Angiopoietin-Like 4 Promotes Glucose Metabolism by Regulating Glucose Transporter Expression in Colorectal Cancer

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Abstract

Purpose

Angiopoietin-like 4 (ANGPTL4) was recently shown to be associated with cancer progression but little is known about its contribution to cancer metabolism. The purpose of this study was to elucidate the role of ANGPTL4 in glucose metabolism in colorectal cancer (CRC).

Methods

Immunohistochemical staining of CRC specimens classified 84 patients into two groups according to ANGPTL4 expression. Clinicopathological characteristics, gene mutation status obtained by next-generation sequencing, and fluorodeoxyglucose (FDG) uptake measured by positron emission tomography/computed tomography (PET/CT) were compared between the two groups. Furthermore, the impact of ANGPTL4 expression on cancer metabolism was investigated by a subcutaneous xenograft mouse model using the ANGPTL4 knockout CRC cell line and glucose transporter (GLUT) expression was evaluated.

Results

There were significantly more cases of T3/4 tumours (94.3% vs. 57.1%, $P < 0.001$) and perineural invasion (42.9% vs. 22.4%, $P = 0.046$) in the ANGPTL4-high group than in the low group. Genetic exploration revealed a higher frequency of *KRAS* mutation (54.3% vs. 22.4%, $P = 0.003$) in the ANGPTL4-high tumours. All the FDG uptake parameters were significantly higher in ANGPTL4-high tumours. In vivo analysis showed a significant reduction in tumor size due to ANGPTL4 knockout with lower expression of GLUT1 and GLUT3, and suppression of AKT phosphorylation.

Conclusion

ANGPTL4 regulates the expression of GLUTs by activating the PI3K-AKT pathway and thereby promoting glucose metabolism in CRC. These findings establish a new functional role of ANGPTL4 in cancer progression and lay the foundation for developing a novel therapeutic target.

Introduction

Angiopoietin-like 4 (ANGPTL4) is a secreted member of the angiopoietin-like protein family, which includes ANGPTL1–7 (Hato et al. 2008). Structurally, it contains a C-terminal fibrinogen-like domain (cANGPTL4) and an N-terminal coiled-coil fragment (nANGPTL4). In recent years, it has been reported that cANGPTL4 participates in cancer progression by elevating adenylate energy charge or activating the ERK pathway (Baba et al. 2017; Teo et al. 2017; Zhu et al. 2016). In fact, high ANGPTL4 expression is

associated with a poor prognosis in some solid cancers, such as gastric, lung, and breast cancer (Baba et al. 2017; Cai et al. 2020; Chen et al. 2018; Zhu et al. 2016). In colorectal cancer (CRC), one report using immunostaining of human CRC specimens showed that postoperative distant metastasis is significantly correlated with high ANGPTL4 expression (Nakayama et al. 2011). However, the detailed mechanism by which ANGPTL4 contributes to CRC progression has not been fully studied.

Physiologically, ANGPTL4 is known to be associated with glucose metabolism and lipid metabolism, and its overexpression has been reported to cause impaired glucose metabolism or hyperlipidaemia (Barchetta et al. 2020; Yoshida et al. 2002). Both tissue expression and serum concentration could be useful markers in clinical practice. In fact, serum ANGPTL4 values of diabetes patients are reported to be significantly higher than those of nondiabetic patients (McCulloch et al. 2020). As such, ANGPTL4 is strongly related to glucose metabolism, although little is known about its contribution to cancer metabolism.

Glucose metabolism is essential for cancer cell proliferation. Enhanced glucose metabolism contributes to the progression of various cancers (Hanahan et al. 2011). Some of the glucose transporter (GLUT) subtypes have been shown to be important for cancer progression and have been demonstrated to be prognostic factors (Szablewski et al. 2013). Especially for CRCs, high expression of GLUT1 or GLUT3 has been reported as a poor prognostic factor (Feng et al. 2017; Kim et al. 2019; Yang et al. 2017). Moreover, it has recently been shown that GLUT1 expression in cancer cells is related to the mutation status of *KRAS*, a major driver gene involved in CRC progression (Yun et al. 2009; Wan et al. 2019).

We herein showed that ANGPTL4 expression in CRCs is associated with the expression of some GLUTs and thereby contributes to promoting glucose metabolism. Our findings will lead to a better understanding of the role of ANGPTL4 in cancer progression and indicate a new potential target for cancer treatment.

Materials And Methods

Human samples

Patients who consecutively underwent surgical resection of CRCs at Keio University Hospital between April 2018 and December 2019 were enrolled in this study. Our inclusion criteria were as follows: pathologically proven primary CRC, preoperative positron emission tomography/computed tomography (PET/CT), and consent to undergo comprehensive genomic testing. According to these criteria, 84 patients were included. The ethics committee of Keio University Hospital approved the study, which was conducted in accordance with the Helsinki Declaration of 1996 (approval number: 20150051).

Immunohistochemical staining

The tissues were embedded in paraffin. The paraffin blocks were cut into 3- μ m thick sagittal sections and subjected to immunohistochemical (IHC) staining, and the expression of ANGPTL4, GLUT1, GLUT3,

phosphorylated PI3K (pPI3K), phosphorylated AKT (pAKT), and CD31 was evaluated. Each section was deparaffinized in xylene and soaked for 30 min at room temperature in 0.3% H₂O₂ methanol to block endogenous peroxidase. Antigen retrieval was performed using 10 mM TE buffer (pH 9.0) in an autoclave at 120°C for 10 min. The sections were blocked in 2% bovine serum albumin (BSA) for 1 hour at room temperature. The primary antibodies against CD31 (ab28364, Abcam, Cam-bridge, UK) were diluted 1:100, ANGPTL4 (#18374, Proteintech Group, Chicago, IL, USA), GLUT3 (#20403, Proteintech Group, Chicago, IL, USA), pPI3K (#4228, Cell Signaling Technology, Danvers, MA, USA), and pAKT (#4060, Cell Signaling Technology, Danvers, MA, USA) were diluted 1:200, and GLUT1 (#21829, Proteintech Group, Chicago, IL, USA) was diluted 1:1000 and applied overnight at 4°C. The primary antibody was visualized using goat anti-rabbit immunoglobulin and the EnVision+ System- HRP Labelled Polymer (Dako North America Inc., Carpinteria, CA, USA), which was applied for 3 min at room temperature. The slides were counterstained with haematoxylin.

ANGPTL4 expression in human samples was evaluated using the Allred score, which is one of the most promising semiquantitative composite scoring systems (Yunokawa et al. 2017). In brief, the proportion and intensity scores were added to obtain a total score ranging from 0 to 8 (Allred et al. 1998). In this study, we randomly selected three fields for each slide and adopted the median scores. We defined an Allred score ≥ 6 as high expression of ANGPTL4.

Next-generation sequencing

In this study, we used an in-house next-generation sequencing (NGS) test that has been previously reported (Hayashi et al. 2020; Saotome et al. 2020). Briefly, genomic DNA was extracted from 10- μ m-thick formalin-fixed, paraffin-embedded (FFPE) tissue sections of tumour specimens, and then targeted amplicon exome sequencing was performed using a 160 cancer-related gene panel as previously described (Hayashi et al. 2020; Saotome et al. 2020). The targeted regions of all 160 genes were specifically enriched using oligonucleotide probes. The enriched libraries were sequenced with a paired-end (150 bp $\times$ 2) sequencing method using the NextSeq sequencing platform (Illumina, San Diego, CA, USA), resulting in a mean depth of 500. The sequencing data were analysed using the GenomeJack bioinformatics pipeline (Mitsubishi Space Software Co., Ltd., Tokyo, Japan) (<http://genomejack.net/>) as previously described (Nakamura et al. 2021). In this study, an actionable gene alteration was defined as a genomic variation that has an established biological role in cancer.

Cell culture

The human colorectal adenocarcinoma cell line HCT116 (CCL-247, ATCC, Manassas, VA, USA) was maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% (v/v) foetal bovine serum and 10,000 units/mL penicillin, 10,000 μ g/mL streptomycin, and 25 μ g/mL Gibco amphotericin B (Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured at 37°C with 5% CO₂.

Western blot analysis

Total cell lysates were extracted using lysis buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 1% sodium deoxycholate, 1 mM beta-glycerophosphate, and 1 µg/ml leupeptin; Cell Signaling Technology, Inc., Danvers, MA, USA). The protein concentration of cell lysates was determined using a Bio-Rad Quick Start Bradford (Bio-Rad, Hercules, CA, USA), and 20 µg of each lysate was resolved on a Ready Gel (Bio-Rad) and transferred to an Immuno-Blot polyvinylidene fluoride membrane (Bio-Rad). The membrane was blocked with 2.5% BSA for 1 hour at room temperature. The primary antibody against ANGPTL4 (#18374, Proteintech Group, Chicago, IL, USA) was diluted 1:750, GLUT1 (#21829, Proteintech Group, Chicago, IL, USA), GLUT3 (#20403, Proteintech Group, Chicago, IL, USA), AKT (#4691, Cell Signaling Technology, Danvers, MA, USA), PI3K (#4257, Cell Signaling Technology, Danvers, MA, USA), pPI3K (#4228, Cell Signaling Technology, Danvers, MA, USA), and β-actin (ab8227, Abcam, Cambridgeshire, UK) were diluted 1:1,000, and pAKT (#4060, Cell Signaling Technology, Danvers, MA, USA) was diluted 1:2000 and applied overnight at 4°C. The membranes were incubated for 60 min with a horseradish peroxidase-conjugated rabbit IgG (H+L) secondary antibody diluted 1:5000 (#65-6120, Thermo Fisher Scientific, Waltham, MA, USA). Finally, the targeted proteins were labelled with the Immobilon® Forte Western HRP substrate (Merck Millipore Co., Darmstadt, Germany) and immediately detected using a Fusion FX system (VILVER, Marne-la-Vallée, France). The protein bands were quantified by densitometry according to the manufacturer's instructions with AlphaView software (ProteinSimple, San Jose, CA, USA), and ANGPTL4, GLUT1, GLUT3, AKT, pAKT, PI3K, and pPI3K expression was evaluated relative to that of β-actin.

Generation of ANGPTL4 knock-out cell line by CRISPR/Cas9

To generate the ANGPTL4 knockout HCT116 cell line, we designed a pair of single guide RNA (sgRNA) sequences (F: 5'-CACCGGAGATGAATGTCCTGGCGCA-3' R: 5'-AAACTGCGCCAGGACATTCATCTCC-3'). Similarly, we designed one pair of sgRNA sequences (F: 5'-CACCGAAAAAGTCCGCGATTACGTC-3' R: AAACGACGTAATCGCGGACTTTTTC) for scrambling as a negative control by FASMAC (Kanagawa, Japan). These sgRNAs were cloned into a lentiCRISPR v2 vector obtained from Addgene (plasmid #52961). ANGPTL4 knockout HCT116 cells (KO) and negative control HCT116 cells (NC) were established by lentiviral infection.

Animal

We used BALB/c nude female mice, which were purchased from CLEA Japan (Tokyo, Japan). The mice were allowed to acclimate for at least 7 days before use and were 8 weeks old at the start of the experimental protocol. The mice were housed in a controlled environment in the laboratory at the Keio University School of Medicine under standard temperature and light and dark cycles. Ethics approval for the present study was provided by the Ethics Committee at the Laboratory Animal Care and Use Committee at Keio University School of Medicine (approval number: 20062), and the study was performed in accordance with the Care and Use of Laboratory Animals (NIH).

Subcutaneous xenograft mouse model

The mice were anaesthetized with 2.0% isoflurane, and 1×10^7 NC or KO in a total volume of 100 μ l phosphate-buffered saline (PBS) and 100 μ l Matrigel (#35423, Corning, Corning, NY, USA) were injected subcutaneously using a 26-gauge needle (n = 5). All mice were sacrificed two weeks after subcutaneous injection, and tumour weights between the two groups were compared.

In silico analysis

The correlation between *ANGPTL4* and *SLC2A* mRNA expression in CRC was evaluated by using data from The Cancer Genome Atlas (TCGA) database. Coexpression analysis was performed, and the results were downloaded from the cBioPortal for Cancer Genomics (<http://www.cbioportal.org/>).

Positron emission tomography/computed tomography

All patients fasted for at least 4 hours prior to PET/CT imaging. Biograph mCT (Siemens Medical Solutions, Knoxville, TN) was used for scanning. Patients were administered 3.7 MBq/kg fluorodeoxyglucose (FDG) and received routine PET/CT 1 hour after administration. For the metabolic parameters, we measured the maximum standardized uptake value (SUVmax), the mean standardized uptake value (SUVmean), metabolic tumour volume (MTV), and total lesion glycolysis (TLG). MTV was defined as the sum of the primary tumour metabolic volumes using an SUVmax of 2.5 as the threshold, and TLG was defined as (SUVmean) $\times$ (MTV). All of these parameters were measured and calculated using Advantage Workstation software (GE Healthcare, Chicago, IL, USA).

Statistical analysis

All results are expressed as the median value (range). All statistical analyses were performed using Stata software (Stata Corporation, College Station, TX, USA). *P* values < 0.05 were considered to indicate statistical significance. Nonparametric Mann-Whitney U tests and χ^2 tests were used to compare 2 groups as appropriate.

Results

High ANGPTL4 expression is associated with advanced pathological factors and *KRAS* mutation

As shown in Fig. 1, tumour samples were classified into ANGPTL4 high or low expression groups by IHC staining. Of the 84 samples, 49 (58%) samples were classified as ANGPTL4 low, and 35 (42%) samples were classified as ANGPTL4 high. The pathological characteristics of the two classified groups are detailed in Table 1. The comparison of Union for International Cancer Control (UICC) tumour-node-metastasis (TNM) classification showed that high ANGPTL4 expression was significantly associated with advanced stage. Especially for tumour invasion, a significantly higher number of T3/4 cases was found in the ANGPTL4-high group than in the ANGPTL4-low group (94.3% vs. 57.1%, *P* < 0.001). Both lymph node metastasis and distant metastasis tended to be more advanced in the ANGPTL4-high group, but the difference was not statistically significant. Of note, there was also a significant difference in perineural invasion (42.9% vs. 22.4%, *P* = 0.046). There were no significant differences in tumour location, histology, lymphatic invasion or venous invasion between the groups.

We next aimed to identify any genetic features of ANGPTL4-high tumours by using NGS data. The comparison of the mutation status of extracted major driver genes is shown in Table 2. There were significantly more *KRAS* mutations in the ANGPTL4-high group than in the ANGPTL4-low group (54.3% vs. 22.4%, $P = 0.003$). Similarly, there were significantly more *FBXW7* mutations in the ANGPTL4-high group (17.1% vs. 4.1%, $P = 0.044$). On the other hand, there were no significant differences in *TP53*, *APC*, *SMAD4*, *PIC3CA*, and *BRAF* mutations between the two groups.

ANGPTL4 knock-out attenuates tumour progression

As more advanced tumours were found in ANGPTL4-high cases in terms of tumour invasion and perineural invasion, we hypothesized that ANGPTL4 positively contributes to tumour progression. To address this hypothesis, we generated an ANGPTL4 knockout cell line by using CRISPR/Cas9 gene editing (Fig. 2A). We used the HCT116 CRC cell line, which expresses significantly higher levels of *ANGPTL4* than other cell lines (Fig.S1). Of note, HCT116 is also known as a *KRAS* mutant cell line, which is considered to be better characterizing human ANGPTL4-high tumours (Buck et al. 2007).

When the ANGPTL4 negative control cell line (ANGPTL4-NC) and knockout cell line (ANGPTL4-KO) were xenografted, the sizes of tumours generated from ANGPTL4-KO were significantly reduced compared with those of ANGPTL4-NC (Fig. 2B, C). The mean total volumes of the tumours showed an approximately 50% reduction (ANGPTL4-NC: 430 mg v.s. ANGPTL4-KO: 196 mg, $P = 0.032$) (Fig. 2D). Notably, ANGPTL4 expression in ANGPTL4-KO tumours was diminished (Fig. 2E). Evaluation of tumour angiogenesis by CD31 staining showed that ANGPTL4-KO tumours are more hypovascular than ANGPTL4-NC tumours.

ANGPTL4 contributes to tumour progression by activating the PI3K-AKT pathway

ANGPTL4 is reported to activate the PI3K-AKT pathway in gastric cancer (Baba et al. 2017). To explore more details of the role of ANGPTL4 in CRC progression, we further analysed the cell lines we generated. As shown in Fig. 3A, phosphorylation of AKT was significantly downregulated in ANGPTL4-KO cells, whereas phosphorylation of PI3K was not significant. We next validated these findings by immunohistochemical staining of xenograft tumours. As shown in Fig. 3B, phospho-AKT expression was markedly reduced in ANGPTL4-KO tumours, whereas phospho-PI3K expression did not change. Taken together, these findings suggest that ANGPTL4 promotes tumour progression by activating the PI3K-AKT pathway via AKT phosphorylation.

ANGPTL4 regulates GLUT expression

According to the result that *KRAS* mutations were more common in the ANGPTL4-high group and the fact that ANGPTL4 physiologically contributes to glucose metabolism, we next aimed to investigate the correlation between ANGPTL4 and GLUT expression. As shown in Fig. 4A, analysis of the TCGA dataset showed that the mRNA expression of *ANGPTL4* was positively correlated with that of *SLC2A1* and *SLC2A3* (*SLC2A1*: $r = 0.23$, $P < 0.001$, *SLC2A3*: $r = 0.49$, $P < 0.001$). We next validated these results by

evaluating the expression of GLUT1 and GLUT3 in our human samples. As shown in Fig. 4B, ANGPTL4-high tumours showed higher expression of both GLUT1 and GLUT3 than ANGPTL4-low tumours. The results from TCGA datasets regarding other *SLC2A* subtypes are shown in Fig.S2. Of note, there were also significant positive correlations between *ANGPTL4* and *SLC2A5*, *SLC2A6*, and *SLC2A14*.

To investigate whether ANGPTL4 expression directly impacts GLUT1 and GLUT3 expression, we utilized a knockout cell line. Western blot analysis showed that the expression of both GLUT1 and GLUT3 was significantly suppressed in the ANGPTL4-KO cell line compared with the ANGPTL4-NC cell line (Fig. 4C). Moreover, immunostaining of GLUT1 and GLUT3 in xenografted tumours showed a reduction in these GLUTs in ANGPTL4-KO tumours (Fig. 4D). These results indicate that ANGPTL4 expression is strongly correlated with several GLUTs and directly influences the expression of GLUT1 and GLUT3.

ANGPTL4 expression is correlated with FDG uptake in human CRCs

To validate our in vitro and in vivo results, we next aimed to evaluate actual glucose uptake in CRC patients. We investigated tumour FDG uptake measured by PET/CT. Fig. 5 shows the comparison of a variety of metabolic parameters between ANGPTL4-high and ANGPTL4-low tumours. The details are as follows: SUVmax (ANGPTL4 high vs. low = 13.28 vs. 7.56, $P < 0.001$), SUVmean (ANGPTL4 high vs. low = 5.33 vs. 3.85, $P < 0.001$), MTV (ANGPTL4 high vs. low = 39.32 ml vs. 11.60 ml, $P < 0.001$), and TLG (ANGPTL4 high vs. low = 214.25 ml vs. 44.67 ml, $P < 0.001$). All parameters were significantly higher in the ANGPTL4-high group, strongly suggesting that ANGPTL4 expression is correlated with glucose metabolism in human CRCs.

Discussion

In the current study, we found that ANGPTL4 expression is related to the progression of CRC, as suggested by the increased levels of tumour invasion and perineural invasion in ANGPTL4-high tumours. Genetic exploration revealed that a higher rate of *KRAS* mutation was found in ANGPTL4-high tumours and was considered to contribute to cancer progression. Furthermore, the expression of several GLUT subtypes, such as GLUT1 and GLUT3, was positively correlated with ANGPTL4 expression, suggesting a pivotal role of ANGPTL4 in glucose metabolism. In fact, all the parameters of tumour FDG uptake measured by PET/CT, which reflect glucose metabolism, were significantly higher in ANGPTL4-high tumours.

In some solid cancers, a relationship between ANGPTL4 expression and cancer progression has been suggested. In scirrhous gastric cancer, ANGPTL4 is uniquely induced by hypoxia, and its expression is essential for tumour growth and resistance to anoikis through the activation of the pro-survival FAK/Src/PI3K-AKT/ERK pathway (Baba et al. 2017). The relationship of ANGPTL4 with the ERK pathway was also demonstrated in a non-small-cell lung cancer study; the study showed that ANGPTL4 contributes to epithelial mesenchymal transition (EMT) induction via the ERK pathway (Zhu et al. 2016). In triple-negative breast cancer, the upregulation of ANGPTL4 reduces the mRNA levels of extracellular matrix (ECM)-related genes, indicating that ANGPTL4 contributes to cancer progression by suppressing

ECM-related proteins (Cai et al. 2020). Our study of CRC is consistent with these past reports; our study showed ANGPTL4 as a cancer progression factor and provides a novel finding that ANGPTL4 impacts glucose metabolism in cancers.

The PI3K-AKT signalling pathway plays an important role in diverse cellular functions, such as cell proliferation, differentiation, angiogenesis and autophagy (Noorolyai et al. 2019). Among the genes related to the pathway, *RAS* genes, including *KRAS*, were the first confirmed human proto-oncogenes that can transform into oncogenes (Luo et al. 2020). In CRC, *KRAS* mutation has been documented as a negative prognostic factor for both overall survival and recurrence-free survival and is now an important biomarker in clinical practice (Tsilimigars et al. 2018; Wan et al. 2019). According to our NGS data, high ANGPTL4 expression is significantly associated with *KRAS* mutations. Taken together, these findings indicate that a highly activated PI3K-AKT pathway induced by both ANGPTL4 overexpression and *KRAS* mutation results in tumour progression. This is compatible with the result of high GLUT expression in ANGPTL4-high tumours since the PI3K-AKT pathway also activates GLUT expression (Papa et al. 2019).

We revealed that ANGPTL4 expression was strongly associated with GLUT1 and GLUT3 expression. Among the several subtypes of GLUTs, these two subtypes are known to be correlated with the progression of CRC, which is consistent with our results (Feng et al. 2017; Kim et al. 2019; Yang et al. 2017). According to TCGA database analysis in the current study, other GLUT coding genes, such as *SLC2A5*, *SLC2A6*, and *SLC2A14*, were also suggested to correlate with ANGPTL4 expression. As little is known about these GLUT proteins in cancers, further study is expected to clarify their roles.

To validate the relationship between ANGPTL4 expression and glucose metabolism from another perspective, we evaluated some measurement values from PET/CT. FDG uptake in tumours is based on the elevated metabolic activity of cancer cells and reflects increased glucose uptake (Watanabe et al. 2020). In fact, positive relationships between FDG uptake and GLUT1 expression in CRC and GLUT3 expression in malignant melanoma have been observed (Gu et al. 2006; Park et al. 2012). Thus, our results strongly suggest that glucose metabolism is highly activated in ANGPTL4-high tumours.

Since knockout of ANGPTL4 in cancer cells attenuated tumour progression in this study, ANGPTL4 is expected to be a novel target for cancer treatment. In fact, a neutralizing antibody against ANGPTL4 has been shown to be effective as a breast cancer treatment in a mouse xenograft model (Kolb et al. 2019). However, there are issues to be elucidated, such as adverse effects when physiological metabolism is suppressed by treatment. In recent years, genetic screening has become widely used as a practice of precision medicine, and the measurement of biomarkers has become easier. Therefore, by evaluating PI3K-AKT pathway activation status and *KRAS* mutation status as biomarkers, we may be able to select patients who are expected to be highly responsive to anti-ANGPTL4 treatment. Similarly, excluding patients who are expected to have adverse effects by genetic screening may be feasible in the near future.

In conclusion, ANGPTL4 expression correlates with glucose metabolism activity and is associated with CRC progression. In particular, GLUT1 and GLUT3 are considered to be regulated by ANGPTL4.

Declarations

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Author Contributions: SM and RS designed and executed the study and wrote the paper. JY and KH executed the in vitro study. MO executed the NGS analysis. LL designed the study. SM, KS, KO, ON, and YK executed the clinicopathological analysis and revised the paper critically.

Data availability: The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Ethics approval: This study was approved by the Ethics Committees of Keio University (ID 20150051), in accordance with the Helsinki Declaration.

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Consent for publication: Written informed consent was obtained from the patients for publication.

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Tables

Table 1 **Pathological characteristics of the two classified groups**

		ANGPTL4 expression		
		Low (n =49)	High (n = 35)	<i>P</i> value
Tumour location	rectum	11 (22.4%)	6 (17.1%)	0.551
T*	3,4	28 (57.1%)	33 (94.3%)	< 0.001
N*	≥ 1	14 (28.6%)	14 (40.0%)	0.237
M*	+	4 (8.1%)	5 (14.3%)	0.371
Stage	III, IV	15 (30.6%)	15 (42.9%)	0.248
Histology	por, sig, muc	5 (10.2%)	4 (11.4%)	0.858
Ly	+	26 (53.1%)	17 (48.6%)	0.685
V	+	32 (65.3%)	23 (65.7%)	0.969
Pn	+	11 (22.4%)	15 (42.9%)	0.046

*UICC tumour-node-metastasis (TNM) classification.

Values are presented as the number of patients (%).

ANGPTL4, angiopoietin-like 4; Ly, lymphatic invasion; V, venous invasion; Pn, perineural invasion; por, poorly differentiated adenocarcinoma; sig, signet-ring cell carcinoma; muc, mucinous adenocarcinoma.

Table 2 Comparison of the mutation status of extracted major driver genes

Mutated genes	ANGPTL4 expression		P value
	Low (n = 49)	High (n = 35)	
<i>KRAS</i>	11 (22.4%)	19 (54.3%)	0.003
<i>TP53</i>	29 (59.2%)	19 (54.3%)	0.655
<i>APC</i>	29 (59.2%)	22 (62.9%)	0.734
<i>FBXW7</i>	2 (4.1%)	6 (17.1%)	0.044
<i>SMAD4</i>	7 (14.3%)	6 (17.1%)	0.721
<i>PIK3CA</i>	3 (6.1%)	6 (17.1%)	0.107
<i>BRAF</i>	10 (20.4%)	6 (17.1%)	0.707

Values are presented as the number of patients (%).

ANGPTL4, angiopoietin-like 4.

Figures

Figure 1

ANGPTL4 expression in human CRC.

Representative images of low (A) and high (B) ANGPTL4 expression in human CRC specimens.

Figure 2

ANGTPL4 knockout in HCT116 cells reduced tumour size in a xenograft model.

- A) Western blot analysis comparing the protein expression of ANGPTL4 between ANGPTL4-negative control (NC) and knockout (KO) HCT116 cells. β -actin was analysed as a loading control.
- B) Images of the mice xenografted with either NC or KO.
- C) Representative images of the tumours generated from NC and KO mice.
- D) Comparison of tumour weight between NC and KO mice (n=5 each). Data are represented as the mean $\pm$ SD. *P < 0.05
- E, F) Representative images of ANGPTL4 (E) and CD31 (F) expression in the tumours generated from NC and KO mice.

Figure 3

ANGPTL4 regulates the PI3K-AKT pathway by promoting AKT phosphorylation.

- A) Western blot analysis comparing the protein expression of PI3K, pPI3K, AKT and pAKT between ANGPTL4-negative control (NC) and ANGPTL4-knockout (KO) cells. β -actin was analysed as a loading control.
- B) Representative images of pPI3K and pAKT expression in the tumours generated from NC and KO mice.

Figure 4

ANGPTL4 expression regulates GLUT proteins in CRC.

- A) Correlation of mRNA expression between *ANGPTL4* and *SLC2A1* and *SLC2A3* according to TCGA database analysis.
- B) Representative images of IHC staining of GLUT1 and GLUT3 in ANGPTL4-high and ANGPTL4-low human CRCs.
- C) Western blot analysis comparing the protein expression of GLUT1 and GLUT3 between ANGPTL4-negative control (NC) and ANGPTL4-knockout (KO) cells. β -actin was analysed as a loading control.
- D) Representative images of GLUT1 and GLUT3 expression in the tumours generated from NC and KO mice.

Figure 5

FDG uptake is associated with ANGPTL4 expression in human CRCs.

Graphs show the comparison of A) SUVmax, B) SUVmean, C) MTV, and D) TLG between ANGPTL4-high and ANGPTL4-low tumours according to the PET/CT measurements. Black horizontal lines show the median values. ***P < 0.001

SUVmax, maximum standardized uptake value; SUVmean, mean standardized uptake value; MTV, metabolic tumour volume; TLG, total lesion glycosis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Fig.S1.pdf](#)
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