의학박사학위논문

대장암에서 FLII 및 THRAP3 발현 및 임상병리학적 중요성

Expression and Clinicopathologic Significance of FLII and THRAP3 in Colorectal Cancer

울산대학교 대학원

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이 논문을 의학박사학위 논문으로 제출함.

2022년 2월

울산대학교 대학원

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감사의 글

되돌아 보면 무엇 하나 온전히 제 힘으로 이룬 것은 없는 듯합니다. 어려서는 부모의 도움으로 살아왔고, 함께 한 누이들 덕에 심심하지 않았고 넉넉하지 않아도 부족함 모르게 지냈습니다.

아무 것도 모르던 인턴으로 아산병원에서 사회 초년을 시작하여 대장항문외과 전문의가 될 때까지 많은 가르침을 주셨던 교수님들께 박사학위 지도를 받을 수 있어 영광이었습니다. 외과의사로서, 저의 롤 모델이시고 대장항문외과 전문의로 역할을 다할 수 있도록 만들어 주신 유창식 교수님께 항상 감사드릴 따름입니다. 새로운 연구 분야에 대한 안목을 제시해 주시고 물심으로 도움 주신 명승재 교수님께 깊은 감사의 뜻을 전합니다. 수련의 시간 동안 존경하고 따르고 싶었던 육정환, 임석병, 김희철 교수님께 다시 한번 배움의 기회를 얻을 수 있어 감사하였습니다. 유전자 분석과 연구에 대한 여러 조언해주신 UNIST 최창현 교수님과 연구실 구성원께 감사의 마음을 전합니다.

제 인생의 가장 큰 선물이며 제가 원하는 외과의사의 삶을 항상 후원해 주고 지지해 주는 인생의 동반자인 아내 미리와 딸 혜령, 혜린과 이 기쁨을 함께 나누며 감사를 전합니다.

대학원 박사과정은 마침표를 찍었으나, 학자로서의 삶은 이제 시작이라 생각합니다. 항상 최선을 다해 부단히 노력하여 임상의로서 어려움에 처한 이들을 위해 공헌함은 물론, 학자 그리고 교육자로서 계속해서 성장하고 발전하도록 노력하겠습니다.

감사드립니다.

2022년 2월 정성민 올림.

국문요약

목적: 본 연구는 대장암 환자에서 Flightless-1(FLII) 및 THRAP3(갑상선 호르몬 수용체 관련 단백질 3) 유전자 발현의 프로파일을 조사하고 두 유전자 발현과 임상병리학적 요인 간의 관계를 확인하는 것을 목적으로 한다.

대상 및 방법: 원발성 대장암으로 진단되어 수술을 받은 총 449명의 환자를 대상으로 하였다. 암 및 정상 조직에서 FLII 및 THRAP3의 발현을 50명 환자의 동결 조직을 이용하여 평가하였고, 이 중 10명은 염증성장질환 관련 대장암 환자이고 40명은 산발성 대장암 환자이다. 이와 더불어 포르말린 고정 파라핀 포매 조직에서 THRAP3의 발현을 산발성 대장암 환자 399명에서 분석하였다. 대장암 조직에서 FLII 및 THRAP3 유전자 발현 수준과 생존 데이터를 포함한 임상병리학적 매개변수 사이의 관계를 조사하였다.

결과: FLII 단백질 발현은 정상 조직에 비해 대장암 조직에서 하향 조절되었고($p<0.001$), 산발성 대장암 그룹에 비해 염증성장질환 관련 대장암 그룹에서 정상과 대장암 조직 모두에서 그 발현이 증가하였다(각각 $p=0.019$ 및 $p=0.013$). THRAP3 단백질 및 mRNA 발현의 수준은 정상 조직에 비해 대장암 조직에서 상향 조절되었다(각각

p<0.001 및 p=0.003). 총 399개의 포르말린 고정 파라핀 포매 조직 샘플 중 29.6%에서 THRAP3에 대해 높은 양성 염색 강도를 나타냈다. THRAP3의 높은 발현은 직장암, 고분화, 면역조직화학염색에서 CEA의 낮은 발현과 관련이 있었다.

결론: 본 연구의 결과는 FLII 및 THRAP3의 발현이 대장암의 발달과 관련이 있음을 시사하며, 두 유전자는 대장암의 예방 및 치료를 위한 약물 개발의 표적으로써 가치를 가진다고 할 수 있다.

주제어: 대장암, FLII, THRAP3, 유전자 발현

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LIST OF ABBREVIATIONS

Adj CTx, adjuvant chemotheraphy

BMI, body mass index

ChREBP, carbohydrate response element binding protein

CI, confidence interval

CRC, colorectal cancer

DM, diabetes mellitus

FLII, Flightless-1

FFPE, formalin-fixed paraffin-embedded

HR, hazard ratio

IBD, inflammatory bowel disease

IHC, immunohistochemistry

IL-6, interleukin-6

MD, moderate differentiation

NLR, neutrophil-to-lymphocyte ratio

OR, odds ratio

PD, poor differentiation

PPARγ, Peroxisome proliferator-activated receptor-γ

PreOp CEA, preoperative carcinoembryonic antigen

THRAP3, thyroid hormone receptor associated protein 3

Tis, carcinoma in situ

TNF-α, tumor necrosis factor-α

WD, well differentiation

MSI, microsatellite instability

INTRODUCTION

Although the incidence of colorectal cancer (CRC) can be reduced through proper screening methods that can detect precancerous lesions, it remains the third most commonly diagnosed cancer and is the second most common cause of cancer-related deaths worldwide [1-3]. In 2018, the Republic of Korea was ranked first in the world for CRC cases, and the country has the highest rate of increase for this cancer worldwide [4]. Despite recent advances in screening programs and CRC patient management, a significant proportion of the total diagnostic cases are diagnosed as stage IV, with a poor prognosis [5]. Of particular worry is the increased incidence of CRC in adults under the age of 50 [6]. Therefore, the continuing socioeconomic burden of colorectal cancer underscores the important need to consider new prevention strategies beyond current screening and therapeutic strategies.

Genes associated with CRC are well known, and several pathways involved in carcinogenesis have been identified [7, 8]. In addition to genetic factors, environmental factors can increase the chances of developing CRC through intricate metabolic and inflammatory mechanisms [9]. Management of these risk factors is not only necessary to reduce the incidence of CRC, but also to understand the signaling pathways involved in the development of CRC, which can be a target for anti-cancer therapies. The association between CRC and inflammation, which promotes all stages of tumorigenesis by inducing DNA damage and mutation [10], is well known in colitis-related CRC [11-13]. Colitis-associated CRC patients are affected at a younger age than sporadic CRC patients and are likely the cause of the long-standing chronic inflammatory condition inherent in inflammatory bowel disease (IBD). Metabolic disorders such as obesity and diabetes

mellitus (DM) are also associated with an increased risk of developing colon cancer [14, 15]. These environmental factors are also likely to have a greater effect on early onset CRC [6]. Many types of tumors show a high rate of aerobic glycolysis, altered fatty acid synthesis, and nucleotide biosynthesis [16]. Metabolic disorders show a very strong association with the proinflammatory state, leading to plasma concentration of inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), being increased in obesity and DM [17, 18].

Peroxisome proliferator-activated receptor-γ (PPARγ) and carbohydrate response element binding protein (ChREBP) have a strong ability to regulate glycolysis and lipogenesis, and their functions are regulated by Flightless-1 (FLII) and/or thyroid hormone receptor associated protein 3 (THRAP3). In our previous study, we found that FLII blocks the interaction between PPARγ and retinoid X receptor α (RXRα) and prevents PPARγ receptor occupancy at the promoter of the target gene [19]. FLII also interacts with ChREBP to downregulate ChREBP-mediated transcription in CRC and hepatocellular carcinoma cells [20]. Mutations in the FLII gene are associated with high expression of enzyme genes and increased insulin resistance [21]. We also reported that THRAP3 can directly interact with the phosphorylation of PPARγ in adipose tissue and stimulate insulin resistance via diabetic gene programming [22].

CRC characteristically has a sequential carcinogenesis process involving several pathways including the chromosomal instability, CpG island methylator phenotype, microsatellite instability, and serrated pathways [8, 23]. In addition to these pathways, carcinogens-induced mechanisms may play a crucial role in cancer initiation [10]. Human carcinogens, defined as chemicals that increase the incidence of cancer and shorten the time of its development [24], include a wide range of substances such as alcohol, tobacco smoking, nitrosamines, aflatoxins,

polycyclic aromatics, physical stressors such as UV, and various kinds of infections [25]. Carcinogens can initiate and stimulate cancer progression through various mechanisms, including DNA damage, inflammation, oxidative stress, cytotoxicity, acute or chronic injury, and apoptosis. The main mechanism for pro-tumorigenic activity due to carcinogens is a genotoxic process that causes DNA damage and mutations in synergy with nongenotoxic processes [11].

Two cellular strategies to combat DNA damage as a result of genotoxicity by carcinogens are either to repair or tolerate DNA damage or to remove cells that harbor DNA damage from the population by death. DNA repair is critical for cancer prevention, as DNA repair prevents genetic mutations in normal cells. The five well-known DNA repair pathways that are critical for cancer prevention caused by genetic mutations include mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ), all of which are active throughout different stages of the cell cycle [26]. RNA processing was recently found to affect DNA damage response (DDR) directly involved in traditional DNA damage repair pathways by interacting with DNA repair proteins, mediated by BRCA1 [27] and BRCA2 [28].

The RNA binding proteins (RNAbps) THRAP3 and BCLAF1 play critical roles in maintaining DNA genomic stability by regulating transcription, mRNA splicing and export of DNA repair proteins in DDR [29]. The DNA damage-induced BRCA1 protein complex increases sensitivity to DNA damage and causes defective DNA repair. A high incidence of somatic mutations in the BRCA1/BCLAF1 mRNA splicing complex has been reported in various cancer types [27]. Deficiencies of BCLAF1 and THRAP3 lead to decreased mRNA splicing, downregulation of export of BCLAF1/THRAP3 target genes, and loss of encoded protein compared to the mild

effects of THRAP3 or BCLAF1 deficiency [29]. THRAP3 deficiency has been observed in several cancer types, suggesting that THRAP3 may function as a tumor suppressor gene through its role in DDR [30-32].

Unrepaired DNA damage ultimately leads to detrimental consequences in the form of chromosomal changes, gene mutations and malignant transformations. Apoptosis is a prominent route of cell inactivation following the induction of DNA damage beyond repair. Apoptosis is executed via three central apoptotic pathways: the extrinsic death receptor, intrinsic mitochondrial, and endoplasmic reticulum (ER) stress pathways. Some conditions, such as a lack of oxygen, changes in pH, low levels of glucose, and the disruption of Ca^{2+} homeostasis can stress the ER, causing the ER to lose its ability to fold proteins correctly. Unfolded proteins in the ER trigger a stress response called the unfolded protein response (UPR) by activating three central ER stress sensors, inositol requiring kinase-1 (IRE1), protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6), which are normally kept in an inactive state. These central sensor proteins are bound to the ER-resident chaperone glucose-regulated protein-78 (GRP78/BiP). Under ER stress, these proteins are released from GRP78/BiP, initiating downstream cascades [33].

FLII is a member of the gelsolin superfamily with an N-terminal leucine-rich repeat domain and a C-terminal gelsolin-like domain [34]. It regulates cell migration, wound healing, and inflammation [35, 36]. The gelsolin family is a major group of Ca^{2+}-dependent actin binding proteins [37]. However, what distinguishes FLII from other members of the gelsolin family is that its actin-binding and actin-serving activities are Ca^{2+}-independent [38]. In a recent study, FLII was found to suppress UPR signaling and apoptosis in the ER stress pathway by

maintaining intracellular Ca^{2+} homeostasis via ryanodine receptors (RyRs). In addition, intracellular Ca^{2+} homeostasis was impaired, and ER stress-induced apoptosis was promoted in FLII-knockdown cells using a CRC cell line [39].

The study of proteins that play an important role in the development and progression of tumors is necessary to develop biomarkers that predict cancer prognosis and to identify new targets for anticancer therapy. FLII and THRAP3 are involved in DNA damage repair and apoptosis, which are important steps in carcinogenesis as well as metabolic disease and inflammation, both of which are strong environmental factors in the development of CRC. Studying the corresponding genes in colitis-related CRC and sporadic CRC, which are considered CRCs that are highly susceptible to environmental factors, is an appropriate research design to understand the molecular and cellular pathways involved in CRC development. In this study, we aimed to investigate the level of FLII and THRAP3 expression in CRC tissues to identify the relationship of that expression with clinicopathological factors and with the prognosis of patients with CRC and ultimately determine its role as a new preventive and therapeutic target.

MATERIALS AND METHODS

Study subjects

A total of 449 study subjects comprised patients who were diagnosed with primary CRC that underwent resection for primary CRC at the Asan Medical Center (AMC). The study was conducted by dividing all subjects into two groups. In Group I, the expression of FLII and THRAP3 was compared in colitis-related cancer and sporadic cancer, and in Group II, clinical data were analyzed to confirm the clinicopathologic significance of THRAP3.

In Group I, fifty participants were selected for western blot and RNA analysis from patients who underwent surgical resection of primary CRC at the AMC between 2011 and 2017. The IBD group consisted of 10 surgically resected primary CRC cases in IBD patients (UC [n=5], CD [n=5]), and the sporadic CRC group consisted of 40 selected surgically resected primary sporadic CRC cases that were matched by age, sex, and TNM stage with the IBD group. All included tumors were pathologically confirmed as adenocarcinomas. None of the patients received preoperative chemotherapy or radiotherapy. Fifty cancer tissue samples and 50 adjacent normal tissue samples from 50 patients enrolled in this study were collected. All samples were obtained on the day of the initial surgical resection of the primary CRC with or without metastasectomy. A portion of the collected cancer tissues was preserved at -80°C, and the adjacent normal tissues were collected from sites at least 5 cm away from the tumor margins. All tissue samples in this study were collected, stored, and provided by the Asan Bio-Resource Center, Korea Biobank Network (2020-14-211). Protocols were submitted to the Institutional Review Board (IBR) for approval.

In Group2, a total of 399 patients who underwent surgical resection of primary CRC at the AMC in 2008 were investigated. The study included resected tissue samples as paraffin-embedded blocks for immunohistochemical staining (IHC) of THRAP3 that were sufficient in amount and stored under favorable conditions. This protocol was used to examine the presence of THRAP3 expression in CRC tissues and to identify the relationship with the results of western blot and RNA analysis in terms of different clinicopathological factors and the prognosis of patients with CRC.

Medical records were reviewed for clinical, radiological, laboratory, and pathological findings, as well as clinical outcomes. Clinical information included age, sex, tumor location, histology, lymphovascular invasion, perineural invasion, nodal status, and follow-up results. All clinical information and demographic characteristics of all the patients were retrieved from their inpatient and outpatient medical records using the Asan Biomedical Research Environ ment (ABLE), a deidentified clinical data warehouse [40, 41]. The study protocols were compliant with the World Medical Association Declaration of Helsinki recommendations and approved by the IRB (2020-0969) of Asan Medical Center. Patient informed consent was waived by the IRB.

Western blot analysis

Colon tissue protein lysates were prepared by homogenizing tissues with RIPA lysis buffer containing 1% sodium deoxycholate, protease inhibitor (Roche), and phosphatase inhibitor cocktail (Sigma Aldrich) on ice. The tissue suspension was vortexed vigorously and centrifuged

for 15 min at 4℃ at 15,000 rpm. The supernatant was collected and the protein concentration was measured using Protein Assay Dye Reagent Concentrate (Bio-Rad). Proteins (40 μg) were separated by SDS-PAGE and transferred to a nitrocellulose blotting membrane (GE Healthcare). The membrane was blocked with 5% skim milk and incubated with primary antibodies at 4°C overnight. The primary antibodies used were as follows: THRAP3 (Santa Cruz Biotechnology, Cat# sc-133250), FLII (Abcam, Cat# ab108594), and β-tubulin (Invitrogen, Cat# MA5-16308). The membrane was incubated with 1:10,000 diluted HRP-conjugated secondary antibody for 1 h at room temperature. Protein levels were detected using chemiluminescent HRP substrate solutions (Advansta). Image Studio Lite version 5.2 was used to measure the protein band intensities. β-Tubulin was used as the loading control. Relative THRAP3 and FLII expression was calculated as THRAP3 or FLII intensity divided by β-tubulin intensity. Relative protein expression levels were compared between non-tumor and tumor tissues of each patient.

Total RNA isolation and relative mRNA expression analysis

Total RNA was extracted from colon tissues by using easy-BLUE™ Total RNA extraction kit (iNtRON). Reverse transcription was performed from 1μg of RNA, by using MultiScribe™ Reverse Transcriptase (Invitrogen). cDNAs were used for quantitative PCR using an 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative Thrap3 mRNA expression was normalized to ACTB and calculated by using the $2^{-\Delta\Delta Ct}$ method. To examine the gene expression changes through carcinogenesis, we analyzed fold change values of Tumor versus Non-tumor tissue of each patient.

Expression of FLII and THRAP3 in public datasets

In addition to the analysis in our study subjects, we performed analysis for FLII and THRAP3 mRNA expression on a gene expression dataset from the Cancer Genome Atlas (TCGA) composed of 376 patient samples with survival information (Supplementary Table 1; https://xenabrowser.net/datapages/). Among them, FLII and THRAP3 expression data were obtained from the normal tissues of 30 patients. The median transcript levels of FLII and THRAP3 genes were used to assign the patient samples to either the high or low expression group. We also performed analysis for FLII and THRAP3 mRNA expression in two cohorts of the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI): GSE100179 and GSE17536.

Statistical analysis

Propensity scores by age, sex, and TNM stage were generated using the R package 'Match It' (The Comprehensive R Archive Network: http://cran.r-project.org) to screen the two groups of study subjects. The relationship between expression and clinical manifestation was analyzed using the chi-square test and Fisher's exact test. Spearman's rank correlation was used for concordant expression analysis, and the other data were analyzed using analysis of variance. Kaplan–Meier survival analysis was used to determine the overall survival (OS) and disease-free survival (DFS), and differences between subgroups were compared using the log-rank test.

Multivariate Cox proportional hazards regression analysis was performed when $p<0.1$ in univariate analysis. Statistical significance was set at $p<0.05$. Statistical analyses were conducted using R version 3.1.2 and STATA for Windows version 17.

RESULTS

In TCGA dataset, FLII expression was significantly lower in cancer compared to normal tissue ($p<0.001$, 8.54±0.42 vs. 7.44±0.90), however THRAP3 expression was not different between normal and cancer tissue ($p=0.99$, 11.44±0.26 vs. 11.44±0.29). According to the Kaplan–Meier analysis, there was no statistically significant difference in OS between the high and low expression groups in either FLII or THRAP3; however, there was a statistically significant positive correlation between the mRNA expression of THRAP3 and FLII ($p<0.001$, β0.83±0.18), suggesting that FLII may interact with THRAP3 in CRC (Supplementary Table 1).

In the GSE100179 dataset of GEO at NCBI, confirming mRNA expression in normal, adenoma, and cancer tissues of 20 patients, FLII expression was highest in normal tissues and significantly decreased stepwise in adenomas and cancers ($p=0.001$, normal vs. cancer). Conversely, THRAP3 expression was lowest in normal tissues and significantly escalated stepwise in adenomas and cancers ($p<0.001$, normal vs. cancer), data is shown in Supplementary Fig 1. In the GSE17536 dataset of GEO at NCBI, confirming mRNA expression in cancer tissues of 177 patients, there was a statistically significant positive correlation between the mRNA expression of THRP3 and FLII ($p=0.002$, β0.208±0.07). There was no statistically significant difference in OS between the high-and low-expression groups in both FLII and THRAP3.

In this study, data of group I for a total of 50 patients consisting of the colitis-related CRC group and the sporadic CRC group are shown in Table 1. The mean age of the patients was 52.9 (range, 21–84 years) at the time of surgery and 28 were male.

Table 1. FLII and THRAP3 expression and clinicopathological characteristics

Variables	Total, n=50	Colitis-related CRC, n=10	Sporadic CRC, n=40	*p* value
Gender (male)	28 (56.0)	6 (60.0)	22 (55.0)	0.532
Age	52.9±16.5 (21-84)	51.6±16.2	53.2±16.7	0.779
BMI	24.1±3.5 (15.8-32.0)	21.7±3.5	24.7±3.3	**0.022**
Obesity (BMI>=25)	121 (30.3)	84 (29.9)	37 (31.4)	0.772
NLR	2.52±1.56	3.43±2.12	2.30±1.33	0.113
PreOp CEA	5.25±8.0	2.29±1.6	5.99±8.8	0.195
FLII protein in normal tissues	0.143±0.065	0.184±0.068	0.133±0.060	**0.019**
FLII protein in CRC tissues	0.095±0.056	0.135±0.558	0.089±0.527	**0.013**
THRAP3 protein in normal tissues	0.143±0.163	0.087±0.090	0.157±0.174	0.344
THRAP3 protein in CRC tissues	0.344±0.275	0.232±0.239	0.372±0.280	0.11
THRAP3 mRNA in normal tissues (n=48)	1.042±0.069	1.055±0.116	1.039±0.577	0.273
THRAP3 mRNA in CRC tissues (n=48)	33.579±181.936	5.30±10.373	39.235±199.185	0.682

Tumor size	5.47±2.35	4.81±1.77	5.64±2.47	0.358
Location				0.068
Colon	28 (56.0)	3 (30.0)	25 (62.5)	
Rectum	22 (44.0)	7 (70.0)	15 (37.5)	
Curability				0.603
R0	46 (92.0)	9 (90)	37 (92.5)	
R1	2 (4.0)	0	2 (5.0)	
R2	2 (4.0)	1 (10)	1 (2.5)	
Adj CTx (n=48)				0.362
No	24 (50)	6 (60)	18 (47.4)	
Yes	24 (50)	4 (40)	30 (52.6)	
Stage				0.799
Tis	3 (6.0)	1 (10)	2 (5.0)	
I	13 (26.0)	2 (20)	11 (27.5)	
II	23 (46.0)	4 (40)	19 (47.5)	
III	6 (12.0)	2 (20)	4 (10)	
IV	5 (10.0)	1 (10)	2 (10)	
Differentiation (n=47)				**0.026**
WD	8 (17)	4 (44.4)	4 (10.5)	
MD	36 (76.6)	4 (44.4)	32 (84.2)	
PD	3 (6.4)	1 (11.1)	2 (5.3)	

Protein expression of FLII and its correlation with clinicopathological factors in CRC and normal colonic tissues (Group I)

We first assessed the protein expression of FLII in colon cancer tissues and adjacent normal tissues by western blotting (Fig. 1A). Expression of FLII showed a positive correlation that increased in cancer tissue as it increased in normal tissue ($p=0.006$, $\beta 0.334 \pm 0.12$). However, our results showed that FLII expression was markedly decreased in cancer tissues compared to that in normal tissues ($p<0.001$, Fig. 1B).

Western blot analyses showed that the protein expression levels of FLII were significantly higher in Colitis-related CRC patients in both cancer tissues and their matched neighboring normal tissues relative to sporadic CRC patients (Table 1). We analyzed the correlation between FLII expression and clinicopathological factors in patients with colon cancer. The expression of FLII in cancer tissues was significantly associated with female gender and the Colitis-related CRC group by adjusted analysis (Table 2). There was no significant difference in survival between the high expression FLII and low expression groups (Fig. 2).

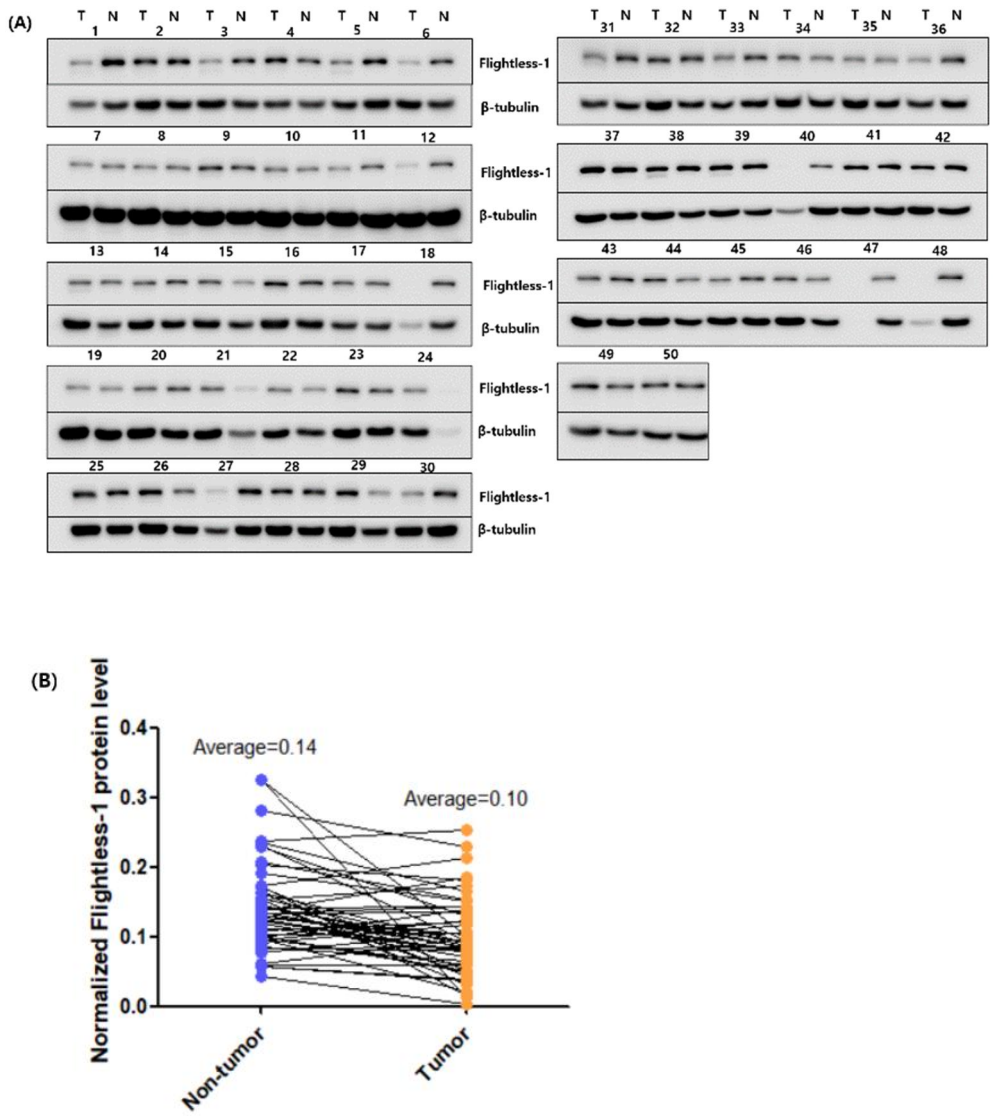


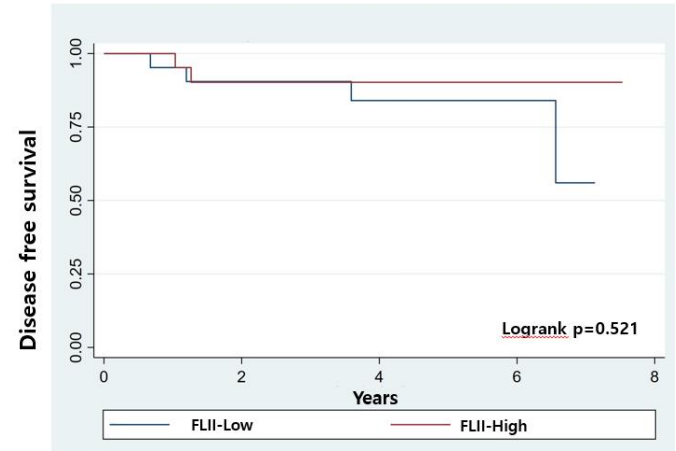
Fig 1. Western blotting for FLII. (A) Western blots of lysates from CRC tissues (T) and normal colonic tissues (N). (B) Normalized expression level of FLII between CRC and normal colonic tissues. FLII expression in CRC tissues was significantly lower than normal colonic tissues ($p<0.001$). It tends to be high as the expression level of FLII in normal tissues is high ($p=0.006$).

Table 2. Association of FLII and THRAP3 expression (n=50) with clinicopathological parameters

Variables	**FLII protein expression in CRC tissues**						**THRAP3 protein expression in CRC tissues**					
	Unadjusted analysis			**Adjusted analysis**			**Unadjusted analysis**			**Adjusted analysis**		
	β	**SEM**	**p-value**	**β**	**SEM**	**p-value**	**β**	**SEM**	**p-value**	**β**	**SEM**	**p-value**
Gender	-0.036	0.015	**0.022**	-0.038	0.0148	**0.016**	0.116	0.075	**0.032**	0.197	0.074	**0.011**
Age			0.908	-0.0001	0.0004	0.82			0.781	-0.001	0.002	0.564
Colitis-related vs. Sporadic CRC	-0.046	0.019	**0.018**	-0.047	0.018	**0.042**			0.189			
BMI	-0.003	0.002	0.098			0.397			0.559			
Obesity			0.334						0.95			
Location			0.734						0.442			
NLR			0.909						0.687			
PreOP CEA									0.343			

Stage	0.003	0.008	0.719		0.818
T stage			0.965		0.884
N stage	0.031	0.0213	0.144	0.936	0.535
M stage	0.034	0.0262	0.199	0.195	0.621
Differentiation			0.883		0.333
Lymphovascular invasion			0.383		0.507
Perineural invasion			0.258		0.776

Bold numbers highlight the statistical significance. Abbreviations: CRC, colorectal cancer; BMI, body mass index, NLR, neutrophil to lymphocyte ratio; PreOP CEA, preoperative carcinoembrionic antige.



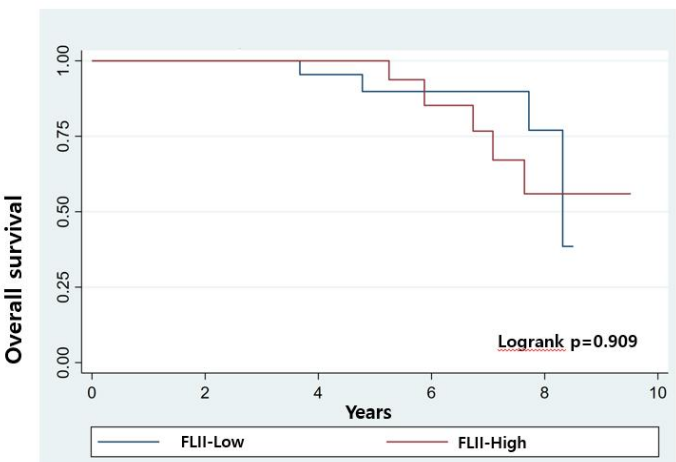


Fig 2. Kaplan-Meier survival curves according to FLII protein expression (Low vs. High). (A) Kaplan-Meier survival curves for disease-free survival. (B) Kaplan-Meier survival curves for overall survival.

Protein and mRNA expression of THRAP3 and its correlation with clinicopathological factors in CRC and normal colonic tissue (Group I)

To examine whether THRAP3 expression levels are correlated with clinicopathological factors in patients with CRC, protein and mRNA expression levels of THRAP3 were evaluated in colonic cancer and normal colonic tissues. There was no difference in the expression of THRAP3 between the Colitis-related CRC and sporadic CRC groups (Table 1). Regression analysis showed that the expression of THRAP3 was only significantly associated with male gender among the clinicopathologic parameters in this cohort (Table 2).

The normalized protein level analysis showed that the protein expression of THRAP3 was significantly higher in cancer tissues than in their matched neighboring normal tissues (Fig. 3). As the protein expression of THRAP3 in normal tissues increased, the protein expression of THRAP3 in tumor tissues also showed a tendency to increase ($p<0.001$, $\beta 1.12 \pm 0.18$). THRAP3 mRNA expression was also significantly higher in cancer tissues (Fig. 4, $p=0.003$), and there was a significant trend between THRAP3 mRNA expression in normal tissue and THRAP3 mRNA expression in cancer tissues ($p=0.001$). Our analysis showed no significant correlation between THRAP3 mRNA expression and clinicopathological parameters. There was no significant difference in survival according to the protein and mRNA levels of THRAP3 in this cohort (Fig. 5).

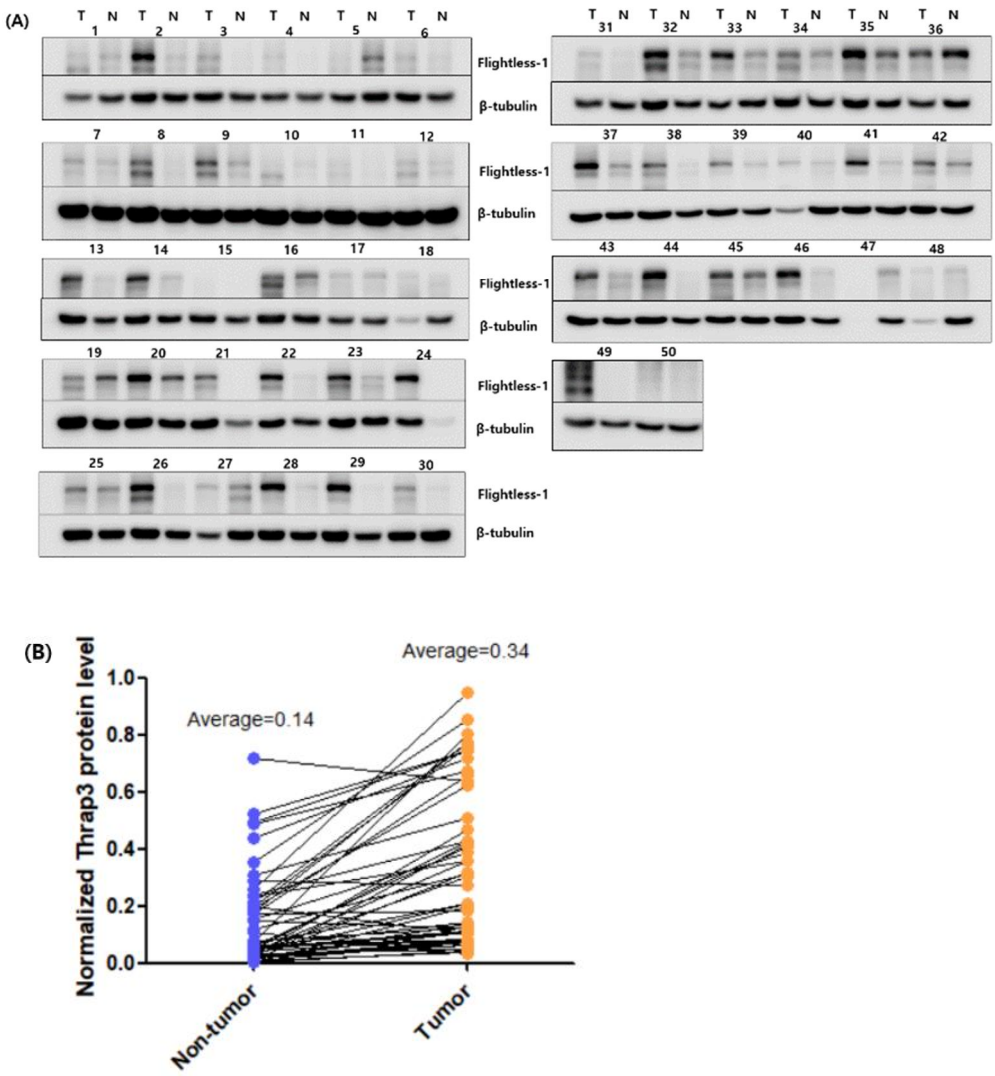


Fig 3. Western blotting for THRAP3. (A) Western blots of lysates from CRC tissues (T) and normal colonic tissues (N). (B) Normalized expression level of THRAP between CRC and normal colonic tissues. THRAP3 expression is significantly increased in CRC tissue than normal colonic tissues ($p<0.001$). The expression level of THRAP3 in CRC tissues tends to be high as the expression level of THRAP3 in normal colonic tissues is high ($p<0.001$).

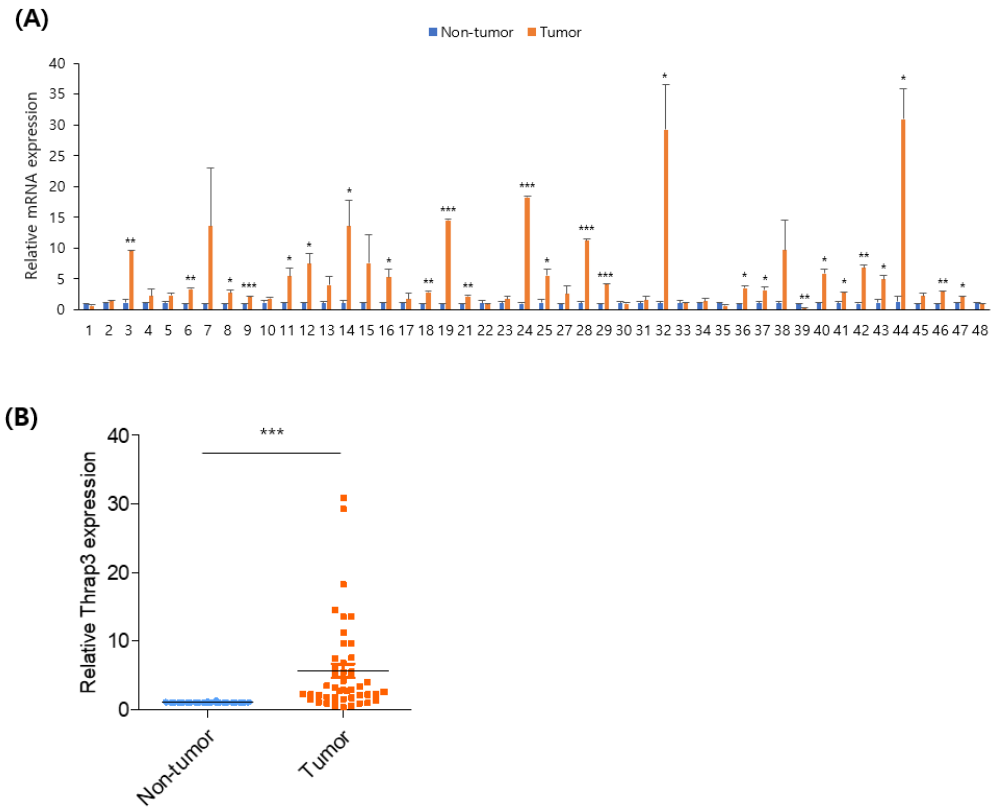


Fig 4. THRAP3 mRNA expression. It is significantly increased in CRC tissues (A and B).

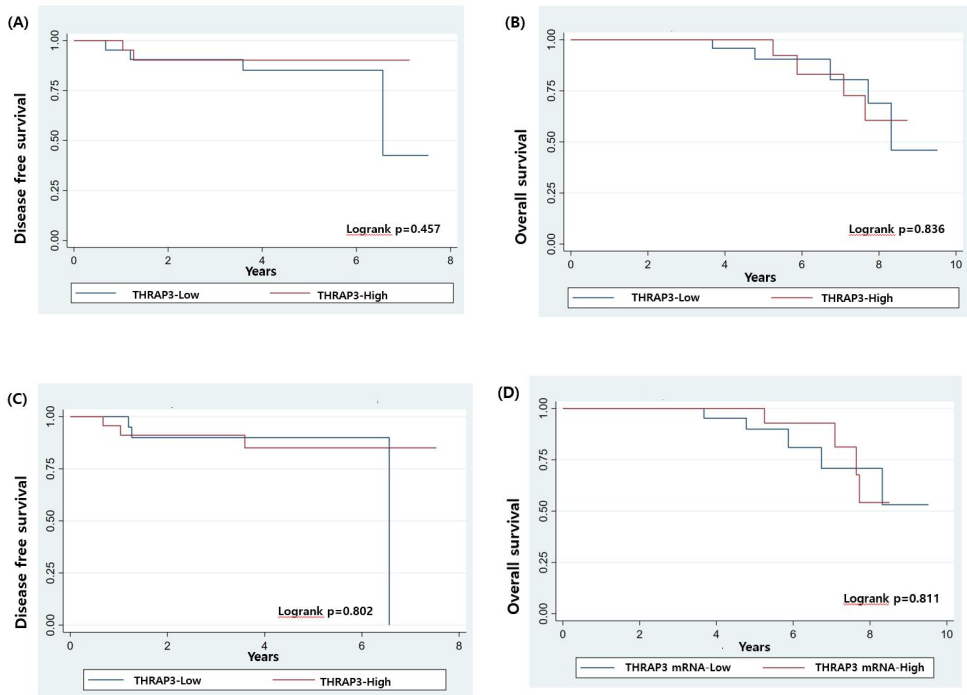


Fig 5. Kaplan-Meier survival curves according to THRAP3 expression. (A and B) Kaplan-Meier survival curves for disease-free survival and overall survival according to THRAP3 ratio (normal colonic tissue to CRC tissue ratio of THRAP3≥3, n=26). (C and D) Kaplan-Meier survival curves for disease-free survival and overall survival according to THRAP3 mRNA ratio (normal colonic tissue to CRC tissue ratio of THRAP3≥3, n=26).

Group II, Relationship between THRAP3 expression on IHC and clinicopathologic variables in colonic cancer

To gain further insight into the role of THRAP3 in colonic cancer specimens, we selected 399 patients from a cohort consisting of primary CRC diagnosed as adenocarcinoma. Next, we performed immunohistochemical analysis of THRAP3 in these patients.

In this cohort, 29.6% (118 of 399) of cancer samples showed highly positive (+++) staining intensity for THRAP3, while four patient samples were negatively stained (0) for THRAP3; 277 patient samples showed weak positive staining (+, ++) for THRAP3. The significantly different parameter between low-and high-positively stained samples was the location of cancer; rectal cancer showed significantly higher rates of THRAP3 expression (Table 3). In multivariate analysis, factors related to high expression of THRAP3 were rectal cancer, good differentiation, and low CEA staining in IHC in this cohort (Table 4). When cross-referencing the immunostaining results of THRAP3 expression with patient survival data, we found that patients with highly positive THRAP3 expression (THRAP3 high) tended to have a better survival rate compared to patients with negative to low positive THRAP3 expression (THRAP3 low), although the difference was not statistically significant (Fig. 6). According to multivariable models for survival, high expression of THRAP3 was not associated with survival (Table 5).

Table 3. Thrap3 expression (IHC) and clinicopathological factors (n=399)

Variables	Total, n=399	THRAP3 staining patterns		*p* value
		THRAP3-low (n=281)	THRAP3-high (n=118)	
Gender (male)	250 (62.66)	174 (61.92)	76 (64.41)	0.64
Age	60.49±10.94 (22-87)	60.68±11.27	59.74±10.13	0.217
Body weight (kg)	63.18±10.35 (40.5-92)	63.97±10.36	63.15±10.35	0.483
Hight (cm)	162.90±8.36 (141.8-182)	163.00±8.50	162.66±8.04	0.355
BMI	23.76±3.11 (14.90-35.66)	23.74±3.16	23.80±3.02	0.561
Obesity (BMI>=25)	121 (30.33)	84 (29.89)	37 (31.36)	0.772
DM	42 (12.46)	31 (13.25)	11 (9.32)	0.511
Location				**0.005**
Colon	253 (63.73)	190 (68.10)	63 (53.39)	
Rectum	144 (36.27)	89 (31.90)	55 (46.61)	
Stage				0.297
I	69 (17.29)	43 (15.30)	26 (22.03)	
II	158 (39.60)	115 (40.93)	43 (36.44)	
III	124 (31.08)	86 (30.60)	38 (32.20)	
IV	48 (12.03)	37 (13.17)	11 (9.32)	
Differentiation				0.103
WD	57 (14.29)	35 (12.46)	22 (18.64)	

MD	326 (81.70)	232 (82.56)	94 (79.66)	
PD	16 (4.01)	14 (4.98)	2 (1.69)	
PreOp CEA	17.77±17.77 (0.3-2380)	21.01±150.38	10.15±36.91	0.223
Tumor size (cm)	5.03±2.20 (0.9-13)	5.07±2.20	4.94±2.20	0.327
Lymphovastular invasion	88 (22.11)	62 (22.14)	26 (22.03)	0.981
Perineural invasion	51 (12.88)	40 (14.39)	11 (9.32)	0.169
Tumor budding (n=396)	165 (41.67)	113 (40.65)	52 (44.07)	0.528
p53 high in IHC (n=391)	272 (69.57)	184 (64.19)	88 (77.88)	0.092
CEA high in IHC (n=392)	207 (52.81)	158 (56.83)	49 (42.98)	**0.013**
MSI high (n=373)	34 (9.12)	24 (9.16)	10 (9.01)	0.652

Categorical variables are presented as number (%), and continuous variables are presented as mean and Standard deviation (±SD) and range (minimum to maximum value). Bold numbers highlight the statistical significance. Abbreviations: IHC, immunohistochemistry; BMI, body mass index; PreOp CEA; preoperative carcinoembryonic antigen; WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; MSI, microsatellite instability.

Table 4. Association between high expression of THRAP3 in IHC and clinicopathological parameters using multiple logistic regression

Characteristics	THRAP3-high (n=118)	Univariate analysis (n=399)			Multivariate analysis (n=399)		
		OR	**(95%CI)**	***P* value**	**OR**	**(95%CI)**	***P* value**
Gender (male)	76 (64.41)	1.113	(0.7115-1.7403)	0.639			
Age	59.74±10.13	0.992	(0.6727-1.7060)	0.434			
Body weight (kg)	63.15±10.35	0.999	(0.9789-1.021)	0.965			
Hight (cm)	162.66±8.04	0.995	(0.9698-1.0211)	0.708			
BMI	23.80±3.02	1.005	(0.9383-1.0774)	0.877			
Obesity (BMI>=25)	37 (31.36)	1.071	(0.6727-1.706)	0.772			
DM	10.68)	0.783	(0.3771-1.6257)	0.512			

Location							
Colon	63 (53.39)						
Rectum	55 (46.61)	1.864	(1.1995-2.8958)	**0.006**	1.817	(1.1503-2.8707)	**0.01**
Stage							
I	26 (22.03)	1.17	(0.9211-1.4871)	0.196	1.058	(0.8191-1.3665)	0.666
II	43 (36.44)						
III	38 (32.20)						
IV	11 (9.32)						
Differentiation							
WD	22 (18.64)	1.717	(1.0281-2.8689)	**0.039**	1.6771	(0.9594-2.9315)	**0.07**
MD	94 (79.66)						
PD	2 (1.69)						
PreOp CEA	10.15±36.91	0.998	(0.9938-1.003)	0.343			

Tumor size	4.94±2.20	0.973	(0.8637-1.0962)	0.652			
Lymphovastular invasion	26 (22.03)	0.994	(0.5915-1.6693)	0.981			
Perineural invasion	11 (9.32)	0.612	(0.3022-1.2382)	0.157			
Tumor budding (n=396)	52 (44.07)	1.15	(0.7445-1.7777)	0.528			
p53 high in IHC (n=391)	88 (77.88)	1.139	(0.9640-1.3451)	0.123	1.1664	0.9821-1.3853)	0.079
CEA in IHC (n=392)							
low	65 (57.02)	1.361	(1.0309-1.7961)	**0.031**	1.4354	(1.0762-1.9146)	**0.014**
high	49 (42.98)						

MSI high (n=373)	10 (9.01)	0.968	(0.6570-1.4270)	0.87

Bold numbers highlight the statistical significance. Abbreviations: OR, odds ratio; CI, confidence interval; IHC, immunohistochemistry; BMI, body mass index; DM, diabetes mellitus; PreOp CEA; preoperative carcinoembryonic antigen; WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; MSI, microsatellite instability.

Table 5. Multivariable Models for Overall Survival and Disease-Free Survival

Characteristics	Univariate analysis						Multivariate analysis					
	OS			DFS			OS			DFS		
	HR	(95%CI)	*P* value	HR	(95%CI)	*P* value	HR	(95%CI)	*P* value	HR	(95%CI)	*P* value
Gender (male)	1.474	1.009-2.153	0.045			0.743			1.134			0.948
Age	1.032	1.014-1.049	<0.001			0.779	1.037	1.006-1.068	**0.018**			0.876
BMI			0.755			0.917						
DM	1.765	1.027-3.034	0.04			0.49	2.66	1.250-5.661	**0.011**			
Obesity (BMI>=25)			0.482			0.57						
Location (colon vs rectum)			0.644			0.295						

T stage	1.858	1.333-2.591	<0.001	2.716	1.632-4.518	<0.001			0.973	1.895	1.055-3.404	**0.032**
N stage (yes or no)	2.48	1.750-3.515	<0.001	4.247	2.526-7.140	<0.001	1.661	0.916-3.012	0.094	2.632	1.500-4.616	**0.001**
M stage	6.823	4.647-10.018	<0.001	3.539	1.888-6.631	<0.001	18.42	2.427-139.87	**0.044**			0.119
Differentiation (WD~PD)	1.846	1.187-2.872	0.007	1.782	0.954-3.328	0.07			0.058			0.748
PreOp CEA	2.048	1.428-2.937	<0.001	1.59	0.9329-2.710	0.088			0.853			0.605
Tumor size (cm)	1.156	1.058-1.263	0.001			0.103			0.109			
Lymphovastular invasion	2.68	1.87-3.836	<0.001	2.297	1.380-3.823	0.001	2.354	1.222-4.534	**0.01**			0.363

Perineural invasion	1.943	1.255-3.001	0.003	2.84	1.632-4.942	<0.001	0.57	1.87	1.057-3.311	**0.032**
p53 high in IHC			0.646			0.956	0.8			
CEA high in IHC	1.434	1.007-2.043	0.046	1.948	1.1667-3.253	0.011	0.104	1.973	1.158-3.362	**0.012**
THRAP3 hign in IHC	0.792	0.533-1.177	0.249	0.767	0.442-1.332	0.347	0.52	1.022	0.567-1.842	0.941

Cox proportional hazard model was used. Bold numbers highlight the statistical significance. Abbreviations: HR, hazard ratio; CI, confidence interval; IHC, immunohistochemistry; BMI, body mass index; DM, diabetes mellitus; PreOp CEA; preoperative carcinoembryonic antigen; WD, well differentiation; MD, moderate differentiation; PD, poor differentiation; MSI, microsatellite instability.

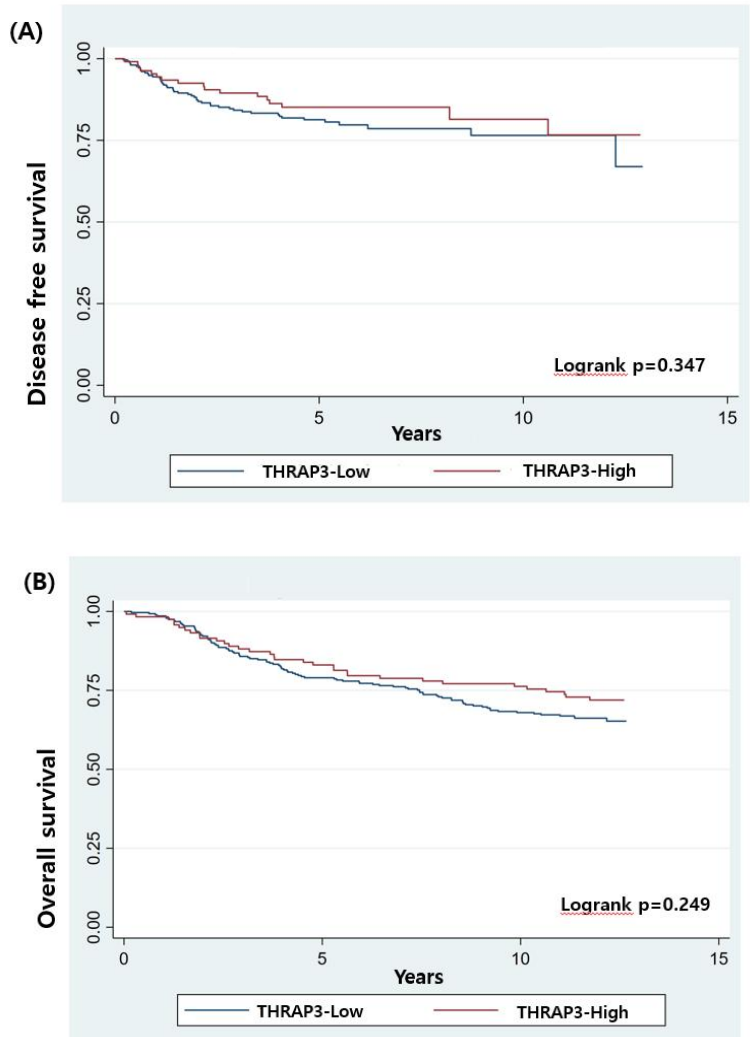


Fig 6. Kaplan-Meier survival curves according to THRAP3 expression in IHC. (A) Disease free survival and (B) Overall survival according to the THRAP3 expression in IHC as obtained by using a Kaplan-Meier analysis (log-rank test). The 5-year and 10-year overall survival rate of patients with high expression of THRAP3 was 83% and 76.3% comparing with 79% and 68% of low expression.

DISCUSSION

In this study, both FLII and THRAP3 expression showed significant differences in CRC tissues compared to normal tissues. FLII expression was decreased in CRC tissues compared to normal tissues, and was significantly higher in both normal and CRC tissues of the colitis-related CRC group compared to the sporadic CRC group. The common expression patterns of FLII in both groups could be allowed FLII to be considered an important molecule in understanding the development of CRC in two groups with different developmental mechanisms. Protein and mRNA expression of THRAP3 was significantly higher in CRC tissues than in normal tissues. However, there was no difference in expression according to the TNM stage of the cancer tissues, which was also confirmed in the GSE17536 dataset (Supplementary Fig. 2). In addition, in the GSE100179 dataset, which confirmed the expression of THRAP3 in adenoma tissues, THRAP3 showed a stepwise increase with tumor progression from adenoma to carcinoma. This increased expression pattern suggests that THRAP3 may play an important role in early development rather than the progression and metastasis of CRC.

Identifying the genes associated with the development of CRC by environmental factors and understanding their signaling pathways will play an important role in discovering new therapeutic and preventive targets for CRC patients. There are environmental factors that have a greater effect on modern people compared to the previous generation, and representative factors include metabolic diseases such as obesity, diabetes mellitus, hyperlipidemia, and exposure to various carcinogens. Studies have shown that CRC incidence has a geographic difference [42], which appears to be due to differences in environmental exposure imposed on the background of genetically determined susceptibility.

A persistent chronic inflammatory condition, a characteristic feature of IBD, is a major predisposing factor for high-grade dysplasia and CRC. Patients with colitis-related CRC tend to develop at a younger age than patients with sporadic CRC. A meta-analysis demonstrated that the overall incidence rate of CRC in patients with UC was 3/1,000 person-years and depended on the extent, duration, and activity of colitis [11]. Although the degree of association is likely to be lower than that of UC, Crohn's disease also revealed a significantly increased risk of CRC [12]. In a recent population-based cohort study, incident CRCs in the UC cohort were 1.29/1,000 person-years with a hazard ratio of 1.66 (95% CI 1.57–1.76), and the time trends of hazard ratios for CRC death decreased over time [13]. This reduction may be due to improvements in treatment regimens that adequately control pancolitis, as well as surveillance strategies to detect precancerous lesions [43]. Several molecular pathways contributing to sporadic CRC, such as inactivation of tumor suppressor genes and mutations in oncogenes including genetic instability, are also involved in the pathogenesis of colitis-associated CRC. However, the typical "normal mucosal-adenoma-dysplasia-carcinoma" sequence in sporadic CRC development is not fully identified in colitis-associated CRC, but rather it occurs in inflamed mucosa and progresses in the "inflammatory-dysplastic-cancer" sequence [44]. Proinflammatory pathways are important in the pathogenesis of dysplasia and carcinoma caused by colitis. These proinflammatory signaling pathways modulate tumorigenesis by increasing inflammatory mediators, upregulating the expression of anti-apoptotic genes, and stimulating cell proliferation and angiogenesis [45]. In our previous study, we demonstrated that FLII suppresses PPARγ-mediated adipogenesis to promote a catabolic state [19] and inhibit apoptosis by regulating Ca2+ homeostasis in the ER [39]. In line with this study, molecular and functional studies indicated that FLII was significantly

elevated in patients and a mouse model of UC, and adversely affected mucosal healing by promoting Th1 and Th2 mediated tissue inflammation and inhibiting the Wnt/β-catenin signaling pathway [46]. The role of the Wnt/β-catenin pathway in CRC is well-known; it induces cell proliferation, angiogenesis and epithelial-mesenchymal transition (EMT) during oncogenesis, reducing oxidative metabolism in the TCA cycle [47]. Our results, in which the level of FLII was significantly increased in the colitis-related CRC group compared to the sporadic CRC group, suggest that FLII induces a persistent colitis state by inhibiting apoptosis while maintaining mucosal inflammation. The decreased level of FLII expression in cancer tissues compared to normal tissues in both groups suggests that maintenance of homeostasis induced by FLII is more likely to be involved in cancer development than cancer progression.

Interestingly, PPARγ and β-catenin have been reported to interact with mechanisms to alter their respective activities [48]; PPARγ is downregulated, while canonical Wnt/β-catenin is upregulated in type 2 DM and cancers [49]. The complex and multiple nature of these two major pathways may partly explain the associations, such as glucose regulation and cell proliferation, commonly observed between metabolic diseases such as DM and CRC. The function of FLII, which is known to inhibit both PPARγ [19]and Wnt/β-catenin signaling pathways [46], seems to make it difficult to understand the action of FLII in cancer. Another major metabolic alteration in which an association between metabolic disease and CRC may be encountered is hyperinsulinemia. Persistent elevation of insulin and free insulin growth factor (IGF-1) promotes cell proliferation and increases the chances of cell transformation, ultimately leading to the development of CRC [50]. Moreover, hyperglycemia is associated with chronic inflammation that promotes cancer growth [51]. In a previous study, we reported that THRAP3 directly

interacts with phosphorylated PPARγ at Ser273 by cyclin-dependent kinase 5 (CDK5) in adipose tissue to stimulate insulin resistance by diabetic genetic programming [22]. The escalation of THRAP3 in adenoma and cancer cells shown in this study supports the hypothesis that cancer development and changes in metabolic status are related.

In our previous study, we found that FLII expression was significantly higher in CRC cell lines and two CRC tissues ($APC^{min/+}$ and colitis-induced mouse model) than in the corresponding normal cell line and non-tumor tissues. In addition, knockdown of FLII significantly repressed xenograft tumor growth and reduced FLII levels, promoting apoptosis in colon cancer cell lines [39]. Based on these results, we hypothesized that FLII is increased in CRC tissues and is involved in cancer progression by regulating apoptosis. However, in this study of 50 CRC patients, the expression of FLII was lower in cancer tissues than in normal tissues. These findings are consistent with the results confirmed in both the TCGA dataset and the GSE100179 dataset, which can compare mRNA expression in normal and cancer tissues. There was no difference in the expression level of FLII according to the TNM stage in this study and both datasets from TCGA and GSE17536. The results of different expression of FLII in previous studies of cell lines and mouse models and in this study of CRC patients enabled the hypothesis that increased expression of FLII may be involved in CRC development rather than cancer progression.

FLII expression has been reported in various tumor types. In a study of 139 clinical breast cancer samples, phosphorylated FLII at Ser436 was highly expressed in cancer tissues and showed an increasing rate with stage. FLII blocks p62-dependent selective autophagy, resulting in increased oxidative stress, ultimately retarding mammary cancer progression. This function of FLII was reinforced by Akt (protein kinase B)-mediated phosphorylation at Ser436 and

inhibited by phosphorylation of Ulk1 (unc51-like kinase 1) at Ser64 [52]. FLII functions in breast cancer as a checkpoint protein for selective autophagy and depends on its phosphorylation at a specific site rather than the total expression level. A study examining the protein and mRNA levels of FLII in clinical prostate cancer specimens showed a significant decrease in FLII expression in prostate cancers, and the high expression of FLII was correlated with improved OS through FLII competitively interfering with the binding between the androgen receptor and ligand [53]. The investigation revealed that FLII was downregulated in lung carcinoma cell lines and its interactome, most of which are RNA-binding proteins involved in RNA post-transcriptional modification and trafficking. FLII knockdown in human bronchial epithelial cell lines (HBE) significantly stimulated their migration and invasion, whereas FLII overexpression inhibited its ability [54]. In addition, FLII acts as a transcriptional coregulator by interacting with various proteins and positively regulates estrogen receptor and thyroid hormone receptor, but suppresses PPARγ, β-catenin, and ChREBP-mediated transcription [19, 20, 55-58]. The expression of FLII was different for each tumor, and the mechanism of action on cancer was also different. The identification of characteristic expression in CRC of FLII would be considered the first step toward finding the function of this gene in CRC.

Importantly, in addition to regulating metabolism by acting directly on PPARγ, THRAP3 is thought to affect tumor progression by regulating DDR through post-transcriptional modifications of pre-mRNA. A previous study demonstrated that cell sensitivity to DNA damaging agents is increased when THRAP3 is depleted using siRNA, and strong phosphorylation of THRAP3 in response to DNA damage suggested a potential role of THRAP3 as a tumor suppressor in parathyroid carcinoma [32]. Oral squamous cell carcinoma (OSCC) is

a tumor that arises through a well-known multistep process driven by the accumulation of carcinogen-induced genetic changes. The highest deletion frequencies (100%) in THRAP3 were detected in seven OSCC patients, with 86% being detected in dysplasia, which also suggested the function of tumor suppressor [30]. THRAP3 is an HBV-DNA integration site, and the change in THRAP3 function is likely to affect cell signaling and cause liver carcinogenesis [31]. Moreover, a significantly lower level of phosphorylated THRAP3 in androgen-independent prostate cancer cell lines has been reported [59]. The phosphorylation state of THRAP3 at S248 and S253 might be involved in the mechanism of androgen-independent prostate cancer cell growth by engaging with RNA splicing and processing [59]. Contrary to the observed THRAP3 deficiency in several cancer types, our study showed that protein and mRNA expression was significantly increased in CRC tissues compared to normal tissues. These results were confirmed more firmly through the mRNA dataset, including TCGA data, and it seems to be a characteristic finding of CRC, distinguishing it from other carcinomas. We also examined THRP3 expression in CRC using IHC staining. THRAP3 was found to be more highly expressed in patients with rectal cancer than in those with colon cancer, and statistically significant differences were observed depending on the differentiation. The expression of THRAP3 showed no statistically significant difference in OS, but the group with high THRAP3 showed a better prognosis than the group with low THRAP3 (Fig. 6).

The relationships between inflammation and cancer are varied and complex, and an important connection linking is DNA damage, which in turn can promote mutations that initiate and promote cancer [26]. A previous study demonstrated that DNA repair is also essential for tolerating inflammation, based on the fact that mice deficient in multiple repair enzymes were

unable to tolerate DSS-induced colitis [60]. Detection and response to DNA damage repeatedly leads to increased inflammation, and the response of one cell to DNA damage can induce damage to surrounding cells through extracellular signaling and epigenetic modification [26]. Therefore, the impact that inflammatory diseases have on the prevalence and uncontrollable progression of cancers should not be taken for granted. FLII increases inflammation and inhibits the Wnt/β-catenin signaling pathway [46], thereby interfering with the healing of damaged tissues and causing a continuous inflammatory response, while maintaining homeostasis by inhibiting apoptosis [39] . This series of processes is expected to increase DDR, and explains the result of our previous study, in which the increase in FLII in all cancers occurred in APC knockdown mice and DSS-induced models [39]. Consistent with this prior knowledge, this study showed that increased expression of THRAP3 correlated significantly and positively with the expression of FLII in CRC tissues. Considering the DDR function of THRAP3, it can be inferred that the increase in CRC tissues is a compensatory action for various DNA damage accumulated during tumor development.

The expression of FLII and THRAP3, which are expected to influence the development and progression of CRC through various mechanisms, such as transcriptional cofactor roles, post-transcriptional regulation of mRNA, and metabolic regulation, was first identified in a clinical CRC patient sample in this study. A pathway involving a direct link between FLII and THRAP3 could not be confirmed, but an association between the two genes was identified. Unfortunately, in this study, mutations or variations of FLII and THRAP3 were not identified, and changes in function according to phosphorylation at specific sites could not be analyzed. Although the expression patterns of FLII and THRAP3 in several CRC datasets were consistent with the

results of our study, the inconsistent expression patterns in various other cancers seem to make it difficult to understand the mechanism of action of these two genes in cancer. In this study as well, THRAP3 expression tended to be higher in male in group I, but there was no gender difference in group II. Furthermore, the difference in THRAP3 expression depending on the location of cancer was not seen in group I, but the ratio of THRAP3-high in rectal cancer was significantly higher in group II. It is difficult to determine the significance of these differences because the methods for confirming the expression of THRAP3 differ between the two groups and there are differences in the selection of subjects. Whether or not there is a difference in THRAP3 expression depending on the location of cancer and gender will need to be confirmed through future studies. Future systematic studies of the protein interactions of FLII and THRAP3 are also necessary for understanding the function of these genes.

In conclusion, we demonstrated that FLII may be associated with the pathogenesis of CRC and its expression level decreased after the development of CRC, and THRAP3 expression was increased more after the onset of CRC. Despite the conflicting expression patterns seen in CRC tissues, FLII and THRAP3 had a significant positive correlation, suggesting that there are pathways that interact with each other. Targeting these two genes, which are expected to act at multiple stages from metabolic disease or carcinogen-induced inflammatory responses to the development of CRC, represents an innovative and effective strategy for the development of preventive and therapeutic agents.

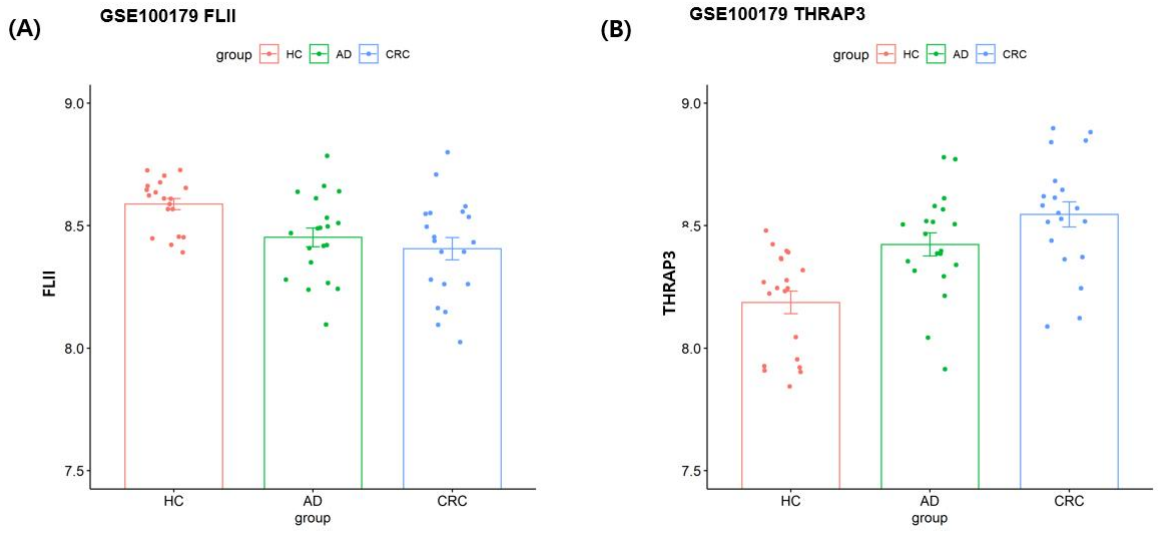
Supplement Table 1. mRNA expression and clinicopathological factors (n=376)

Variables	Total, n=376	FLII expression		*p* value	THRAP3 expression		*p* value
		Low	High		Low	High	
Gender (male)	207 (55.1)	107 (57.0)	100 (53.2)	0.468	110 (58.5)	97 (51.6)	0.178
Age	64.5±13.1 (31-90)	65.15±12.8	63.9±13.3	0.378	65±12.5	64.1±13.7	0.535
BMI (n=279)	28.0 ±6.1 (14.0-52.1)	28.1±6.3	28.0±6.0	0.874	28.4±5.9	27.7±6.4	0.386
Obesity (BMI>=25)	190 (68.1)	94 (66.7)	96 (69.6)	0.604	100 (75.2)	90 (61.6)	**0.015**
Location, n=362				0.667			0.423
Colon	274 (75.7)	138 (76.7)	136 (74.5)		136 (73.9)	138 (77.5)	
Rectum	88 (24.3)	42 (23.3)	46 (25.3)		48 (26.1)	40 (22.5)	
Stage, n=370				0.713			0.221
I	59 (16.0)	31 (16.7)	28 (15.2)		30 (16.1)	29 (15.8)	
II	141 (38.1)	72 (38.7)	69 (37.5)		64 (34.4)	77 (41.9)	

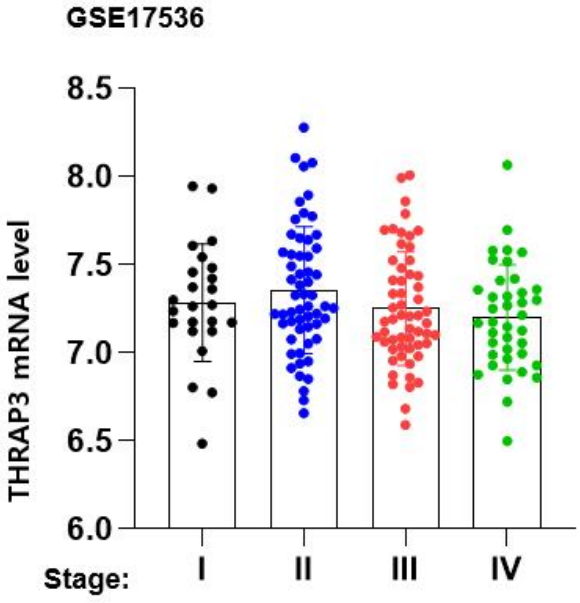
III	117 (31.6)	54 (29.0))	63 (34.2)		59 (31.7)	58 (31.5)	
IV	53 (14.3)	29 (15.6)	24 (13.0)		33 (17.7)	20 (10.9)	
T stage, n=375				0.596			0.68
0	1 (0.3)	0 (0)	1 (0.5)		0	1 (0.5)	
1	10 (2.7)	7 (3.7)	3 (1.6)		7 (3.7)	3 (1.6)	
2	57 (15.2)	29 (15.5)	28 (14.9)		28 (15)	29 (15.4)	
3	258 (68.8)	129 (67.4)	132 (70.2)		128 (68.5)	130 (69.1)	
4	49 (13.1)	25 (13.4)	24 (12.8)		24 (12.8)	25 (13.3)	
N stage, n=373				0.438			
negative	206 (55.2)	107 (57.2)	99 (53.3)		97 (51.9)	109 (58.6)	
postitive	167 (44.8)	80 (42.8)	87 (46.8)		90 (48.1)	77 (41.4)	
M stage, n=370				0.451			**0.05**
0	319 (86.2)	157 (84.9)	162 (87.6)		153 (82.7)	166 (89.7)	

1	51 (13.8)	28 (15.1)	23 (12.4)		32 (17.3)	19 (10.3)	
MSI, n=368	57 (14.29)			0.656			**<0.001**
Stable to low	315 (85.6)	159 (86.4)	156 (84.8)		176 (95.7)	139 (75.5)	
High	53 (14.4)	25 (13.6)	28 (15.2)		8 (4.4)	45 (24.5)	
PreOp CEA	77.7 ± 573 (range 0.2-7868)	45±247	104±741	0.428	51.6±261	104±772	0.48

Categorical variables are presented as number (%), and continuous variables are presented as mean and Standard deviation (±SD) and range (minimum to maximum value). Bold numbers highlight the statistical significance. Abbreviations: TCGA, the cancer genomeatlas; BMI, body mass index; PreOp CEA; preoperative carcinoembryonic antigen; MSI, microsatellite instability.



Supplementary Fig 1. FLII and THRAP3 expression in dataset. (A) FLII expression in GSE100179 dataset. (B) THRAP3 expression in GSE100179 dataset.



Supplementary Fig 2. Expression level of THRAP3 did not differ according to the TNM stage.

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ABSTRACT

Purpose: This study aimed to investigate Flightless-1 (FLII) and thyroid hormone receptor associated protein 3 (THRAP3) expression profiles in patients with colorectal cancer (CRC) and to identify the relationship between expression and clinicopathological factors.

Materials and Methods: A total of 449 patients who were diagnosed with primary CRC and underwent surgery were included. The expression of FLII and THRAP3 in cancer and normal tissues was evaluated in 50 patients, of which 10 had colitis-related CRC and 40 had sporadic CRC. Expression of THRAP3 in formalin-fixed paraffin-embedded (FFPE) tissues was analyzed in 399 patients with sporadic CRC. The relationship between the level of FLII and THRAP3 expression in CRC tissues and clinicopathologic parameters with survival data was investigated.

Results: Our findings demonstrated that FLII protein levels were downregulated in CRC tissues compared to normal tissues ($p<0.001$) and increased in both normal and tumor tissues of the colitis-related CRC group compared to the sporadic CRC group ($p=0.019$ and $p=0.013$, respectively). THRAP3 protein and mRNA levels were upregulated in CRC tissues compared to normal tissues ($p<0.001$ and $p=0.003$, respectively). Of the total 399 FFPE samples, 29.6% showed highly positive staining

intensity for THRAP3. High expression of THRAP3 was associated with rectal cancer, well differentiation, and low expression of CEA in staining.

Conclusions: Results suggest that expression of FLII and THRAP3 is associated with the development of CRC, and these genes could be considered as therapeutic targets for preventive and therapeutic agents.

Keywords: Colorectal cancer, FLII, THRAP3, Gene expression