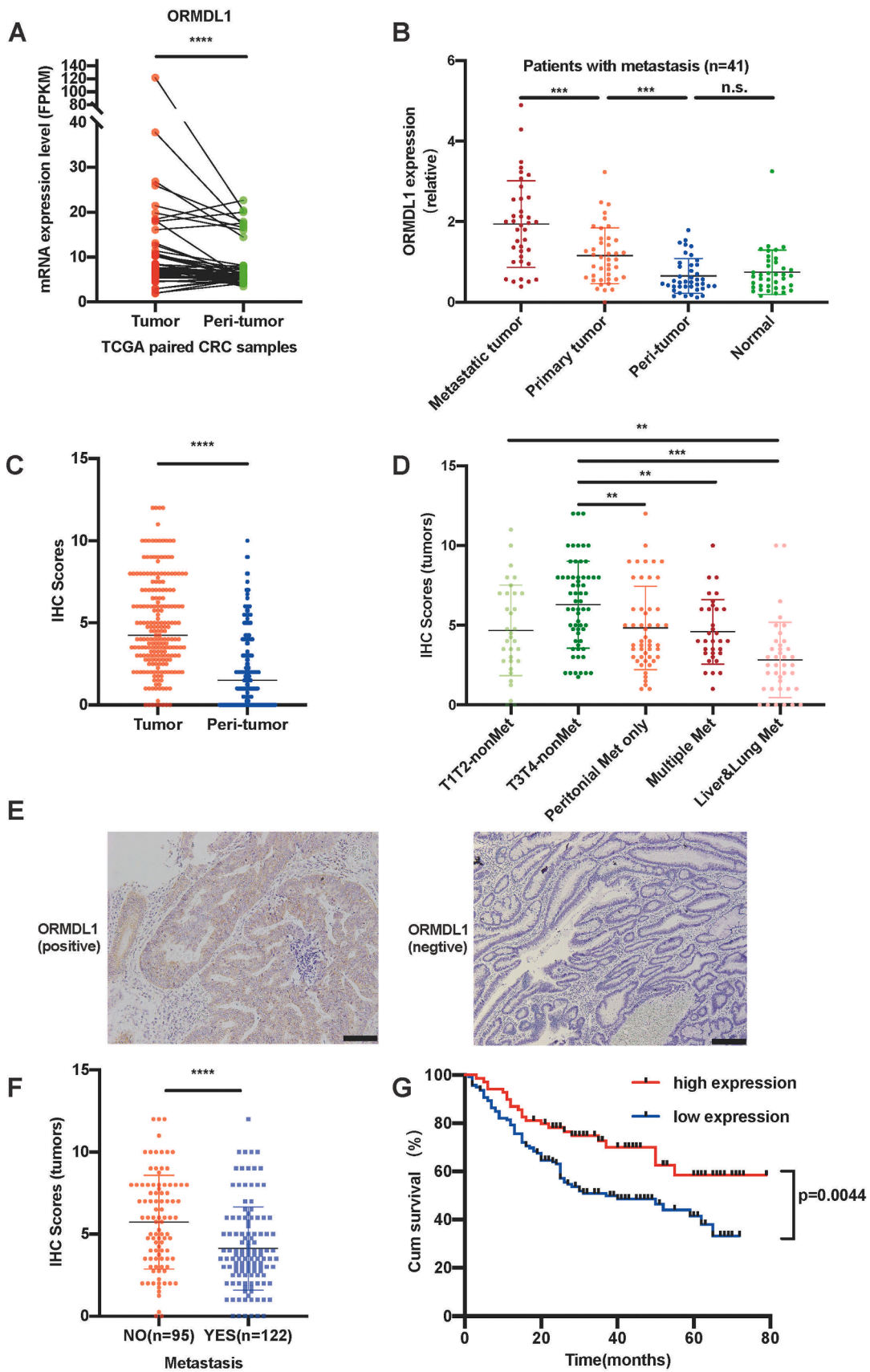
#### ORMDDL1 expression was increased in CRC and associated with favorable outcomes

Analysis of TCGA data (50 CRC cases with self-matched peritumor tissue) showed that the mRNA level of ORMDDL1 was obviously higher in tumor tissues than in peritumor tissues (Fig. 1a). We also examined the ORMDDL1 mRNA level in primary tumors, peritumors (~2 cm from tumors), normal tissues (~5 cm from tumors) and metastatic tumors among 41 CRC patients independently. We found that ORMDDL1 expression was increased in CRC primary tumors compared with peritumor tissues and normal tissues (Fig. 1b). Moreover, it expressed significantly higher levels in metastatic tissues (Fig. 1b).

To further investigate the association between ORMDDL1 expression and clinicopathologic characteristics of the patients, we performed IHC analysis based on the paraffin-embedded tissue microarray of 217 CRC patients in our center. ORMDDL1-positive staining was concentrated mainly in the cytoplasm of CRC tissues; in contrast, the adjacent normal glands presented negative or low levels (Fig. 1c). IHC scoring revealed that similar to the ORMDDL1 mRNA level, the ORMDDL1 protein level was upregulated in tumors compared to adjacent tissues (Fig. 1d). IHC scores were lower in the metastatic patients, but different metastatic groups expressed different levels of ORMDDL1 (Fig. 1e). Among the cohort, patients with metastasis were significantly lower than those without metastasis (Fig. 1f). According to the ROC curve analysis, we selected the IHC score of 6.3 as the cutoff value which can best separate the cohort into two groups: high ( $n = 69$ ) and low ( $n = 141$ ) ORMDDL1 expression. Among all the clinical characteristics analyzed, we found that the depth of invasion, distant metastasis, TNM stage and nerve invasion were related to ORMDDL1 expression (Table 1). The multivariate analysis showed that ORMDDL1 expression was not an independent predictor for CRC patient OS (Table 2). Kaplan-Meier analysis showed that the high expression group had better overall survival ( $P = 0.004$ ) (Fig. 1g).

#### Knockdown of ORMDDL1 expression promoted proliferation and invasion but inhibited migration of CRC cells

To explore the potential role of the ORMDDL1 gene in CRC cellular behavior, we first estimated the ORMDDL1 expression level in 8 CRC cell lines (Fig. 2a). HCT116 and DLD1 cells were chosen for all following cell-based experiments. ORMDDL1 was successfully silenced in HCT116 and DLD1 cells by siRNA transfection (Fig. 2b). Real-time monitoring of cell growth by RTCA indicated that knockdown of ORMDDL1 expression promoted HCT116 or DLD1 proliferation (Fig. 2c, d). Colony formation assays also confirmed that ORMDDL1 knockdown increased colony numbers, either in both HCT116 cells (Fig. 2e) and DLD1 cells (Fig. 2f).



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**Fig. 1. ORMDL1 expression was increased in CRC and associated with favorable outcomes.**

(a) The mRNA expression level of ORMDL1 was significantly higher in CRC tissues than in peritumor tissues in the TCGA cohorts.  $n = 50$ , \*\*\*\* $P < 0.0001$ , two-tailed Student's  $t$ -test.

(b) The mRNA expression level of ORMDL1 in CRC primary tumors, and matched peritumor, metastatic tumor and normal tissues of patients. \*\*\* $P < 0.001$ , ns not significant ( $P > 0.05$ ), two-tailed Student's  $t$ -test.

(c) IHC scores of ORMDL1 expression levels in tissue microarrays of tumor and peritumor tissues from patients. \*\*\*\* $P < 0.0001$ , two-tailed Student's  $t$ -test.

(d) IHC scores of ORMDL1 expression level in a tissue microarray of T1/T2-nonmetastasis group, T3/T4-nonmetastasis group, peritoneal metastasis group, liver and lung metastasis group and multiple metastasis group. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , two-tailed Student's  $t$ -test.

(e) IHC staining images of ORMDL1 in a tissue microarray of paired samples of CRC tissues (left image) and adjacent tissues (right image). The scale bar is 200  $\mu\text{m}$ .

(f) IHC scores of ORMDL1 expression levels in the tissue microarray of patients with or without metastasis. \*\*\*\* $P < 0.0001$ , two-tailed Student's  $t$ -test.

(g) Kaplan-Meier survival curves of overall survival based on IHC scores of ORMDL1 expression in the tissue microarray.  $P = 0.0044$ , log-rank test.

**Table 1**

Correlation between the expression of ORMDL1 and the clinicopathological features of CRC patients.

Characteristics	ORMDL1		$p$ value
	Low expression ( $n = 141$ )	High expression ( $n = 69$ )	
Age			1.000
< 60	72 (51.1)	35 (50.7)	
$\geq 60$	69 (48.9)	34 (49.3)	
Sex			0.077
Female	55 (39.0)	36 (52.2)	
Male	86 (61.0)	33 (47.8)	
Location			0.694
Colon	119 (84.4)	56 (81.2)	
Rectum	22 (15.6)	13 (18.8)	
Histological grade			0.041
Well	27 (19.1)	12 (17.4)	
Moderately	75 (53.2)	48 (69.6)	
Poorly	39 (27.7)	9 (58.6)	
Depth of invasion			0.024
T1	5 (3.5)	5 (7.2)	
T2	10 (7.1)	7 (10.1)	
T3	60 (42.6)	15 (21.7)	
T4	66 (46.8)	42 (60.9)	
Lymph node metastasis			0.307
N0	55 (39.0)	33 (47.7)	
N1	54 (38.3)	26 (37.7)	
N2	32 (22.7)	10 (14.5)	
Distant metastasis			< 0.001
M0	51 (36.3)	46 (66.7)	
M1	90 (63.8)	23 (33.3)	
TNM stage			< 0.001
I	11 (7.8)	8 (11.6)	
II	14 (9.9)	14 (20.3)	
III	15 (10.6)	21 (30.4)	
IV	101 (71.6)	26 (37.7)	
Vein invasion			0.859
yes	31 (22.1)	14 (20.3)	
no	109 (77.9)	55 (79.7)	
Nerve invasion			< 0.001
yes	102 (73.4)	22 (31.9)	
no	37 (26.6)	47 (68.1)	
Tumor size			0.131
$\leq 5$ cm	100 (70.9)	56 (81.2)	
$> 5$ cm	41 (29.1)	13 (18.8)	

Interestingly, while knockdown of ORMDL1 enhanced the invasion behavior of CRC cells (Fig. 2g, h), knockdown of ORMDL1 inhibited the migration capability of CRC cells (Fig. 2i, j).

#### Overexpression of ORMDL1 attenuated proliferation and invasion but promoted migration of CRC cells

To further determine the effect of ORMDL1 expression on CRC cells, we transfected HCT116 and DLD1 cells with an ORMDL1 overexpression vector, and the overexpression level of ORMDL1 was confirmed

**Table 2**

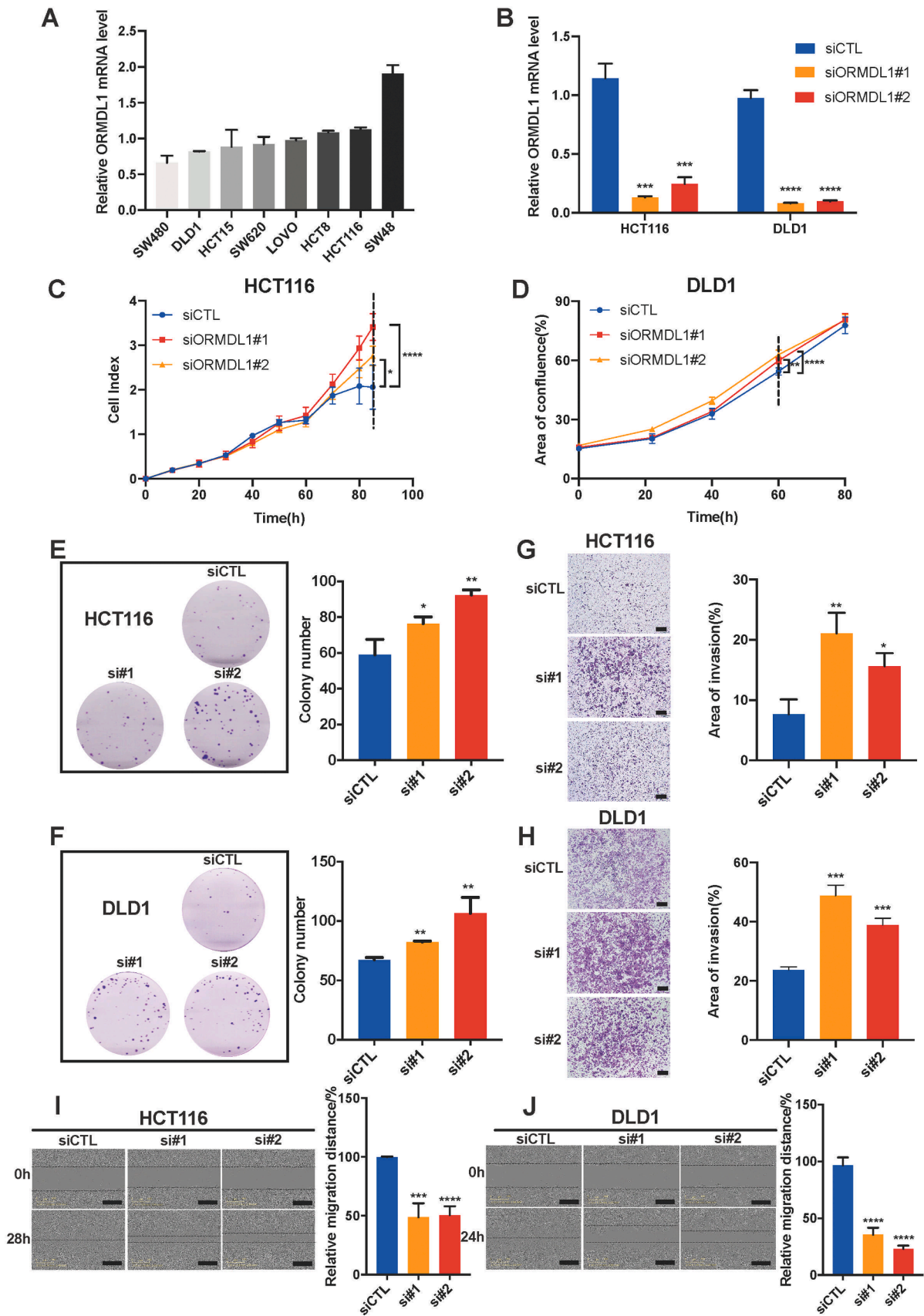
Univariate and multivariate analysis of different prognostic parameters of the CRC patients in the cohort.

Factors	Univariate analysis HR (95%CI)	$p$ value	Multivariate analysis	
			HR (95%CI)	$p$ value
Age( $< 60/\geq 60$ )	0.685 (0.456–1.029)	0.069		
Sex(female/male)	0.796 (0.527–1.203)	0.279		
Location(colon/rectum)	0.716 (0.401–1.276)	0.257		
T stage(T1 + T2/T3 + T4)	0.173 (0.068–0.442)	0.000		
N stage(N0/N1 + N2)	0.321 (0.188–0.548)	0.000		
Distant metastasis (absent/present)	0.085 (0.042–0.169)	0.000	1.928 (5.953–23.899)	< 0.001
TNM stage (I + II/III + IV)	0.193 (0.092–0.402)	0.000		
Histological grade (well+moderate/poor)	0.406 (0.262–0.629)	0.000	2.390 (1.532–3.729)	< 0.001
Vein invasion(no/yes)	0.515 (0.327–0.811)	0.004		
Nerve invasion(no/yes)	0.566 (0.369–0.868)	0.009		
Tumor size( $\leq 5/> 5$ cm)	0.613 (0.404–0.930)	0.021		
ORMDL1 expression (low/high)	1.978 (1.226–3.192)	0.005		

(Fig. 3a). The proliferation of CRC cells seemed to be suppressed by ORMDL1 overexpression (Fig. 3b), consistent with the results from the colony formation assay (Fig. 3c). Interestingly, while overexpression of ORMDL1 attenuated the invasion behavior of CRC cells (Fig. 3d), overexpression of ORMDL1 promoted the migration capability of CRC cells (Fig. 3e). These results suggest that ORMDL1 expression plays nonuniform roles in the proliferation, migration and invasion behavior of CRC cells.

#### Knockdown of ORMDL1 expression can promote tumor growth in vivo

Based on the clinicopathologic analysis and *in vitro* assays above, we continued to investigate further whether knockdown of ORMDL1 affects tumor growth *in vivo* by performing tumor-forming experiments in nude mice. Tumor volumes were recorded every 3 days, and the results showed that knockdown of ORMDL1 (shORMDL1) promoted the proliferation of cancer cells *in vivo* to some extent (Fig. 4a). The shORMDL1 group showed larger tumor formation, but without statistical significance in terms of tumor weights (Fig. 4b). We used qRT-PCR assays to confirm that ORMDL1 was successfully knocked down in the tumor cells (Fig. 4c). HE staining showed that the dissected tumors were indeed tumorous and not inflammatory tissue (Fig. 4d, upper panel). IHC staining for Ki67 showed more intense Ki67 signals in the shORMDL1 group (Fig. 4d, lower panel and quantification, Fig. 4e), suggesting that



(caption on next page)

**Fig. 2. Knockdown of ORMDL1 promoted proliferation and invasion but inhibited the migration of CRC cells.**

- (a) The relative mRNA expression level of ORMDL1 in human cell lines was analyzed by qRT-PCR.  
 (b) The knockdown efficiency of ORMDL1 in HCT116 and DLD1 cells was analyzed by qRT-PCR. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , two-tailed Student's  $t$ -test.  
 (c) Proliferation assay by RTCA showed that downregulation of ORMDL1 can promote the proliferation ability of HCT116 cells. \*\*\*\* $P < 0.0001$ , \* $P < 0.05$ , two-way ANOVA.  
 (d) Proliferation assay by IncuCyte showed that downregulation of ORMDL1 can promote the proliferation ability of DLD1 cells. \*\*\* $P < 0.0001$ , \* $P < 0.05$ , two-way ANOVA.  
 (e,f) Images and statistical analysis of colony formation assays in two cell lines. \* $P < 0.05$ , \*\* $P < 0.01$ , two-tailed Student's  $t$ -test.  
 (g,h) Images and statistical analysis of wound invasion assays in two cell lines. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . The scale bar is 200  $\mu\text{m}$ .  
 (i,j) Images and statistical analysis of wound healing assays in two cell lines. \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . The scale bar is 400  $\mu\text{m}$ .

cancer cells proliferated vigorously to form a tumor.

*ORMDL1 affected expression level of Rho GTPase*

To explore the potential mechanism through which ORMDL1 executes its function during the progression of CRC, we performed transcriptome RNAseq analysis in cells with ORMDL1 knockdown or overexpression. By filtering for significantly expressed genes ( $|\text{fold change}| \geq 1.5$ ,  $P < 0.05$ ,  $FDR < 0.05$ ). We identified 76 differentially expressed genes (DEGs) in the overexpression group (oeORMDL1 vs oeControl, Supplemental Table 1) and 2329 DEGs in the knockdown group (siORMDL1 vs siControl, Supplemental Table 2). As the DEG list in the overexpression group was too small to be subjected to enrichment analysis (usually 10% of the whole transcriptome), we focused only on DEGs in the knockdown group in the following analysis, which included 1222 upregulated genes and 1107 downregulated genes (volcano plot, Fig. 5a). Within the DEGs of the knockdown group comparison, MET, TRAIL signaling and integrin signaling were as the most significantly enriched pathways for the upregulated genes (Fig. 5b). We also confirmed some of the most DEGs by qRT-PCR and Western blotting. Classical EMT markers, including E-cadherin,  $\alpha$ -SMA, ZO-1, Slug, Snail, and ZEB1 did not change when ORMDL1 was knocked down (Fig. 5c). Interestingly, the best-known Rho GTPases, RhoA, RhoB and RhoC, were clearly influenced by ORMDL1 expression to different extents (mRNA level, Fig. 5d; protein level, Fig. 5e). The level of activated RhoA (GTP-bound) was also increased when ORMDL1 was knocked down (Fig. 5f).

*ORMDL1 expression was enhanced by DTT-induced ER stress*

Members of the ORMDL family are best known to regulate sphingolipid and ceramide synthesis, and were reported to affect ER homeostasis or to be induced by elevated or dysregulated ER stress. However, downstream effector proteins (CHOP, BiP) in the ER stress response pathway did not seem to change when either ORMDL1 was overexpressed or knocked down (Fig. 6a). HCT116 or DLD1 cells were treated with DTT to induce an ER stress state. ORMDL1 was induced by DTT in a dose-dependent manner in two cell lines (Fig. 6b, c). ER stress can also activate mitochondrial dependent apoptosis by inducing mitochondrial ATP depletion. But the cellular ATP levels did not change when ORMDL1 was overexpressed or knocked down (Fig. 6d).

**Discussion**

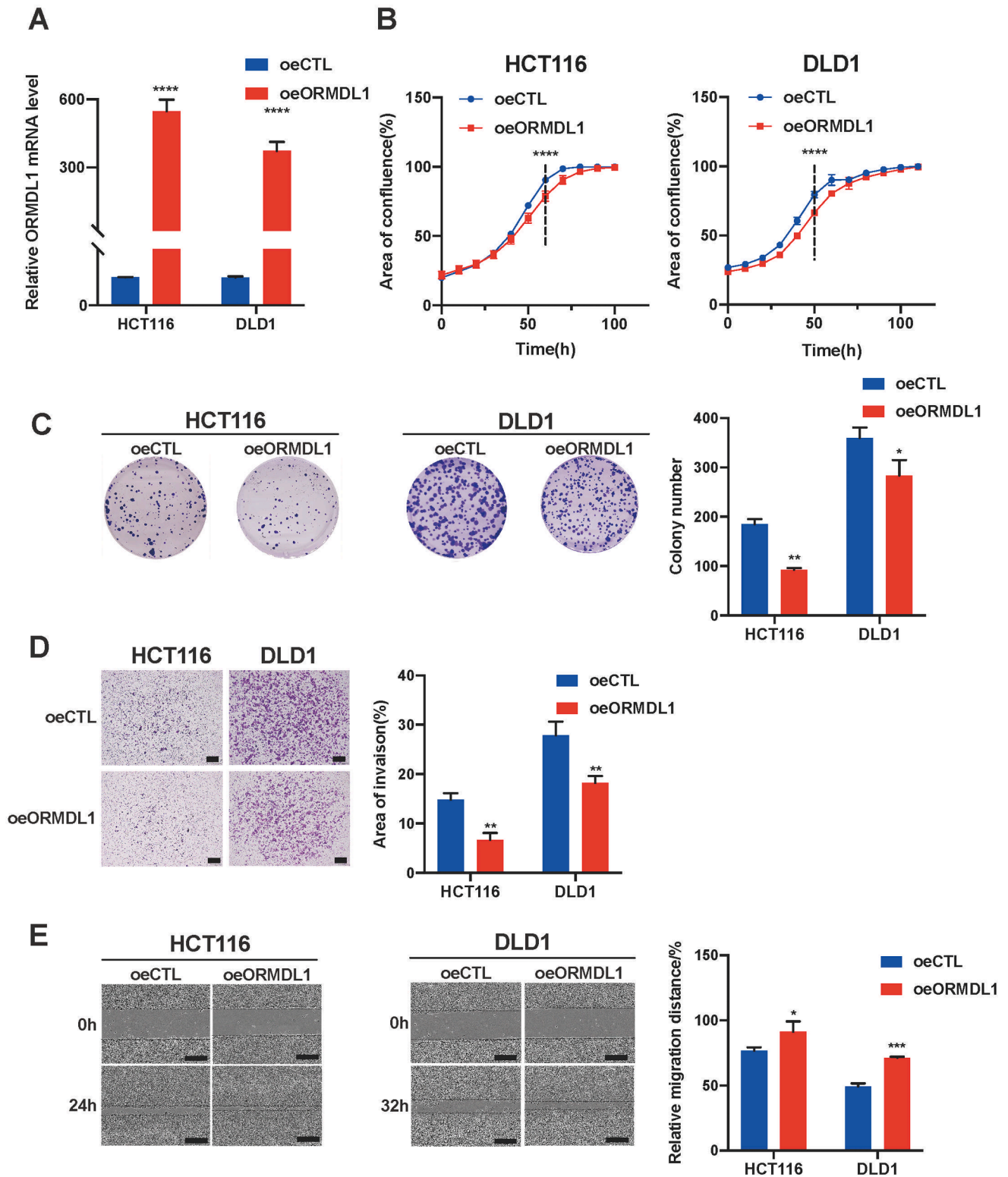
The human ORMDL gene family has three members ORMDL1, ORMDL2 and ORMDL3, all encoding transmembrane proteins anchored in the endoplasmic reticulum [25]. This gene family seems to be very conserved across eukaryotic cells, from singled cell budding yeast to mammalian cells [25]. Genome-wide association studies (GWAS) showed that there was a significant association between mutations in ORMDL genes (ORMDL3 in particular) and asthma [29]. Later, ORMDL3 was identified as an inducible lung epithelial gene regulating metalloproteases, chemokines, OAS, and ATF6, establishing the milestone molecular basis for its functions in the allergic response [30]. Another milestone finding was that proteins of the ORMDL family were shown to be negative homeostasis regulators of serine palmitoyl transferase (SPT),

which is the rate-limiting enzyme for the biosynthesis of new sphingolipids [31,32]. Within these three genes, ORMDL3 and ORMDL1 seem to be prevalent players in either the allergic response or sphingolipid regulation. Although the genomic locus of ORMDL3 has been revealed to be associated with childhood acute lymphoblastic leukemia by GWAS [33], little is known about its roles in the context of other diseases, *i.e.*, human cancer. ORMDL3 was reported to be involved in triple-negative breast cancer cell proliferation regulated by the upstream factor long noncoding RNA SOX21-AS1/miR-520a-5p [34]. Yet, the direct link between ORMDL and cancer progression needs to be explored.

In our study, by examining the expression level of ORMDL1 in the TCGA cohort as well as in our cohort, we found that ORMDL1 was obviously upregulated in CRC tumor tissues compared with adjacent normal tissues. In tissues with metastasis, expression of ORMDL1 was lower compared with tissues without metastasis according to IHC staining scores. Among all the clinical characteristics analyzed, we found that the depth of invasion, distant metastasis, TNM stage and nerve invasion were associated with low ORMDL1 expression. Interestingly, patients with higher expression of ORMDL1 were less likely to develop metastasis and therefore had better OS. These interesting phenomena all indicated that high expression of ORMDL1 is associated with better clinical outcomes so that it could potentially serve as a marker for screening patients with better prognosis. It is worth investigating whether combining ORMDL1 expression and other biomarkers (PD-L1, MSI *etc.*) would have better stratification for CRC patients.

This study is only a starting point to bring attention to ORMDL1 functions in the context of cancer. Pan-cancer analysis indicated that the prognostic value of ORMDL1 seems to be cancer-dependent [35]. Detailed molecular mechanism dissection will shed light on the direct link between ORMDL1 and cancer development, for example, how ORMDL1 regulates Rho GTPase. In summary, we found that ORMDL1 could promote the migration and suppress the invasion and proliferation of CRC cells, suggesting that ORMDL1 modulates cancer cell aggressiveness in multiple ways. Rho GTPase could be influenced by ORMDL1 expression. These Rho GTPase are crucial upstream regulators for coordinating the remodeling of cytoskeleton in space and time. ORMDL1 is an ER membrane-bound protein and the direct molecular link to downstream effectors should be uncovered.

Despite its critical role in sphingolipid metabolism, it might also affect the survival of cancer cells by coregulating serine palmitoyl transferase long chain base subunit 1 (SPTLC1) in the tumor microenvironment and helping SPTLC1 be a tumor suppressor protein in ccRCC [27]. Based on the relationship between SPTLC1 and the ORMDL family, we hypothesized that ORMDL1 could also work on CRC progression. Previous studies have shown that autophagy of ORMDL1 plays an important role in regulating free cholesterol (FC) metabolism and stimulating sphingomyelin biosynthesis [21,36]. The human abdominal cavity has a variety of cellular substances. The triacylglycerol phosphate produced in fat cells can be decomposed into glycerol and free fatty acids by lipase in the body. We hypothesized that this FC-rich microenvironment provides suitable soil for peritoneal transfer of CRC. When FC is loaded in CRC cells, ORMDL1 binds to autophagosomes and then causes autophagy, resulting in a decrease in ORMDL1 in tumor cells. Thus, we hypothesized that ORMDL1 may also be an important hub for regulating FC the influencing the ER stress and apoptosis.



**Fig. 3. Overexpression of ORMDL1 attenuated proliferation and invasion but promoted migration of CRC cells.**

(a) Overexpression of ORMDL1 in HCT116 and DLD1 cells was analyzed by qRT-PCR. \*\*\*\* $P < 0.0001$ , two-tailed Student's  $t$ -test.

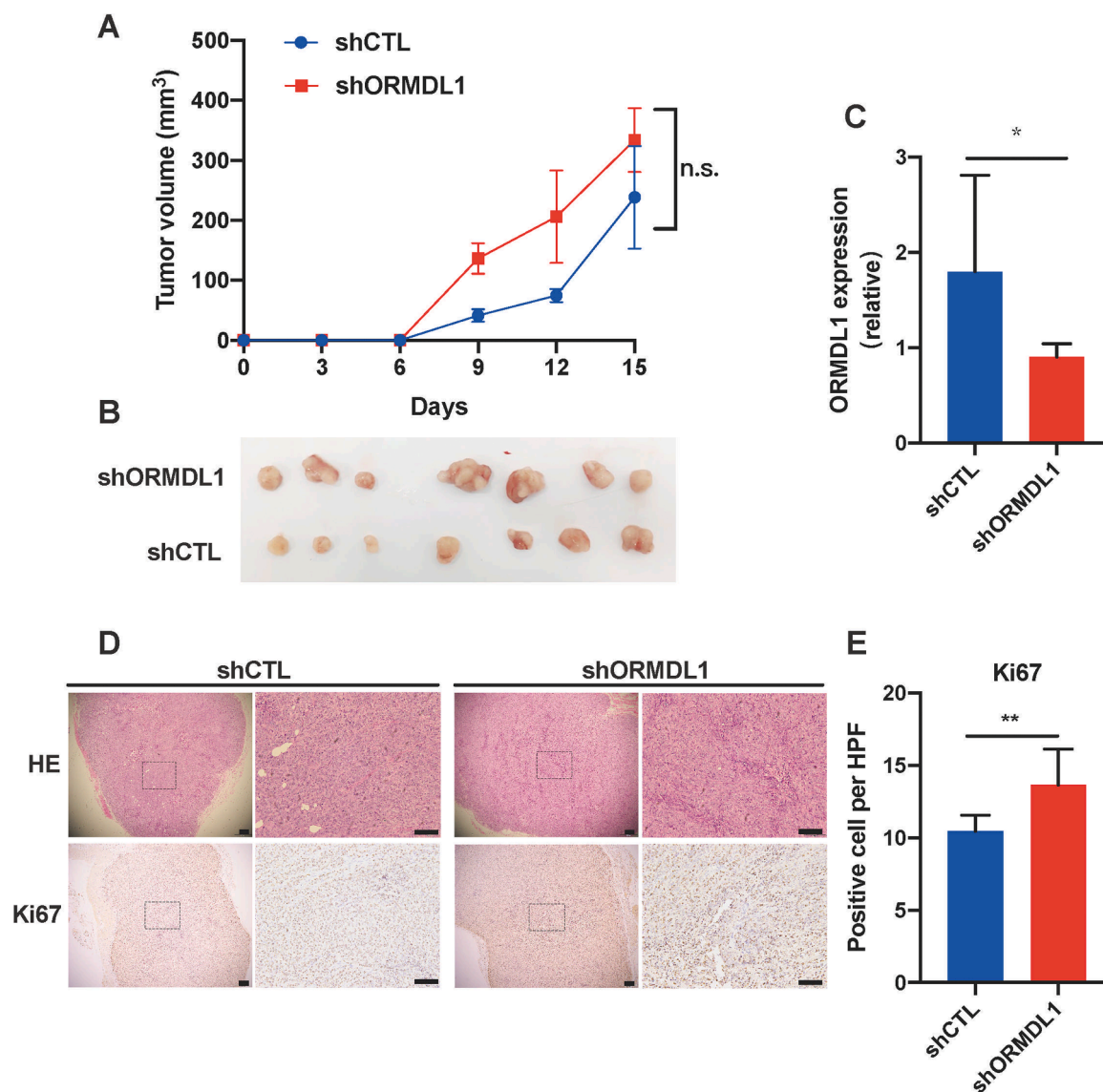
(b) Proliferation assays recorded by IncuCyte showed that overexpression of ORMDL1 promoted proliferation in CRC cells, \*\*\*\* $P < 0.0001$ , two-way ANOVA.

(c) Images and statistical analysis of colony formation assays showed that overexpression of ORMDL1 inhibited the colony formation ability of CRC cells. \* $P < 0.05$ , \*\* $P < 0.01$ , two-tailed Student's  $t$ -test.

(d) Images and statistical analysis of invasion assays indicated that overexpression of ORMDL1 inhibited CRC cell invasion. \*\* $P < 0.01$ , two-tailed Student's  $t$ -test. The scale bar is 200  $\mu\text{m}$ .

(e) Images and statistical analysis of wound-healing assays showed that overexpression of ORMDL1 can promote the migration ability of CRC cells. \* $P < 0.05$ , \*\*\* $P < 0.001$ , two-tailed Student's  $t$ -test. The scale bar is 400  $\mu\text{m}$ .





**Fig. 4. Knockdown of ORMDL1 expression can promote tumor growth *in vivo*.**

(a) Stable ORMDL1 knockdown cells were subcutaneously implanted into a nude mouse tumor model as described in the Methods. Tumor volumes were recorded and calculated ( $n = 7$ ).

(b) After 15 days, mice were sacrificed and tumors were collected and weighed.

(c) Dissected tumors from mice were subjected to qRT-PCR analysis to determine ORMDL1 levels.

(d) Dissected tumors were subjected to HE staining and Ki67 IHC staining. The scale bar is 50  $\mu$ m.

(e) Ki67-positive cells per 20 x high power field (HPF) were calculated.

Neither overexpression nor knockdown of ORMDL1 seemed to change the expression of downstream effector proteins in the ER stress response. It is desirable to perform these experiments after the cells are challenged with ER stress first (e.g., usnic acid or DTT treatment). Again, these chemicals very likely introduce stress to the cells in many aspects, and the assays need to be conducted under strict conditions, also in normal cell lines. Another possibility could be that ER stress caused by DTT might induce expression changes in multiple proteins, known or unknown regulators in the ER stress response. These proteins might work together to execute the ER stress response, which is indicated by increased expression of markers, such as CHOP/BiP/ATF-4.

Notably, very few DEGs were identified when performing transcriptome changes for oeORMDL1 vs oeControl. The overexpression level seemed to be reasonable based on qRT-PCR. Unfortunately, we were not able to detect the protein level by Western blotting, as no commercial monoclonal antibody is available. We can only infer that

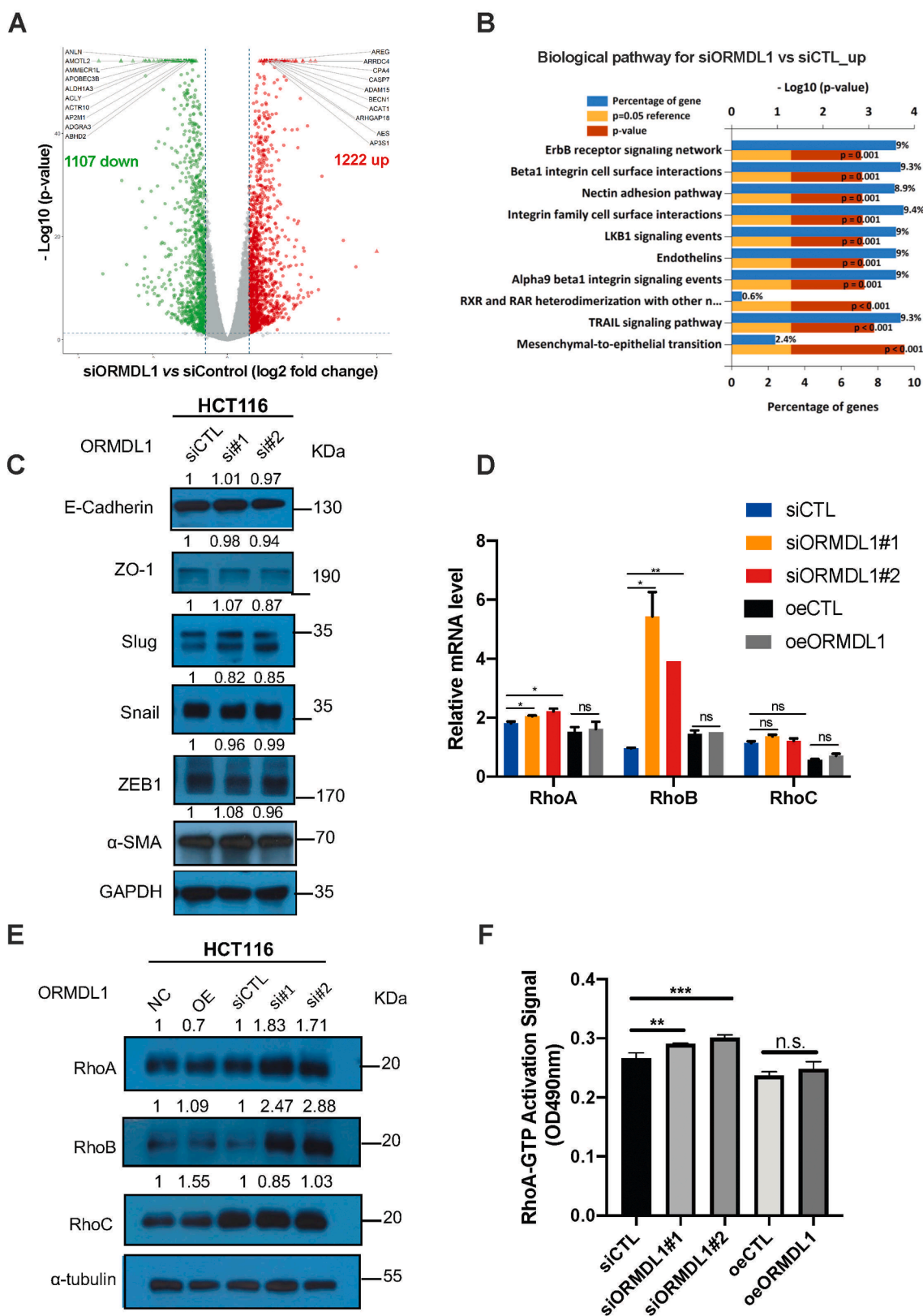
ORMDL1 was overexpressed by using an ORMDL3 antibody that could potentially recognize ORMDL1–3 [24]. As ORMDL1 is a membrane-bound protein, cofactors or receptors inside the cell may be needed for downstream signal transduction.

## Conclusion

ORMDL1 was upregulated in CRC, and patients with higher ORMDL1 expression had longer OS, suggesting that ORMDL1 expression may serve as a biomarker for predicting favorable outcomes. ORMDL1 may participate in cytoskeleton regulation and ER stress response.

## Ethics approval and consent to participate

Our study involving human specimens was approved by the Institutional Ethics Committee of Sixth Affiliated Hospital, Sun Yat-sen



**Fig. 5. ORMDL1 affected expression level of Rho GTPase.**

(a) Volcano plot for genes identified by RNA sequencing, with the X axis for the  $\log_2$ -fold change (siORMDL1 vs siControl) and the Y axis for the  $-\log_{10}$  P value.

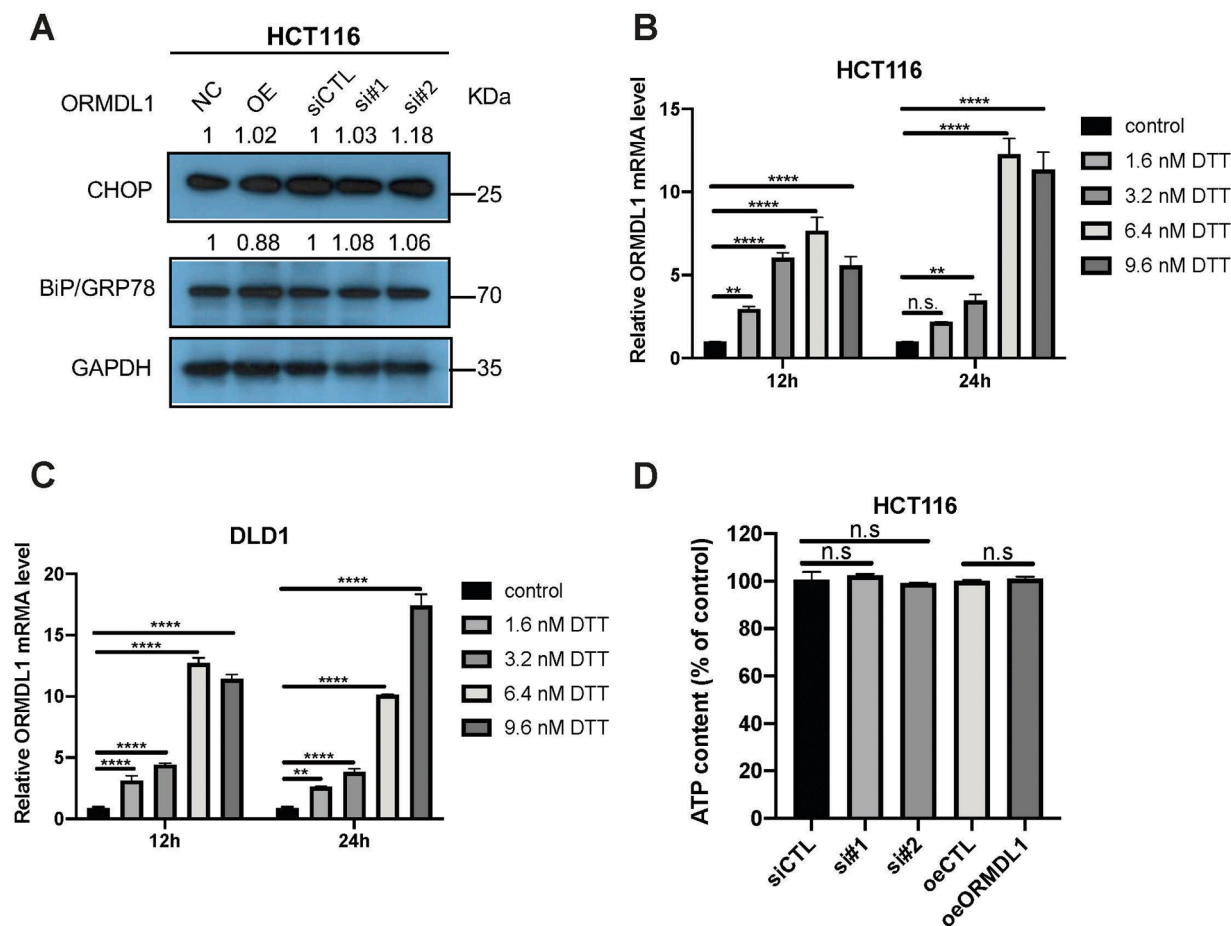
(b) The list of upregulated genes in (a) was subjected to pathway enrichment analysis and the top 10 enriched pathways are shown.

(c) Protein level of classical EMT-related markers were determined by Western blotting in HCT116 cells with ORMDL1 knockdown.

(d) qRT-PCR to confirm Rho family genes whose expression level was affected when ORMDL1 was overexpressed or knocked down in HCT116 cells.

(e) Western blotting and semiquantitative analysis of the Rho family gene when ORMDL1 was overexpressed or knocked down in HCT116 cells.

(f) Activated-RhoA levels in HCT116 cells with ORMDL1 overexpression or knockdown. \*\*P < 0.01, \*\*\*P < 0.001.



**Fig. 6.** ORMDL1 expression was enhanced by DTT-induced ER stress.

(a) Western blotting to detect the expression level of ER stress response-related proteins in HCT116 cells with ORMDL1 overexpression or knockdown. (b) The expression of ORMDL1 in HCT116 cells treated with DTT.  $**P < 0.001$ ,  $****P < 0.0001$ . (c) The expression of ORMDL1 in DLD1 cells treated with DTT.  $**P < 0.001$ ,  $****P < 0.0001$ . (d) ATP levels in HCT116 cells with ORMDL1 overexpression or knockdown.

University. The related ethical approval code is 2020ZSLYEC-065. The animal study was approved by the Laboratory Animal Center of The Sixth Affiliated Hospital of Sun Yat-sen University (SYXK(YUE) 2018-0190).

#### Consent for publication

Not applicable.

#### Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

#### CRedit authorship contribution statement

**Qian Wang:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Wanjun Liu:** Formal analysis, Writing – review & editing. **Si Chen:** Data curation. **Qianxin Luo:** Data curation, Writing – review & editing. **Yichen Li:** Data curation. **Shaoyong Peng:** Data curation, Writing – review & editing. **Huaiming Wang:** Data curation, Writing – review & editing. **Xiaoxia Liu:** Methodology, Writing – review & editing. **Daici Chen:** Supervision, Writing – original draft, Writing – review & editing, Conceptualization.

#### Declaration of Competing Interest

The authors declare that they have no competing interests.

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2021.101171](https://doi.org/10.1016/j.tranon.2021.101171).

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