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OLA1 promotes colorectal cancer tumorigenesis by activation of HIF1 α /CA9 axis

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Abstract

Background: Obg-like ATPase 1 (OLA1) is a highly conserved GTPase, which was over expressed in a variety of malignant tumors, but its role in colorectal cancer (CRC) was poorly studied.

Patients and methods: Three public CRC gene databases were applied for OLA1 mRNA expression detection. The clinical data of 111 CRC patients were retrospectively collected from the Second Affiliated Hospital of Zhejiang University (SAHZU) for OLA1 protein expression and Kaplan-Meier Survival analysis. OLA1 stably knocked out CRC cell lines were conducted by CRISPR-Cas9 for experiments in vitro and in vivo.

Results: OLA1 was highly expressed in 84% CRC compared to matched surrounding tissues. Patients with OLA1 high expression had a significantly lower 5-year survival rate (47%) than those with OLA1 low expression (75%). OLA1 high expression was an independent factor of poor prognosis in CRC patients. OLA1-KO CRC cell lines showed lower ability of growth and tumorigenesis in vitro and in vivo. By mRNA sequence analysis, we found 113 differential express genes in OLA1-KO cell lines, of which 63 were hypoxic related. HIF1 α was a key molecule in hypoxic regulation. Further molecular mechanisms showed HIF1 α /CA9 mRNA and/or protein levels were heavily downregulated in OLA1-KO cell lines, which could explain the impaired tumorigenesis. According to previous studies, HIF1 α was a downstream gene of GSK3 β , we verified GSK3 β was over-activated in OLA1-KO cell lines.

Conclusion: OLA1 was a new gene that was associated with carcinogenesis and poor outcomes in CRC by activation of HIF1 α /CA9 axis, which may be interpreted by GSK3 β .

Keywords: Colorectal cancer, Tumorigenesis, OLA1, HIF 1 α , CA9

Text

Introduction

Colorectal cancer (CRC) is one of the most frequent gastrointestinal malignant tumors and is also the third leading cause of cancer-associated death worldwide [1, 2]. Various genes involved in the development of cancer

pathogenesis provide novel avenues to understand the underlying molecular mechanisms that contribute to the development of CRC [3]. However, the effect of genes on the tumorigenesis of CRC is still being elucidated.

OLA1 belongs to the translation-factor (TRAFAC) related class, Obg family, and YchF subfamily of *P*-loop GTPases [4, 5]. The YchF/OLA1 proteins are highly conserved from bacteria to humans. Unlike other Obg family members, OLA1 possess both GTPase and ATPase activities [6, 7]. We have previously reported that mouse embryos lacking OLA1 have immature and small organs at birth and in the later growth and development. It probably indicates its potential role in tumor development

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[8]. As reported, OLA1 was over expressed in a variety of malignant tumors including CRC, and was an important gene in regulating the growth and metastases in lung cancer and breast cancer [9–11]. However, its role in CRC was poorly studied.

In solid tumors, hypoxia is a prevalent occurrence. Hypoxia-inducible factor 1 (HIF-1) is required for regulating the cellular response to hypoxia by activating hundreds of genes through transcription [12].

HIF-1 is a heterodimer comprised mainly of two subunits called HIF-1 α and HIF-1 β . The HIF-1 α subunit is activated by hypoxia signals and forms a functional HIF-1 with 1 β whose transcription factor specifically recognizes hypoxia response elements in the target gene promoters, resulting in transcriptional activation, whereas the HIF-1 β subunit is persistently produced in cells and serves a structural function. Cancer cells generate HIF-1 dramatically increased in hypoxic circumstances, promoting anaerobic glycolysis and angiogenesis, which leads to cell proliferation, apoptosis and invasive phenotype [13, 14]. CA9 is one of the target genes of HIF1 α [15, 16] which is frequently over expressed in various of malignancies. CA9 appears to be involved in tumor growth and metastatic dissemination in vitro and clinical studies, and overexpression of CA9 is linked to a poor prognosis in a variety of cancers [17, 18]. In CRC, CA9 is proved to promote tumor growth and necrosis in vivo [19].

In the study, we found that OLA1 was highly expressed in most CRC samples by immunohistochemistry, which was associated with poor prognosis. To explore the molecular role of OLA1 in CRC, we constructed two OLA1-KO CRC cell lines Hct116 and Lovo, deprivation of OLA1 led to lower ability of growth and tumorigenesis in vitro and in vivo. By mRNA sequence analysis, we found 113 differential expression genes in OLA1-KO and control cell lines, 63 of which were hypoxia-related. HIF1 α was a key molecule in hypoxic regulation. Further molecular mechanisms showed HIF1 α /CA9 mRNA and/or protein levels were heavily downregulated in OLA1-KO cell lines, which could explain the impaired tumorigenesis.

Materials and methods

Sampling

Public database: Three independent public CRC gene databases, TCGA-RNAseq dataset ($n=50$), GSE31737 ($n=40$) and GDS4382 ($n=17$), were applied for OLA1 mRNA expression detection. Clinical cohort: 111 patients from the Second Affiliated Hospital of Zhejiang University (SAHZU) were enrolled. All patients were confirmed CRC by histopathological examination and had a follow-up over 5 years. Primary tumor specimens

and matched adjacent non-tumorous tissues were collected for further OLA1 immunohistochemistry (IHC). All human materials were obtained with informed consent and the study was approved by the Ethical Review Committee of SAHZU (2021-LYS-0689).

Clinical data collection

The expression quantity of OLA1 mRNA, normalized by log₂, was extracted from TCGA-RNA dataset, GSE31737 and GDS4382. The relative expression between CRC and paired adjacent normal tissue was analyzed within the three datasets respectively. Both primary tumor and adjacent non-tumorous tissues from SAHZU were performed OLA1 IHC. The patients were divided into OLA1 high (IHC-score: 2 or 3) group and OLA1 low (IHC-score: 0 or 1) group by IHC-score according to DAB dye depth.

Cell culture

Human colon cancer cell lines HCT116 and Lovo were purchased from American Type Culture Collection (ATCC). Cell lines were cultured under standard conditions in 1640 medium (Gibco) with 10% fetal bovine serum (FBS, Hyclone), 10 units/ml penicillin, and 10 mg/ml streptomycin at 37°C and 5% CO₂ in a humidified incubator (Thermo Scientific) and regularly tested for mycoplasma contamination.

Establishment of OLA1-KO CRC cell line by CRISPR/Cas9

Stable OLA1-KO cell lines (HCT116-KO, Lovo-KO) were established using CRISPR/Cas9. Designed CRISPR/Cas9 target OLA1 gRNA OLA1-gRNA1: ACGTTCACCTAC CCAACATTTGG and OLA1-gRNA2: ATTTTCAGAT GCCCCCTAAAAAGG, conducted the knockout vector and transfected into Hct116 and Lovo cells by electrotransfection. Screening monoclonal cells by PCR. The knockout efficiency was verified by western blot analysis.

In vitro cell proliferation, colony formation assays

The cell proliferation assay was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kyushu, Japan). Experimental and control cells were both placed into the 96-well plate with a density of 1000 cells/well, and 10 μ l of CCK-8 was added into 90 μ l of the culture medium per well. After incubating at 37°C for 2h, and the density was measured at 450 nm.

For the colony formation assay, experimental and control cells were plated into a 6-well plate with a density of 1000 cells/well and 500 cells/well respectively. Cells were incubated at 37°C for 2 weeks. The colonies were fixed and stained using 20% methanol and 0.1% crystal violet, and the number of colonies (> 50 cells) was counted. All assays were replicated three times.

In vivo assays for tumor formation

This was conducted by subcutaneous tumor formation in nude mouse. 1×10^6 experimental and control colon cancer cells were suspended in 100 μ l PBS and subcutaneously injected into nude mouse (female nude mouse, 10 per group). The tumor sizes were measured every 3 days as soon as the tumors were measurable. The mice were sacrificed after 28 days and the tumors were harvested for further analysis.

mRNA sequence analysis

Total mRNA isolated from OLA1-KO and control cells. DNase I was used to digest double-stranded and single-stranded DNA in total RNA, then, magnetic beads were purified to recover the reaction products, RNase H or Ribo-Zero method (Human, mouse, plants) (Illumina, USA) was used to remove the rRNA. Purified mRNA from previous steps was fragmented into small pieces with fragment buffer at the appropriate temperature. Then, the first-strand cDNA was generated in First Strand Reaction System by PCR, and the second-strand cDNA was generated as well. The reaction product was purified by magnetic beads, afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to carry out end repair. The cDNA fragments with adapters were amplified by PCR, and the products were purified by Ampure XP Beads. Library was validating on the Agilent Technologies 2100 bioanalyzer for quality control. The double-stranded PCR products above were heated denatured and circularized by the splint oligo sequence. The single-stranded circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 (thermos Fisher Scientific, MA, USA) to make DNA nanoball (DNB) which had more than 300 copies of one molecular, DNB were loaded into the patterned nanoarray and single end 50 bases reads were generated on BGISEQ500 platform (BGI-Shenzhen, China) [20].

Western blot

Equal amounts of cellular protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat milk for 1 h and incubated with primary and secondary antibodies. Immunoreactivity was visualized using chemiluminescence ECL reagents (Pierce, Rockford, IL). The densitometry analysis was measured by Image-Pro Plus 6.0 (Media Cybernetics). All antibodies used in these studies were as follows: anti-OLA1, (Sigma-Aldrich,1:500), anti-GSK3 β , (cell signaling technology,1:1000), anti-P-GSK3 β , (cell signaling

technology,1:1000), anti-CA9(sigma-Aldrich,1:500), anti-GAPDH (cell signaling technology,1:5000), anti-STAT3, (cell signaling technology,1:1000), and anti-rabbit/mouse IgG (cell signaling technology,1:5000), peroxidase-linked secondary antibody. The images were collected by Azure multifunctional molecular imaging system (AZURE600) and scanner.

IHC staining

IHC was performed for OLA1 on 90 paired formalin-fixed, paraffin-embedded samples and 111 formalin-fixed, paraffin-embedded colorectal cancer samples. In a nutshell, sections were deparaffinized, rehydrated and pretreated in a pressure cooker in 1 mM EDTA buffer pH 8.0. Slides were then incubated with a polyclonal antibody against OLA1, diluted 1:800 (Sigma, #HPA027524), CA9, diluted 1:500 (sigma-Aldrich), HIF1 α , diluted 1:500 (cell signaling technology), Ki67, diluted 1:1000 (cell signaling technology) at 37 °C for 2 h. Slides were rinsed three times with PBS containing 0.05% Tween20 and developed with the EnVision-System (DAKO, #K4003) according to the manufacturer's protocol. Finally, they were counterstained with hematoxylin, diluted 1:10(Merck; #1.09249.0500) for 60–90 s. Two researchers masked sections and analyzed them independently. The image of IHC was collected by light microscope.

Real-time quantitative reverse transcription-polymerase chain reaction

The Qiagen RNeasy Mini kit was used to extract total RNA from the cells according to the manufacturer's guidelines. We evaluated the quality and quantity of the RNA by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). The quality and amount of the RNA was assessed by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). The PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara Biotechnology, Dalian, China) was used to generate the cDNA we needed. The Step-One-Plus Real-Time PCR System (Life Technologies, Foster, CA, USA) was utilized for quantitative real-time RT-PCR (qRT-PCR), whose data were analyzed via Step-One software V2.1. Genetic expression was measured with the TaqMan test system and the following keys and probes: ola1 forward: 5'-TGGACAAGTATGACCCAGGT-3', reverse: 5'-GCTGCAAACCCAGCCTTAATG-3'; ca9 forward: 5'-TACAGCTGAACTTCCGAGCG-3', reverse: 5'-CTAGGCTCCAGTCTCGGCTA-3'; gapdh forward: 5'-CATGAG AAGTATGACAACAGCCT-3', reverse: 5'-AGTCCTTCCACGATACCAAAGT-3'.

Animal and embryo studies

Generation of mouse strains carrying *Ola1* null mutations has been described previously [8]. Time matings were set up by housing a single male with one or two females. At day 18.5 embryonic age, pregnant females were euthanized using CO₂ asphyxiation and the embryos were dissected. Thermo Scientific T-PER tissue protein extraction (No.78510) and Thermo Scientific Halt Protease Inhibitor Cocktail was used. Scissor 1g of soft tissues of mouse embryo (excluded skeletal, skin, brain, lung, liver), add 30ul T-PER reagent with cocktail and homogenize, lysis on ice for 45 to 60 min, then centrifuge the sample at 10,000×g for 5 min to pellet tissue debris, finally, Collect supernatant for western blot.

Statistical analysis

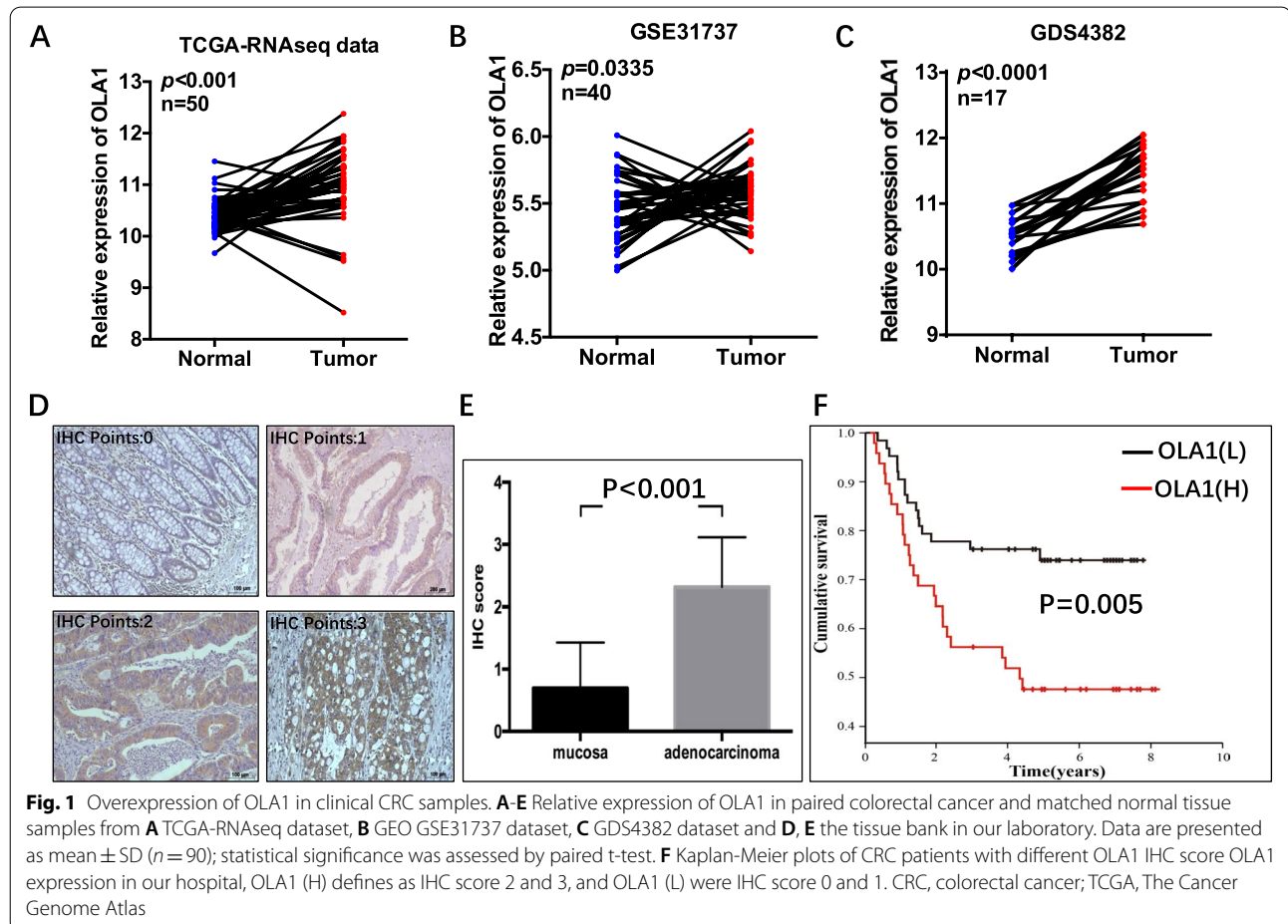
All of the results came from at least three separate experiments. For comparisons between two groups, the data were reported as mean ± SD and the Student's t test was utilized. The population results are shown as percentages, medians, and 95% confidence intervals (CI). The population results are shown as percentage, medians,

and 95% confidence interval (CI). The Kaplan-Meier estimator technique was used to produce overall survival (OS) curves. The univariate and multivariate Cox's proportional hazards models were used to investigate prognostic factors. A statistically significant difference was defined as $P < 0.05$. The ratio of the relative expression of the indicated gene from the tumor patients was plotted and applied with the spearman correlation test. A p value of 0.05 was considered statistically significant. In the graphed data *, **, and *** denote p values of < 0.05 , 0.01 and 0.001, respectively. All of the statistical analyses were performed by using SPSS18.0.

Results

OLA1 was upregulated in CRC and linked to a poor prognosis

OLA1 gene copy number was dramatically increased in CRC, according to the Cancer Genome Atlas (TCGA) and the GEO database (Fig. 1A-C). However, the higher OLA1 copy numbers were identified in all stages of CRC and were unconnected with tumor stage. In the samples from the TCGA dataset (Fig. 1A), GEO



GSE31737 dataset (Fig. 1B), and GDS4382 dataset (Fig. 1C), the expression level of OLA1 in matched-paired CRC and normal tissue samples was investigated. The results demonstrated tumor tissue samples had a considerable up-regulation of OLA1 in these datasets.

To validate the role of OLA1 in colorectal cancer in these databases, OLA1 expression was analyzed in CRC tissues by IHC (Fig. 1D). Results showed that 84% (76/90) primary CRC tumors had higher OLA1 expression (Fig. 1E) compared to the matched surrounding tissues. However, there was no significant correlation between OLA1 expression and other clinicopathological variables, such as patient gender, age, tumor size, and TNM stage ($P > 0.05$, Table 1). Patients with high OLA1 expression showed a considerably poorer survival rate than those with low OLA1 expression, according to a Kaplan–Meier analysis (Fig. 1F, Table 2). CRC patients with high OLA1 expression had a 5-year survival rate of 47%, which was substantially lower than those with low OLA1 expression (75%). To assess whether OLA1 high expression was an independent prognostic factor, all variables that may affect the OS were included in a Cox's proportional hazards model (Table 3). Independent prognostic factors were: T stage [hazard ratio (HR) 2.092; 95% CI (1.090–4.031); $P = 0.026$], N stage [HR 1.383; 95% CI (1.018–1.878); $P = 0.038$], M stage [HR 4.407; 95% CI (2.033–9.552); $P < 0.001$], and OLA1 high expression [HR 2.616; 95% CI (1.328–5.153); $P = 0.005$].

In addition, we analyzed the OLA1 protein expression in eight colorectal cancer cell lines by western blot, and the result indicated that OLA1 was high expression in most of these cell lines (Fig. S1). Together, these results clearly showed that OLA1 was overexpressed in CRC tissues and CRC cells, suggesting that OLA1 might play a role in CRC carcinogenesis and development.

Knockout of OLA1 inhibited growth of CRC cells in vitro

The prevalence of OLA1 upregulation raises an intriguing possibility that OLA1 overexpression may be a cancer-promoting event in CRC. To verify this hypothesis, OLA1 was stably knocked out by CRISPR/Cas9 in Lovo and Hct116 cells expressing high levels of OLA1 protein. The KO efficiency was confirmed by western blotting (Fig. 2A). The CCK8 assays showed that knocking out OLA1 significantly reduced cellular proliferation (Fig. 2B and C). Colony formation assays were also used to investigate the role of OLA1 in cell proliferation (Fig. 2D). The colony formation ability was significantly impaired when these two cells lacked of OLA1. Collectively, these data demonstrated that OLA1 can promote colorectal cancer cell growth in vitro.

Table 1 Baseline of patients with different OLA1 expression level

	OLA1 Low (N = 63)	OLA1 High (N = 48)	<i>p</i>
Age	58.84 ± 14.21	49.56 ± 15.12	0.797
Gender			0.497
Male	34(54.0%)	29(60.4%)	
Female	29(46.0%)	19(39.6%)	
Tumor location			0.577
Right colon	20(31.7%)	11(22.9%)	
Left colon	13(20.6%)	12(25.0%)	
Rectum	30(47.6%)	24(52.1%)	
T stage			0.147
T1	4(6.3%)	3(6.2%)	
T2	16(25.4%)	7(14.6%)	
T3	22(34.9%)	27(56.2%)	
T4	21(33.3%)	11(22.9%)	
N stage			0.702
N0	36(57.1%)	22(45.8%)	
N1	14(22.2%)	13(27.1%)	
N2a	4(6.3%)	4(8.3%)	
N2b	9(14.3%)	9(18.8%)	
M stage			0.159
M0	57(90.5%)	39(81.2%)	
M1	6(9.5%)	9(18.8%)	
Tumor size			0.938
≤ 3 cm	17(27.0%)	14(29.2%)	
3–5 cm	31(49.2%)	22(45.8%)	
> 5 cm	15(23.8%)	12(25.0%)	
Tumor differentiation			0.425
Poor	4(6.3%)	2(4.2%)	
Moderate	25(39.7%)	25(52.1%)	
Well	24(38.1%)	15(31.2%)	
Mucous	7(11.1%)	6(12.5%)	
Signet ring	3(4.8%)	0(0.0%)	
Chemotherapy			0.721
No	44(69.8%)	32(66.7%)	
Yes	19(30.2%)	16(33.3%)	

OLA1 promoted the growth of CRC cells in mouse models in vivo

The nude mice's flanks were injected with the same number of OLA1-KO and control CRC cells Hct116 and Lovo. Every 3 days, tumor formation was tracked and tumor sizes were measured. Tumors were established on 12 days after implantation, as evidenced by the fact that OLA1-KO cells developed much more slowly than control cells, and tumor sizes in these mice were significantly less than those in control mice (Fig. 2E). IHC also confirmed the extent of OLA1 depletion in the tumors (Fig. 3). To sum

Table 2 Univariate analysis of prognostic factors for overall survival

	Patient		Kaplan-Meier, Log-Rank test	
	N	%	5-year survival	P-value
Age (years)				0.270
≤ 40	13	12	46%	
40–70	76	68	65%	
≥ 70	22	20	64%	
Gender				0.181
Male	63	57	70%	
Female	48	43	58%	
Tumor location				0.126
Right colon	31	28	53%	
Left colon	25	23	72%	
Rectum	55	50	66%	
Tumor differentiation				0.119
Well	6	5	69%	
Moderate	50	45	68%	
Poor	39	35	35%	
mucous	13	12	60%	
Signet ring	3	3	35%	
Tumor size (cm)				0.009
≤ 3	31	28	80%	
3–5	53	48	65%	
>5	27	24	44%	
T stage				0.000
T1	7	6	100%	
T2	23	21	95%	
T3	49	44	53%	
T4	32	29	45%	
N stage				0.000
N0	58	52	82%	
N1	27	24	52%	
N2a	8	7	50%	
N2b	18	16	28%	
M stage				0.000
M0	96	86	73%	
M1	15	14	8%	
Chemotherapy				0.001
Yes	35	32	42%	
No	76	68	76%	
OLA1 score				0.005
0 + 1	63	57	78%	
2 + 3	48	43	48%	

up, these findings demonstrated low OLA1 expression were sufficient to suppress CRC cell growth, strongly suggesting that OLA1 acted as a tumor promoter in CRC.

Table 3 Multivariate analysis for overall survival in colorectal cancer

	Overall survival		
	HR	95% CI	P-value
Gender	0.717	0.352–1.462	0.360
Age	0.833	0.425–1.632	0.594
OLA1 score	2.616	1.328–5.153	0.005
Tumor location	1.285	0.828–1.994	0.263
T stage	2.092	1.090–4.031	0.026
N stage	1.383	1.018–1.878	0.038
M stage	4.407	2.033–9.552	0.000
Tumor size	1.292	0.767–2.177	0.336
Tumor differentiation	1.070	0.739–1.548	0.720
Chemotherapy	1.390	0.694–2.783	0.353

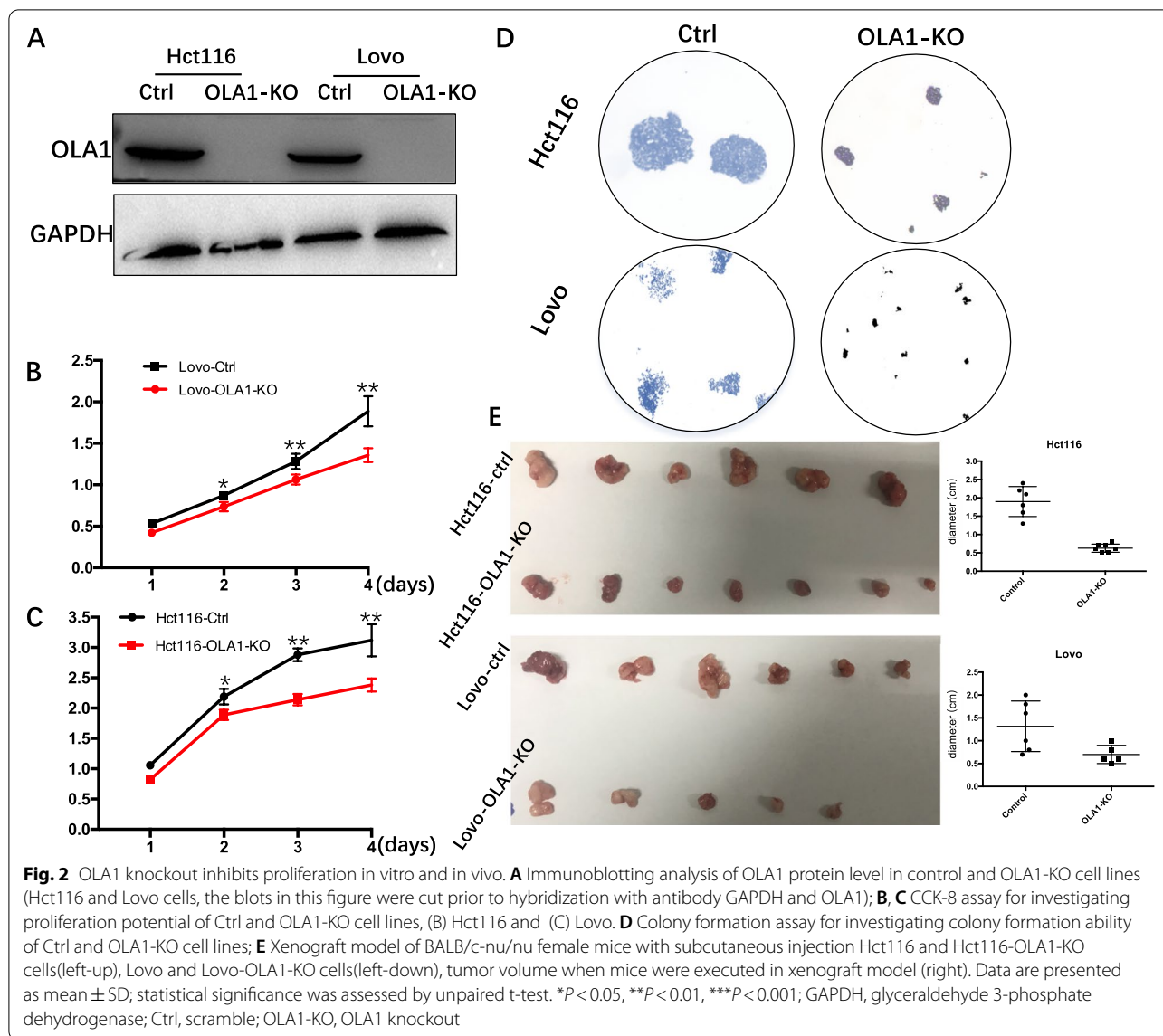
HR Hazard ratio, CI confidence interval

Modulation of hypoxia-related gene expression by OLA1 in CRC cells

In order to clarify the underlying mechanisms of OLA1 in CRC, we performed mRNA sequencing in OLA1-KO and control cells. Biological replicates were 2 pairs of colon cancer cell line Hct116 and Lovo. Gene expression was analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array. The mRNA expression of OLA1 was validated through qRT-PCR. As showed in Fig. 4A, 113 genes were changed in both pairs of cell lines. Functional annotations of these genes indicated that there were a number of differentially expressed genes with functions related to key cellular processes, including regulation of cell proliferation, invasion, cell cycle, glucose metabolism and formation of nucleosome structure (Fig. 4B). Interestingly, 63 genes were hypoxia-related genes among these 113 common changed genes (Fig. 4C). Overall, these data suggested that OLA1 promoted the tumorigenesis of CRC by upregulating expression of proliferation, invasion and cell cycle related genes.

CA9 and HIF1 α were downregulated in OLA1-KO CRC cell lines

HIF1 α , which was a key molecule in hypoxic regulation, was examined by IHC in OLA1-KO xenografts, and the result showed that HIF1 α was downregulated in OLA1-KO groups. Meanwhile, Ki67 and necrosis were also decreased in OLA1-KO groups (Fig. 3). CA9 was a target gene of HIF1 α , and was the most upregulated gene in the mRNA sequence. By qRT-PCR, western blot and IHC, we found CA9 level was heavily downregulated in OLA1-KO cell lines both in mRNA and protein level. (Fig. 3, and 5A–B). These results indicated that OLA1 depletion inactivated HIF1 α signaling which caused growth retardation.



Overactivation of GSK3 β in OLA1-KO cell lines inhibited HIF1 α signaling

Ola1^{-/-} knockout mice had been generated in our previous study, a large majority of which were found dead on the day of birth. Ola1^{-/-} embryos were significantly smaller in size than their Ola1^{+/+} and Ola1^{+/-} controls as measured on E18.5. We collected the tissue and extracted protein of E18.5, GSK3 β expression was much heavier in Ola1^{-/-} mice than the controls (Fig. 5C). Further, in our study, we validated the GSK3 β activity in control and OLA1-KO CRC cell lines, and GSK3 β was over-activated in OLA1-KO cell lines (Fig. 5B). Our previous study showed that OLA1 directly interacted with GSK3 β and inhibited its activity [21, 22], which is also proved by other study [23, 24]. Meanwhile, GSK3 β can

phosphorylate HIF1 α and facilitate its degradation [25], which can explain why HIF1 α decreased in OLA1-KO CRC cell lines.

Discussion

OLA1 is a member of the GTPase protein family; unlike other members, it possesses both GTPase and ATPase activities, and can bind and hydrolyze ATP more efficiently than GTP. OLA1 participates in cell proliferation, oxidative response, protein synthesis and tumorigenesis [6]. According to previous studies, OLA1 can interact with BRCA1 to regulate the centrosome number, which promotes the proliferation of breast cancer cells [9]; OLA1 deficiency causes p21 accumulation which leads to stunted growth and frequent perinatal lethality in mouse

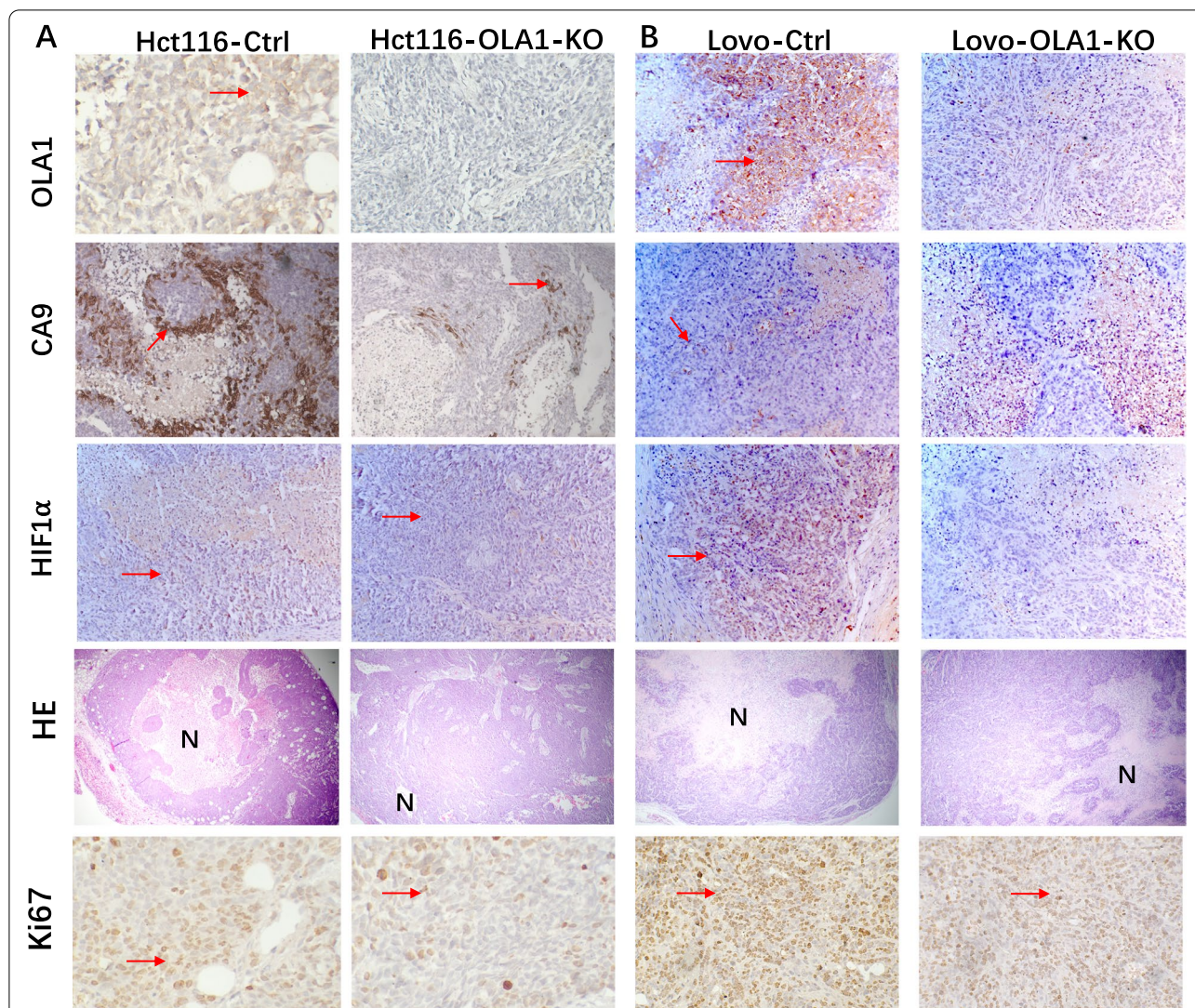


Fig. 3 OLA1 expression is associated with increased CA9, necrosis and proliferation in HCT116 and Lovo xenografts. Representative immunohistochemical images of HCT116 (A) and Lovo (B), control and OLA1-KO xenografts. The expression of OLA1, CA9, HIF1, Ki67, and the fraction of necrosis between the control and OLA1-KO groups were substantially different. The expression staining of OLA1, CA9, HIF-1, and Ki-67 were depicted in dark brown. The dark pink stain in H&E indicates living tissue, while the light pink stain indicates necrosis. SD is represented by the error bars. Necrosis is indicated by the letter N. Positive staining is indicated by red arrows

embryonic development [8]. All these data indicates that the function of OLA1 is tightly related to growth and proliferation.

In the study, we found that OLA1 was an important gene in CRC. The expression of OLA1 was obviously elevated in GEO and TCGA databases and also the database in our own center. The result was in accordance with previous studies [10]. Meanwhile, the study first illustrated that elevated expression of OLA1 indicated poor prognosis in CRC. By Cox regression model, OLA1 high expression was the second highest risk factor of CRC, only to distant metastases. The

clinical significance of OLA1 gave us more confidence to explore its molecular mechanisms in CRC, like BRAF and KRAS [26, 27]. Further experiments confirmed OLA1 deficiency injured the ability of proliferation, colony formation and tumorigenesis in CRC cell lines, which were accordance with the clinical data.

To explore the molecular mechanism of OLA1 in CRC, mRNA sequence was conducted in OLA1-KO CRC and control cell lines. In these two pairs of colon cancer cell lines (Hct116 and Lovo), we found CA9 mRNA decreased in both Hct116 and Lovo OLA1-KO cell lines.

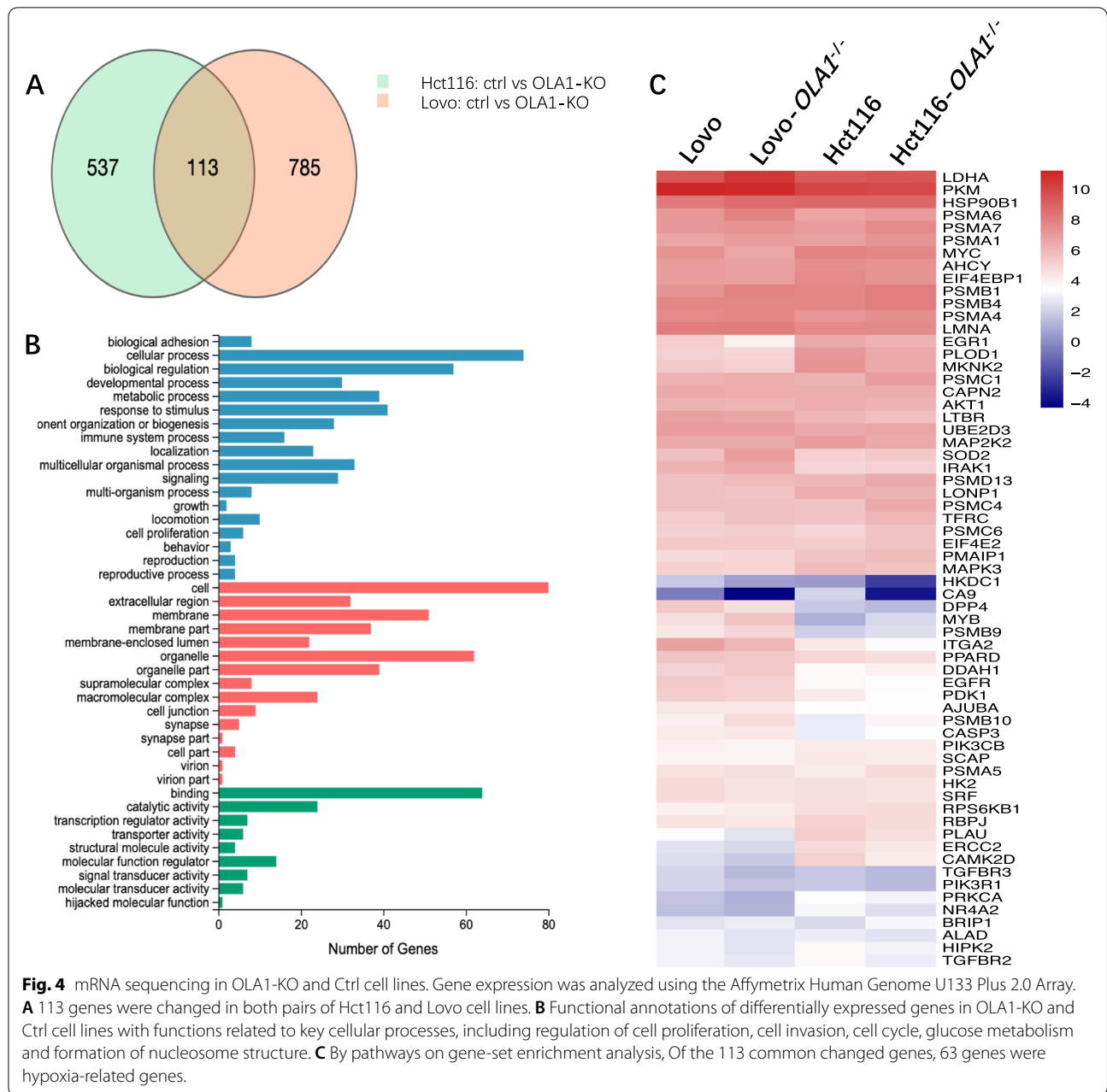
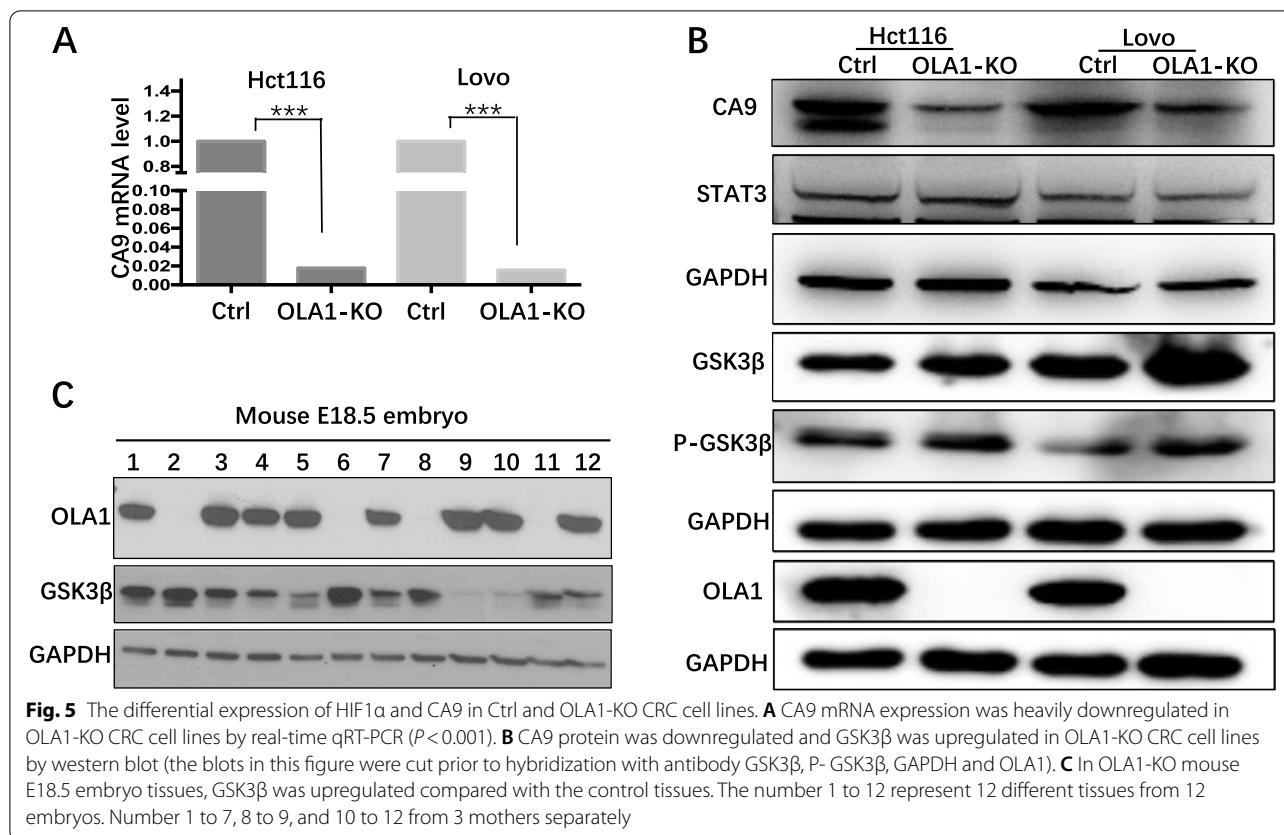


Fig. 4 mRNA sequencing in OLA1-KO and Ctrl cell lines. Gene expression was analyzed using the Affymetrix Human Genome U133 Plus 2.0 Array. **A** 113 genes were changed in both pairs of Hct116 and Lovo cell lines. **B** Functional annotations of differentially expressed genes in OLA1-KO and Ctrl cell lines with functions related to key cellular processes, including regulation of cell proliferation, cell invasion, cell cycle, glucose metabolism and formation of nucleosome structure. **C** By pathways on gene-set enrichment analysis, Of the 113 common changed genes, 63 genes were hypoxia-related genes.

CA9 is an important gene in carcinogenesis. In CRC samples studied by cDNA microarray, CA9 was found to be the most upregulated gene [28]. In CRC, CA9 showed heavier expression in carcinomas than in benign lesions [29, 30]. The ratio of CA9 positive cells in the normal intestinal epithelia is as low as 10% [31]. The expression pattern of CA9 was just in accordance with OLA1 in CRC.

CA9 is also a marker for poor clinical outcome in most cancer types [10, 19, 28, 30]. The strong staining intensity of CA9 is an adverse prognostic factor

in rectal cancer [32]. In CRC cell lines, CA9 promotes tumor growth and necrosis in vivo [19]. Tumor necrosis is a promising prognostic factor in colorectal cancer [33]. We observed the same phenomenon in our study. In xenograft, controlled tumors showed a gradient of CA9 expression with highest levels adjacent to frank necrosis, while in OLA1-KO tumors, CA9, necrosis and Ki67 decreased obviously. High expression of OLA1 indicated poor prognosis maybe related with more necrosis and Ki67 caused by increased CA9.



CA9 is mainly regulated by HIF1 α , where the HRE/HIF1 α -binding site is located at 3 of 10 position in the promoter region of this gene [15]. HIF1 α also contributes to intestinal tumorigenesis [34]. We tested HIF1 α protein by western blot in OLA1 control and KO cell lines and by IHC in OLA1 knockout nude mice subcutaneous tumors. We failed to detect it by western blot, which might ascribe to the short life time of HIF-1 α under normoxic cell culture conditions [35–38]. But the IHC results confirmed HIF1 α expression decreased in OLA1-KO groups which was in accordance with CA9, Ki67 and necrosis. At the same time, we found an interesting phenomenon. In Hct116 cell lines, HIF1 α increased less than CA9 while in Lovo cell lines, HIF1 α increased more than CA9. This was also reported by Solmaz Sobhanifar et al. [35]. This can be ascribed to long lifetime of CA9 and short lifetime of HIF1 α . Since CA9 is a target gene of HIF1 α , maybe there is a feedback loop between CA9 and HIF1 α .

However, our study did not illustrate how OLA1 influenced HIF1 α /CA9 axis. The possible mechanism was the activity of HIF1 α was regulated by a variety of genes including GSK3 β , and GSK3 β can directly interact with OLA1 [39]. OLA1 may upregulate HIF1 α expression by GSK3 β which needs further study.

This study proposed the possibility that OLA1 could be a new gene that it was associated with carcinogenesis and poor outcomes in CRC by facilitating HIF1 α /CA9 signaling. This process may be interpreted by GSK3 β .

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12885-022-09508-1>.

Additional file 1.

Acknowledgments

All authors would like to express their sincere thanks to the participants who provided colorectal tissue samples for this study and ensuring the successful completion of the study.

Code availability

SPSS18.0.

Authors' contributions

YL, XK and JH contributed equally to this article. YL and KD designed the study. YL, JZ and LZ wrote the manuscript. XK, JH and YX contributed to collection of the data. DX contributed to the critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the National Natural Science Foundation of China (81902818).

Availability of data and materials

The sequence data generated in this study are publicly available in The Cancer Genome Atlas (TCGA) -RNAseq dataset, in Gene Expression Omnibus (GEO) at GSE31737 and GDS4382. Further inquiries can be directed to the corresponding author.

Declarations

Ethics approval and consent to participate

All human participants, human material, and human data were obtained with informed consent and the study was approved by the Ethical Review Committee of the Second Affiliated Hospital of Zhejiang University (2021-LYS-0689). All methods were performed in accordance with the relevant guidelines and regulations.

All experimental protocols were approved by Animal Experimentation Ethics Committee of the Second Affiliated Hospital of Zhejiang University. All methods were carried out in accordance with relevant guidelines and regulations and are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Received: 9 November 2021 Accepted: 5 April 2022

Published online: 19 April 2022

References

- Parkin DM. Global cancer statistics in the year 2000. *Lancet Oncol.* 2001;2(9):533–43.
- Siegel RL, Miller KD, Jemal A. Cancer statistics, 2020. *CA Cancer J Clin.* 2020;70(1):7–30.
- Farooqi AA, et al. Overview of the oncogenic signaling pathways in colorectal cancer: mechanistic insights. *Semin Cancer Biol.* 2019;58:65–79.
- Gracia DF, et al. Characterization of a novel Obg-like ATPase in the protozoan *Trypanosoma cruzi*. *Int J Parasitol.* 2009;39(1):49–58.
- Koller-Eichhorn R, et al. Human OLA1 defines an ATPase sub-family in the Obg family of GTP-binding proteins. *J Biol Chem.* 2007;282(27):19928–37.
- Chen H, et al. OLA1 regulates protein synthesis and integrated stress response by inhibiting eIF2 ternary complex formation. *Sci Rep.* 2015;5:13241.
- Zhang J, et al. OLA1, an Obg-like ATPase, suppresses antioxidant response via nontranscriptional mechanisms. *Proc Natl Acad Sci U S A.* 2009;106(36):15356–61.
- Ding Z, et al. OLA1, a translational regulator of p21, maintains optimal cell proliferation necessary for developmental progression. *Mol Cell Biol.* 2016;36(20):2568–82.
- Gomez V, Hergovich A. OLA1 in centrosome biology alongside the BRCA1/BARD1 complex: looking beyond centrosomes. *Mol Cell.* 2014;53(1):3–5.
- Sun H, et al. DOC45, a novel DNA damage-regulated nucleocytoplasmic ATPase that is overexpressed in multiple human malignancies. *Mol Cancer Res.* 2010;8(1):57–66.
- Yoshino Y, et al. RACK1 regulates centriole duplication by controlling localization of BRCA1 to the centrosome in mammary tissue-derived cells. *Oncogene.* 2019;38(16):3077–92.
- Sobhanifar S, et al. Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. *Cancer Res.* 2005;65(16):7259–66.
- Al Tameemi W, et al. Hypoxia-modified Cancer cell metabolism. *Front Cell Dev Biol.* 2019;7:4.
- Majmundar AJ, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell.* 2010;40(2):294–309.
- Kaluz S, et al. Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: a one transcription factor (HIF-1) show? *Biochim Biophys Acta.* 2009;1795(2):162–72.
- Wykoff CC, et al. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res.* 2000;60(24):7075–83.
- Chia SK, et al. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. *J Clin Oncol.* 2001;19(16):3660–8.
- Hui EP, et al. Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. *Clin Cancer Res.* 2002;8(8):2595–604.
- McIntyre A, et al. Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy. *Clin Cancer Res.* 2012;18(11):3100–11.
- Zhang Y, Liu L, Ren L. RNA-sequencing-based transcriptome analysis of cantharidin-induced myocardial injury. *J Appl Toxicol.* 2020;40(11):1491–7.
- Bai L, et al. OLA1 contributes to epithelial-mesenchymal transition in lung cancer by modulating the GSK3β/snail/E-cadherin signaling. *Oncotarget.* 2016;7(9):10402–13.
- Xu D, et al. Obg-like ATPase 1 regulates global protein serine/threonine phosphorylation in cancer cells by suppressing the GSK3β-inhibitor 2-PP1 positive feedback loop. *Oncotarget.* 2016;7(3):3427–39.
- Narasimhan G, et al. OBG-like ATPase 1 inhibition attenuates angiotensin II-induced hypertrophic response in human ventricular myocytes via GSK-3beta/beta-catenin signalling. *Clin Exp Pharmacol Physiol.* 2019;46(8):743–51.
- Lu J, et al. HIV p17 enhances T cell proliferation by suppressing autophagy through the p17-OLA1-GSK3β axis under nutrient starvation. *J Med Virol.* 2021;93(6):3607–20.
- Flügel D, et al. Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1alpha and mediates its destabilization in a VHL-independent manner. *Mol Cell Biol.* 2007;27(9):3253–65.
- Smeby J, et al. CMS-dependent prognostic impact of KRAS and BRAFV600E mutations in primary colorectal cancer. *Ann Oncol.* 2018;29(5):1227–34.
- Richman SD, et al. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. *J Clin Oncol.* 2009;27(35):5931–7.
- Talvinen K, et al. Biochemical and clinical approaches in evaluating the prognosis of colon cancer. *Anticancer Res.* 2006;26(6c):4745–51.
- Saarnio J, et al. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. *Am J Pathol.* 1998;153(1):279–85.
- Saarnio J, et al. Immunohistochemistry of carbonic anhydrase isozyme IX (MN/CA IX) in human gut reveals polarized expression in the epithelial cells with the highest proliferative capacity. *J Histochem Cytochem.* 1998;46(4):497–504.
- Takahashi H, et al. Characteristics of carbonic anhydrase 9 expressing cells in human intestinal crypt base. *Int J Oncol.* 2016;48(1):115–22.
- Korkeila E, et al. Expression of carbonic anhydrase IX suggests poor outcome in rectal cancer. *Br J Cancer.* 2009;100(6):874–80.
- Pollheimer MJ, et al. Tumor necrosis is a new promising prognostic factor in colorectal cancer. *Hum Pathol.* 2010;41(12):1749–57.
- Rohwer N, et al. Non-canonical HIF-1 stabilization contributes to intestinal tumorigenesis. *Oncogene.* 2019;38(28):5670–85.
- Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem.* 1997;272(36):22642–7.
- Huang LE, et al. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A.* 1998;95(14):7987–92.
- Kallio PJ, et al. Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. *J Biol Chem.* 1999;274(10):6519–25.

38. Sutter CH, Laughner E, Semenza GL. Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. *Proc Natl Acad Sci U S A*. 2000;97(9):4748–53.
39. Flügel D, Görlach A, Kietzmann T. GSK-3 β regulates cell growth, migration, and angiogenesis via Fbw7 and USP28-dependent degradation of HIF-1 α . *Blood*. 2012;119(5):1292–301.

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