



## Apoptosis, necroptosis and autophagy in colorectal cancer: Associations with tumor aggressiveness and p53 status



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### ABSTRACT

**Objective:** Cleaved caspase-3 (CC3), phosphorylated-mixed-lineage kinase domain-like protein (p-MLKL), and microtubule-associated protein-1 light chain-3B (LC3B) have pivotal functions in apoptosis, necroptosis, and autophagy, respectively. *In vitro* studies have shown that interaction of these proteins are complex and their roles in cancer can be influenced by many factors. However, these findings are not adequately assessed in human tissues. Here, we determined CC3, p-MLKL, and LC3B expression in colorectal cancers (CRCs), and assessed their associations with clinicopathological parameters, and with *KRAS* and p53 status.

**Methods:** We immunohistochemically assessed 113 CRC specimens for levels of CC3, p-MLKL, LC3B, and p53. *KRAS* gene status was analyzed using the Scorpion- amplification refractory mutation system.

**Results:** High levels of CC3 (CC3<sup>High</sup>) and LC3B (LC3B<sup>High</sup>) were detected in 38% and 35% of the 113 CRCs, respectively, but no or only a few p-MLKL-positive cells were observed in any of the tumors. CC3<sup>High</sup> was significantly associated with high pT status ( $P = 0.03$ ), vascular invasion ( $P = 0.03$ ) and high pStage ( $P = 0.04$ ) and was marginally associated with lymph node ( $P = 0.06$ ) and distant metastases ( $P = 0.06$ ). LC3B<sup>High</sup> was also significantly associated with high pT status ( $P = 0.02$ ) and lymphatic invasion ( $P = 0.002$ ), and was marginally associated with nerve plexus invasion ( $P = 0.06$ ). In combined analysis, compared with CC3<sup>Low</sup>/LC3B<sup>Low</sup> tumors, tumors that were either CC3<sup>High</sup>, LC3B<sup>High</sup>, or both were significantly associated with high pT status ( $P = 0.0007$ ), lymphatic invasion ( $P = 0.03$ ), vascular invasion ( $P = 0.003$ ), distant metastasis ( $P = 0.04$ ) and high pStage ( $P = 0.04$ ). LC3B<sup>High</sup> was significantly associated with a mutant-type expression pattern of p53 ( $P = 0.003$ ).

**Conclusion:** To the best of our knowledge, this is the first study to examine the combination of CC3/LC3B and p-MLKL expression in clinical CRC samples and to correlate these expression data with clinicopathological parameters and *EGFR* and p53 status. Our results suggest that necroptosis is a rare process in CRC, apoptosis and autophagy are upregulated in aggressive CRCs, and p53 mutation may lead to the upregulation of autophagy.

### 1. Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers worldwide. Most CRCs are either invasive or metastatic at the time of presentation. Understanding the correlation between molecular alterations and clinicopathological characteristics of CRC may contribute to better management of the disease.

Apoptosis affects tissue homeostasis and carcinogenesis by removing unwanted or damaged cells from the body [1]. It can be

induced by various extracellular and intracellular stimuli, resulting in coordinated activation of caspases, a family of cysteine proteases [2,3]. Caspase proforms are activated thorough proteolytic cleavage in either the intrinsic (mitochondrial) or extrinsic (death receptor-mediated) pathway [4,5]. The intrinsic and extrinsic pathways share common downstream features. Caspase-3 is a major executioner caspase and is activated (cleaved) directly by caspases-8, -9 or -10 in the extrinsic and intrinsic pathways. Cleaved caspase-3 (CC3) cleaves various intracellular proteins, leading to disruption of cytoskeletal integrity, cell

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**Table 1**  
Associations between CC3 and LC3B levels and clinicopathological parameters.

Variables	<i>n</i>	CC3			LC3B			
		Low	High	<i>P</i> value	Low	High	<i>P</i> value	
Age (years)	< 60	34	22 (65)	12 (35)	0.43	23 (68)	11 (32)	0.46
	≥ 60	79	48 (61)	31 (39)		51 (65)	28 (35)	
Sex	Male	69	45 (65)	24 (35)	0.86	46 (67)	23 (33)	0.70
	Female	44	25 (57)	19 (43)		28 (64)	16 (36)	
Tumor location	Right colon	26	17 (65)	9 (35)	0.38	14 (54)	12 (46)	0.21
	Left colon	41	28 (68)	13 (32)		26 (63)	15 (37)	
	Rectum	46	25 (54)	21 (46)		34 (74)	12 (26)	
Histological grade	Well/moderate	110	67 (61)	43 (39)	0.23	73 (66)	37 (34)	0.27
	Poor	3	3 (100)	0 (0)		1 (33)	2 (67)	
pT status	pT1 + pT2	51	37 (73)	14 (27)	0.03*	39 (76)	12 (24)	0.02*
	pT3 + pT4	62	33 (53)	29 (47)		35 (56)	27 (44)	
Lymphatic invasion	Negative	66	42 (64)	24 (36)	0.40	51 (77)	15 (23)	0.002*
	Positive	47	28 (60)	19 (40)		23 (49)	24 (51)	
Vascular invasion	Negative	51	37 (73)	14 (27)	0.03*	37 (73)	14 (27)	0.11
	Positive	62	33 (53)	29 (47)		37 (60)	25 (40)	
Nerve plexus invasion	Negative	94	58 (62)	36 (38)	0.56	65 (69)	29 (31)	0.06**
	Positive	19	12 (63)	7 (37)		9 (47)	10 (53)	
Lymph node metastasis	Negative	59	41 (69)	18 (31)	0.06**	41 (69)	18 (31)	0.23
	Positive	54	29 (54)	25 (46)		33 (61)	21 (39)	
Distant metastasis	Negative	100	65 (65)	35 (35)	0.06**	67 (67)	33 (33)	0.26
	Positive	13	5 (38)	8 (62)		7 (54)	6 (46)	
pStage	I + II	55	39 (71)	16 (29)	0.04*	40 (73)	15 (27)	0.08
	III + IV	58	31 (53)	27 (47)		34 (59)	24 (41)	

Parentheses: percentage.

CC3: cleaved caspase-3; LC3B: microtubule-associated protein-1 light chain-3B.

\* Statistically significant.

\*\* Marginally significant.

shrinkage, nuclear condensation, and activation of endonucleases [2,3]. Immunohistochemical (IHC) assays with antibodies for CC3 can identify apoptotic cells in archival material [6,7]. In addition, CC3 can be observed in the nuclei and cytoplasm of cells without morphological apoptotic features, which indicates earlier detection of the apoptotic cascade compared with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method.

Necrosis has long been regarded as an accidental and unregulated cell death. However, accumulating evidence indicates that necrosis can also be regulated through a defined signal transduction pathway called “necroptosis” [8]. Necroptosis is a normal physiological process, but is often impaired during the pathogenesis of several diseases, including cancer [9,10]. A critical characteristic of necroptosis is the formation of “necrosomes” that require activation by receptor-interacting protein kinase (RIP)-1 and RIP3. RIP3 phosphorylates mixed-lineage kinase domain-like protein (MLKL) to promote necroptosis. Therefore, MLKL is identified as a key downstream effector of necroptosis signaling [11,12].

Autophagy is a catabolic process that eliminates unnecessary, injured or aged proteins and organelles and subsequently recycles degraded products to maintain cell homeostasis [13]. During autophagy, cytoplasmic components are sequestered and engulfed by double-membrane structures known as autophagosomes. Autophagosomes fuse with lysosomal vesicles, in which the sequestered components are degraded by lysosomal hydrolases [13]. Initiating autophagy upregulates expression of microtubule-associated protein-1 light chain-3 (LC3), which is essential for autophagosome formation. LC3 has three isoforms: LC3A, LC3B, and LC3C. During autophagosome formation, LC3B undergoes lipidation and is recruited to the autophagosome membrane. Accumulation of LC3B in autophagosomes is often used as a marker for autophagy [14].

The RAS signaling pathway affects malignant progression of various cancers including CRC. *KRAS* is the main mutated RAS gene in CRCs and is found in about 40% of CRCs [15]. The hot spots for *KRAS* mutations are in codons 12 and 13 of exon 1 [16]. *KRAS* mutations lead to continuous activation of downstream pathways, including the RAS–MEK–ERK and phosphatidylinositol 3-kinase–AKT pathways [16]. Lack of *KRAS* mutation is critical to the effectiveness of anti-epidermal growth factor receptor therapy in patients with CRC [17].

The tumor suppressor protein, p53, has a variety of anticancer functions [18]. It is activated in the presence of cellular stress, including DNA damage, hypoxia, and oncogene activation. p53 induces cell-cycle arrest, to allow either cell repair or apoptosis to remove the damaged cell (mainly via the intrinsic pathway) [19]. The p53 gene is mutated in at least 50% of human cancers, resulting in disruption of tumor suppressor functions [20,21].

Many *in vitro* studies have shown that the interaction between apoptosis, necroptosis and autophagy is extremely complex and their roles in cancer can be influenced by many factors, such as tissue and cell types, tumor stage, type of oncogenic mutation, extent of damage or stress, and levels of intratumoral oxygen or nutrients [22]. However, these findings are not adequately assessed in human samples. In the present study, therefore, we assessed patterns of apoptosis, necroptosis, and autophagy in CRCs by determining the expression of CC3, phosphorylated-MLKL (p-MLKL), and LC3B in tumor tissues using specific antibodies, and we analyzed their associations with clinicopathological parameters and *KRAS* and p53 status.

## 2. Materials and methods

### 2.1. Patients and tissue samples

We obtained CRC tissue samples from the archives of the Department of Diagnostic Pathology, Fukuoka Sanno Hospital and Takagi Hospital, Japan, which had come from 113 patients with CRC who underwent surgery between 2009 and 2016. None of the patients underwent radiotherapy or chemotherapy prior to surgery. This study was approved by the ethics committees of Fukuoka Sanno Hospital and Kobe University Graduate School of Health Sciences (reference numbers FS-127 and 425, respectively). All patients provided written informed consent for the use of their tissue samples for this study.

Tumors were classified by TNM classification [23]. Clinicopathological parameters included age, sex, tumor location, histological grade, pT status, lymphatic and vascular invasion, nerve plexus invasion, lymph node and distant metastases, and pStage (Table 1). Lymphatic and venous invasions were evaluated by D2-40 immunostaining and Victoria blue-hematoxylin–eosin (HE) staining, respectively.

### 2.2. Immunostaining

Surgical specimens were fixed in neutral buffered formalin and embedded in paraffin. Representative paraffin blocks from each specimen were selected for IHC by a pathologist (M.T.) and contained invasive edges and viable tumors. Four- $\mu\text{m}$ -thick sections were cut serially and mounted on aminopropyltriethoxysilane-coated glass slides (Matsunami Glass, Osaka, Japan). The sections were deparaffinized with xylene and rehydrated with an ethanol gradient. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min. Heat-induced antigen retrieval using a pressure cooker was performed for 10 min. Optimal soaking solutions were determined by preliminary experiments as follows: 1 mM ethylenediaminetetraacetic acid solution, pH 8.0 for CC3 and p-MLKL, and 10 mM citrate buffer, pH 6.0 for LC3B and p53. After pressure cooking, the sections were left at room temperature (RT) to cool in the soaking solution for 30 min, then rinsed in running tap water, followed by 10 mM phosphate buffered saline (PBS; pH 7.2). The sections were then incubated with anti-CC3 (Asp175) rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-p-MLKL (Ser358) rabbit monoclonal antibody (Clone EPR9514; Abcam, Cambridge, UK), anti-LC3B rabbit polyclonal antibody (Cell Signaling Technology), or anti-p53 mouse monoclonal antibody (clone DO-7; Nichirei Bioscience, Tokyo, Japan) overnight at RT. The sections were then rinsed in PBS, and incubated for 1 h at RT with anti-rabbit or anti-mouse horseradish peroxidase polymer (Histofine Simple Stain MAX-PO, Nichirei Bioscience). The sections were developed with diaminobenzidine solution (Dako, Glostrup, Denmark), and counterstained with Mayer's hematoxylin. Negative controls were produced by replacing the primary antibody with PBS. Positive staining controls were included in each run as follows: surface epithelial cells from non-tumorous colorectal tissue (internal positive control) for CC3, basal cell skin cancer for p-MLKL, nerve plexus cells in colorectal tissue (internal positive control) for LC3B, and pancreatic cancer for p53.

### 2.3. Immunostain assessment

The immunostained sections were reviewed independently by three investigators (F.S., M.S. and S.K.), who were blinded to patients' clinicopathological characteristics. Cytoplasmic or cytoplasmic/nuclear staining for CC3 and cytoplasmic staining for p-MLKL and LC3B were considered positive. We selected 10 hot spots (areas with the highest density of positive cells) from tumor tissues using an optical microscope at  $\times 100$  magnification. We evaluated only tumor cell clusters in viable areas; necrotic areas were excluded. In these areas, total tumor cells,

and CC3-, p-MLKL- and LC3B-positive tumor cells were counted at  $\times 400$  magnification. To obtain percentages of positively stained tumor cells, numbers of CC3-, p-MLKL- and LC3B-positive cells were divided by the total number of tumor cells. The average of the 10 hot spots was taken to represent the mean percentage of positive tumor cells in each tumor. Based on the mean percentage of positive tumor cells, CC3 and LC3B were regarded to be highly expressed (CC3<sup>High</sup> and LC3B<sup>High</sup>) when staining was observed in  $> 10\%$  of tumor cells, and as lowly expressed (CC3<sup>Low</sup> and LC3B<sup>Low</sup>) when staining was observed in  $\leq 10\%$  of tumor cells. Based on our previous study [24], the trimmed mean value was used to define these border values. p-MLKL consistently showed no staining, or positive staining in only a few tumor cells; therefore, all tumors were regarded to have low p-MLKL expression (p-MLKL<sup>Low</sup>). Necrotic areas were excluded from the evaluation.

Nuclear p53 staining of target cells was considered positive. p53 mutations lead to stabilization or loss of p53 protein, and both positive staining in the majority of tumor cells and completely negative staining are associated with p53 mutation [25]. Therefore, positive staining in  $\leq 70\%$  of tumor cells was considered to be the wild-type expression pattern of p53 protein (p53<sup>WT-ex</sup>), and negative or positive staining in  $> 70\%$  of tumor cells was the mutant-type expression pattern (p53<sup>MT-ex</sup>).

### 2.4. Analysis of KRAS gene status

The TheraScreen K-RAS Mutation Kit (Qiagen N.V., Venlo, Netherlands) was used to detect somatic mutations in KRAS genes. One paraffin block containing a high density of tumor cells was selected from each case, from which H-E-stained slides were reviewed by a pathologist (M.T.). For each case, 10 unstained, 10- $\mu\text{m}$  thick sections were cut and mounted on aminopropyltriethoxysilane-coated slides. The region with the highest density of tumor cells was selected for microdissection and its targets were confirmed by direct microscopic visualization. Tumor DNA was extracted from the microdissected fragments. Real-time polymerase chain reaction was performed to analyze seven mutations (Gly12Ala, Gly12Asp, Gly12Arg, Gly12Cys, Gly12Ser, Gly12Val, and Gly13Asp). An amplification refractory mutation system was used for allele-specific amplification, which was detected using Scorpion primers. Based on gene analysis, tumors were classified into KRAS wild-type (KRAS<sup>WT</sup>) or KRAS mutant-type (KRAS<sup>MT</sup>).

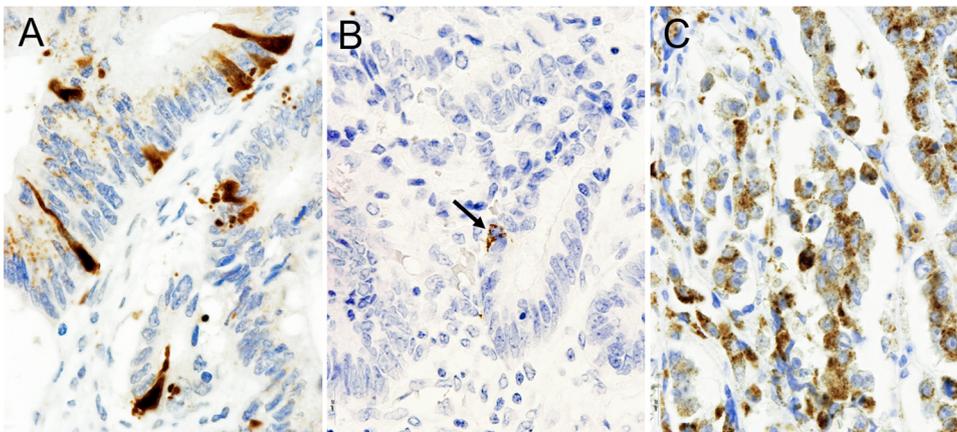
### 2.5. Statistical analysis

All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University; Saitama, Japan). The chi-square test or Fisher's exact test was used to evaluate associations of CC3 or LC3B expression with clinicopathological parameters or with KRAS and p53 status. The Kruskal-Wallis test was used to analyze associations between CC3 and LC3B expression and tumor location. Strength of correlation between CC3 and LC3B expression was assessed by Spearman's rank correlation coefficient.  $P < 0.05$  was considered significant.

## 3. Results

### 3.1. Patients and tumor characteristics

The patients' median age was 64.4 years (range: 41–86 years); 34 (30%) were  $< 60$  years old and 79 (70%) were  $\geq 60$  years old. They included 69 men and 44 women. Of the 131 tumors, 26 (23%) were localized in the right colon, 41 (36%) in the left colon, and 46 (41%) in the rectum; 110 tumors (97%) were low-grade (well or moderately differentiated) and 3 (3%) were high-grade (poorly differentiated); 33 tumors (29%) were pT1, 18 (16%) were pT2, 29 (26%) were pT3, and 33 (29%) were pT4. Lymphatic and vascular invasion were seen in 47 (42%) and 62 (55%) patients, respectively; 59 patients (52%) were



**Fig. 1.** Representative immunostaining patterns for CC3, p-MLKL, and LC3B in CRC tissue samples. (A) High CC3 expression, (B) low p-MLKL expression, (C) high LC3B expression. Although several cancer cells show cytoplasmic and/or nuclear staining for CC3 (A) and cytoplasmic staining for LC3B (C), p-MLKL expression is seen in only one cancer cell (arrow) (B).

lymph node-negative, and 54 (48%) were lymph node-positive; 100 patients (88%) had no distant metastasis at the time of surgery, and 13 patients (12%) had distant metastasis; 39 patients (35%) had stage I, 16 (14%) had stage II, 52 (46%) had stage III, and 6 (5%) had stage IV disease.

### 3.2. CC3, p-MLKL, and LC3B expression in CRC tissues

Fig. 1 shows representative staining for CC3, p-MLKL, and LC3B. Staining for CC3 was either cytoplasmic or cytoplasmic/nuclear (Fig. 1A). Most tumor cells with CC3<sup>+</sup> nuclear staining showed characteristics of apoptotic nuclei, such as chromatin condensation and margination. In addition, apoptotic bodies were usually CC3<sup>+</sup>. Immunostaining for p-MLKL and LC3B was invariably granular cytoplasmic (Fig. 1B, C).

Among the 113 tumors, 43 (38%) were classified as CC3<sup>High</sup> and 70 (62%) as CC3<sup>Low</sup>; 39 (35%) were classified as LC3B<sup>High</sup> and 74 (65%) as LC3B<sup>Low</sup>. However, all tumors were observed to be p-MLKL<sup>Low</sup>, with no or only a few positive cells. No correlation was detected between CC3 and LC3B levels ( $r = -0.06$ ,  $P = 0.50$ ). Few differences in CC3 and LC3B levels were detected between invasive and non-invasive components within the same tumors.

Epithelial cells on the surfaces of normal colorectal mucosa were positive for CC3. CC3 was also expressed in lymphocytes in the germinal centers of submucosal lymphoid follicles. No p-MLKL<sup>+</sup> cells were found in normal tissues. Nerve plexus cells consistently showed moderate to strong LC3B immunostaining, whereas normal colorectal mucosa lacked LC3B expression.

### 3.3. Association between CC3 and LC3B expression and clinicopathological parameters

We examined associations between CC3 and LC3B levels and clinicopathological parameters, including patients' age and sex, tumor location, histological grade, pT status, lymphatic and vascular invasion, nerve plexus invasion, lymph node and distant metastases, and pStage (Table 1). CC3<sup>High</sup> was associated with high pT status ( $P = 0.03$ ), vascular invasion ( $P = 0.03$ ), high pStage ( $P = 0.04$ ), and marginally with lymph node ( $P = 0.06$ ) and distant metastases ( $P = 0.06$ ). LC3B<sup>High</sup> was also associated with high pT status ( $P = 0.02$ ), lymphatic invasion ( $P = 0.002$ ), and marginally with nerve plexus invasion ( $P = 0.06$ ).

We further analyzed associations of combined CC3 and LC3B expression with parameters for tumor aggressiveness (Table 2). Patients with either CC3<sup>High</sup>, LC3B<sup>High</sup> or both tumors were significantly more likely to have high pT status ( $P = 0.0007$ ), lymphatic invasion ( $P = 0.03$ ), vascular invasion ( $P = 0.003$ ), distant metastasis ( $P = 0.04$ ), and high pStage ( $P = 0.04$ ), compared to patients with CC3<sup>Low</sup>/LC3B<sup>Low</sup> tumors.

### 3.4. Association between CC3 and LC3B levels and KRAS and p53 status

Of the 69 tumors (out of 113) for which *KRAS* gene status was evaluable, 41 (59%) were *KRAS*<sup>WT</sup> and 28 were *KRAS*<sup>MT</sup> (codon 12:  $n = 23$ ; codon 13:  $n = 5$ ). Of the 113 tumors, 26 (23%) were p53<sup>WT-ex</sup> and 87 (77%) were p53<sup>MT-ex</sup>. Although neither CC3 nor LC3B expression was associated with *KRAS* mutational status, LC3B<sup>High</sup> was significantly associated with p53<sup>MT-ex</sup> ( $P = 0.003$ ) (Table 3). No association between CC3 expression and p53 status was detected.

## 4. Discussion

The interaction between apoptosis, necroptosis and autophagy is extremely complex, and the pathway chosen by a cell will vary under different circumstances, such as levels of oxygen or nutrients and the extent of damage or stress [22]. However, a particular pathway ultimately predominates over others, and secondary pathways are only activated if the dominant pathway is attenuated. For example, apoptosis is the first choice in most settings, and necroptosis acts only as an alternative to ensure cell death is inevitable if the apoptotic machinery fails [22]. Although autophagy is activated by ATP depletion, necroptosis is induced if autophagy fails to maintain necessary energy levels [22]. In the present study, we first found that CC3<sup>High</sup> and LC3B<sup>High</sup> were detected in 38% and 35% of the 113 CRCs, respectively, but no or only a few p-MLKL-positive cells were observed in any of the tumors. These results suggest that necroptosis is a rare process in CRC.

Next, we found that CC3<sup>High</sup> was significantly associated with high pT status ( $P = 0.03$ ), vascular invasion ( $P = 0.03$ ), high pStage ( $P = 0.04$ ), and marginally with lymph node ( $P = 0.06$ ) and distant metastases ( $P = 0.06$ ). Another study also found CC3<sup>High</sup> to be significantly associated with distant metastasis and high pStage and with shorter survival in CRC patients [26]. We found LC3B<sup>High</sup> was significantly associated with high pT status ( $P = 0.02$ ), lymphatic invasion ( $P = 0.002$ ), and marginally with nerve plexus invasion ( $P = 0.06$ ). Another study reported LC3B expression to be significantly associated with tumor differentiation, tumor margin, vascular and nerve plexus invasion, lymph node metastasis, and pStage [27]. In addition, a recent study demonstrated a statistical trend for shorter overall survival in patients with LC3B<sup>+</sup> CRC [28].

We also analyzed CC3 and LC3B data in combination. Compared with CC3<sup>Low</sup>/LC3B<sup>Low</sup> tumors, tumors with either CC3<sup>High</sup>, LC3B<sup>High</sup> or both were significantly associated with high pT status ( $P = 0.0007$ ) and vascular invasion ( $P = 0.003$ ). Statistical significance was also more robust for the combination of CC3 and LC3B status than with CC3 alone (only  $P = 0.03$  for either pT status or vascular invasion) or LC3B alone ( $P = 0.02$  for pT status,  $P = 0.11$  for vascular invasion). Tumors with either CC3<sup>High</sup>, LC3B<sup>High</sup> or both were also associated with lymphatic invasion ( $P = 0.03$ ), distant metastasis ( $P = 0.04$ ), and high pStage

**Table 2**  
Associations between combined CC3/LC3B expression and parameters of tumor aggressiveness.

Variables		n	Either CC3 <sup>High</sup> , LC3B <sup>High</sup> or both	Both CC3 <sup>Low</sup> and LC3B <sup>Low</sup>	P value
Histological grade	Well/moderate	110	64 (58)	46 (42)	0.63
	Poor	3	2 (67)	1 (33)	
pT status	pT1 + pT2	51	21 (41)	30 (59)	0.0007*
	pT3 + pT4	62	45 (73)	17 (27)	
Lymphatic invasion	Negative	66	33 (50)	33 (50)	0.03*
	Positive	47	33 (70)	14 (30)	
Vascular invasion	Negative	51	22 (43)	29 (57)	0.003*
	Positive	62	44 (71)	18 (29)	
Nerve plexus invasion	Negative	94	53 (56)	41 (44)	0.23
	Positive	19	13 (68)	6 (32)	
Lymph node metastasis	Negative	59	30 (51)	29 (49)	0.07
	Positive	54	36 (67)	18 (33)	
Distant metastasis	Negative	100	55 (55)	45 (45)	0.04*
	Positive	13	11 (85)	2 (15)	
pStage	I + II	55	27 (49)	28 (51)	0.04*
	III + IV	58	39 (67)	19 (33)	

Parentheses: percentages.  
CC3: cleaved caspase-3; LC3B: microtubule-associated protein-1 light chain-3B.  
\* Statistically significant.

**Table 3**  
Associations between CC3 and LC3B levels and KRAS and p53 status.

Variables	n	CC3			LC3B			
		Low	High	P value	Low	High	P value	
KRAS	WT	41	25 (61)	16 (39)	0.11	25 (61)	16 (39)	0.81
	MT	28	12 (43)	16 (57)		15 (54)	13 (46)	
p53	WT-ex	26	15 (58)	11 (42)	0.39	23 (88)	3 (12)	0.003*
	MT-ex	87	55 (63)	32 (37)		51 (59)	36 (41)	

Parentheses: percentages.  
CC3: cleaved caspase-3; LC3B: microtubule-associated protein-1 light chain-3B;  
WT: wild type; MT: mutant type; WT-ex: wild-type expression pattern; MT-ex: mutant-type expression pattern.  
\* Statistically significant.

(P = 0.04). These results suggest that apoptosis and autophagy affect CRC progression. However, because few studies have assessed IHC expression of CC3 and LC3B in CRC, the clinicopathological significance of CC3 and LC3B in CRC needs further investigation.

Hypoxic and nutrient-limiting tumor environments lead to elevated invasion and metastasis in many cancers. Apoptosis can also be a response to hypoxia and low nutrient conditions [29]. Thus, increased CC3 expression may be simply a secondary event in aggressive CRCs. Conversely, some studies have suggested that apoptotic cancer cells can increase proliferation of viable cancer cells by activating caspase-3 [30,31]. The mechanism and role of increased CC3 expression in aggressive CRCs remains inconclusive.

In hypoxic and nutrient-limited conditions, cancer cells require autophagy, both to provide nutrients and oxygen by recycling cellular constituents, and to clear accumulated damaged organelles; autophagy can thus protect cancer cells from undergoing apoptosis, and promote their survival [32–34]. In addition, a critical role for autophagy in motility, invasion, and metastasis has been demonstrated. For example, autophagy promotes focal adhesion degradation and cancer cell motility through direct interaction of LC3B with paxillin, a core component of focal adhesions [35].

As described above, in advanced cancers, increased autophagy may be a mechanism for tumor survival or even tumor promotion. Thus, inhibiting autophagy could be a promising anticancer therapeutic strategy. Indeed, several clinical trials have been designed to assess the

effectiveness of conventional anticancer agents combined with autophagy inhibitors, such as hydroxychloroquine and chloroquine [36]. For example, hydroxychloroquine, combined with vorinostat, was active, safe and well tolerated in patients with refractory CRC [37]. Our results suggest that autophagy inhibitors might be effective for CRCs with high pT classification or positive lymphatic invasion because of upregulated autophagy.

A novel finding in this study is that LC3B<sup>High</sup> was significantly associated with p53<sup>MT-ex</sup> (P = 0.003). *In vitro* studies with human cancer cells reported the role of p53 in controlling autophagy. Although p53 can induce autophagy in response to DNA damage or p53 overexpression, physiological levels of p53 can inhibit autophagy in other cellular contexts [38]. In addition, p53 can downregulate LC3B in nutrient-deprived cancer cells [39]. In other words, p53 mutation can disrupt the ability to downregulate autophagy, which implies that mutant p53 increases autophagy, leading to more aggressive CRC. Conversely, CC3 expression showed no association with p53 status. The main reason for this may be that the p53-independent apoptotic pathway is also activated in CRCs. p53 mainly induces apoptosis via the intrinsic pathway, whereas death receptors can induce apoptosis independently of p53 [40]. For example, Fas-mediated apoptosis is induced in colorectal cancer cell lines regardless of their p53 status [41].

In conclusion, this immunohistochemical study produced the following novel findings: in human CRC tissues, no or only a few p-MLKL-positive cells were observed, tumor aggressiveness was more robustly associated with the combined status of CC3<sup>High</sup> and LC3B<sup>High</sup> than with CC3<sup>High</sup> alone or LC3B<sup>High</sup> alone, and LC3B<sup>High</sup> was associated with p53<sup>MT-ex</sup>. These results indicate that necroptosis is a rare process in untreated CRC, apoptosis and autophagy are highly activated in aggressive CRC, and p53 mutation may lead to the upregulation of autophagy. However, our study is descriptive and lacks functional investigation. Therefore, to understand the role of combined CC3 and LC3B in CRC progression more fully, additional studies, including analysis of patient survival, are needed.

**Declaration of interests**

The authors declare no conflict of interest.

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## Authors' contribution

F.S. and S.K. designed the study, interpreted the data, and wrote the manuscript. F.S. and M.S. performed the immunostaining and statistical analyses. M.T. performed the histopathological diagnosis. H.O. participated in the design of the study and aided in data analysis. All authors read and approved the final manuscript.

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