pISSN 1598-2998, eISSN 2005-9256

Cancer Res Treat. 2019;51(4):1518-1526

https://doi.org/10.4143/crt.2019.008

Original Article

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Loss of LKB1 Protein Expression Correlates with Increased Risk of Recurrence and Death in Patients with Resected, Stage II or III Colon Cancer

Maria Sfakianaki, BSc[1]
Chara Papadaki, PhD[1]
Maria Tzardi, MD, PhD[2]
Maria Trypaki, BSc[1]
Sardar Alam, MSc[1]
Eleni D. Lagoudaki, MD[1]
Ippokratis Messaritakis, PhD[1]
Odysseas Zoras, MD, PhD[3]
Dimitris Mavroudis, MD, PhD[1,4]
Vassilis Georgoulias, MD, PhD[5]
John Souglakos, MD, PhD[1,4]

[1]*Laboratory of Translational Oncology, School of Medicine, University of Crete, Heraklion, Departments of* [2]*Pathology,* [3]*Surgical Oncology, and* [4]*Medical Oncology, University General Hospital of Heraklion, Iraklio,* [5]*School of Medicine, University of Crete, Heraklion, Greece*

Correspondence: John Souglakos, MD, PhD
Department of Medical Oncology,
University General Hospital of Heraklion,
Voutes and Stavrakia, PO BOX 1352, Heraklion,
Crete 71110, Greece
Tel: 30-2810-392783
Fax: 30-2801-392857
E-mail: oncsec@med.uoc.gr

Received January 2, 2019
Accepted March 17, 2019
Published Online March 20, 2019

Purpose

The purpose of this study was to investigate the prognostic significance of liver kinase b1 (LKB1) loss in patients with operable colon cancer (CC).

Materials and Methods

Two hundred sixty-two specimens from consecutive patients with stage III or high-risk stage II CC, who underwent surgical resection with curative intent and received adjuvant chemotherapy with fluoropyrimidine and oxaliplatin, were analyzed for LKB1 protein expression loss, by immunohistochemistry as well as for *KRAS* exon 2 and $BRAF^{V600E}$ mutations by Sanger sequencing and *TS*, *ERCC1*, *MYC*, and *NEDD9* mRNA expression by real-time quantitative reverse transcription polymerase chain reaction.

Results

LKB1 expression loss was observed in 117 patients (44.7%) and correlated with right-sided located primaries (p=0.032), and pericolic lymph nodes involvement (p=0.003), $BRAF^{V600E}$ mutations (p=0.024), and *TS* mRNA expression (p=0.041). Patients with LKB1 expression loss experienced significantly lower disease-free survival (DFS) (hazard ratio [HR], 1.287; 95% confidence interval [CI], 1.093 to 1.654; p=0.021) and overall survival (OS) (HR, 1.541; 95% CI, 1.197 to 1.932; p=0.002), compared to patients with LKB1 expressing tumors. Multivariate analysis revealed LKB1 expression loss as independent prognostic factor for both decreased DFS (HR, 1.217; 95% CI, 1.074 to 1.812; p=0.034) and decreased OS (HR, 1.467; 95% CI, 1.226 to 2.122; p=0.019).

Conclusion

Loss of tumoral LKB1 protein expression, constitutes an adverse prognostic factor in patients with operable CC.

Key words
LKB1, *KRAS*, *BRAF*, MSI, Prognosis, Stage II-III

Introduction

Colorectal cancer (CRC) causes high morbidity and mortality rates; however, both declined the last four decades in Western countries [1]. Cancer prevention could be attributed through screening at an early stage and more effective treatment modalities. Moreover, it was shown that patients with high-risk stage II and stage III colon cancer (CC) who received the current standard treatment according to the National Comprehensive Cancer Network guidelines had a benefit on their survival rates [2].

For stage III disease, combination chemotherapy with a backbone of fluoropyrimidine and oxaliplatin (FOLFOX, CAPOX) is the current standard of care, since it leads to prolongation of both disease-free survival (DFS) and overall survival (OS) [3]. The results for the addition of oxaliplatin in OS and DFS for patients with stage II CC with high-risk features (such as T4 tumors, obstruction or perforation, and vessel invasion) showed a marginal but significant [3]. Despite

that, it has long been recognized patients' individual risk of recurrence varies widely even in patients with the same stage in CC. Since today, microsatellite instability (MSI) status is the only biomarker used in daily clinical practice [4].

Several studies have reported that germline inactivating mutations in liver kinase b1 (LKB1) to be the primary cause syndromes, such as the Peutz-Jeghers syndrome, which confer an increased risk of cancer development [5,6]. The LKB1 also known as serine threonine kinase 11 (STK11), was initially discovered as a tumor suppressor gene and it has been implicated on initiation and progression of neoplastic diseases [5]. It controls a wide range of diverse cellular processes through phosphorylation of the adenosine monophosphate-activated protein kinase (AMPK) protein when cellular energy levels are depleted promoting ATP [6]. Furthermore, loss of LKB1 has been shown to influence cell polarity, epi-thelial-to-mesenchymal transition, apoptosis, angiogenesis, and cell cycle inhibition [7]. Also, negatively regulates the mammalian target of rapamycin signaling [5] and mediates p53 activation [8]. LKB1 has been most intensively studied using lung cancer mouse models [9]. Finally, the results of a first comprehensive meta-analysis suggested that decreased LKB1 expression significantly contributed to shorter OS in solid tumor patients [10]. However, additional studies rela-ted to specific tumor types and perspectives are required to verify the clinical utility of decreased LKB1 levels in solid tumors.

Based on the above-mentioned data we conducted a retrospective biomarkers-based studies, in order to elucidate the clinicopathological features and prognostic significance of LKB1 loss of expression in high-risk stage II and stage III CC, treated with oxaliplatin and fluoropyrimidine combination adjuvant chemotherapy.

Materials and Methods

1. Patients' population

Two hundred and two formalin-fixed, paraffin-embedded samples from consecutive patients with stage III or high-risk stage II CC treated with FOLFOX or CAPOX has been studied.

2. Specimens' selection, DNA and RNA extraction

The most enriched in cancer cells areas were selected by a pathologist (M.T.) and afterwards, serial sections of 5 μm were stained by nuclear Fast Red (Sigma-Aldrich, St. Louis, MO). Micro-dissection, using a piezoelectric micro-dissector (Eppendorf, Hamburg, Germany) was performed in cases with lower than 80% of neoplastic cells in the examined section. Extraction of nucleic acids (DNA and RNA) was performed, according to the manufacturer's protocol as previously described [11].

3. *KRAS* and *BRAF* mutational analysis

KRAS exon 2 and $BRAF^{V600E}$ mutation mutations analysis was carried out by Sanger sequencing after polymerase chain reaction (PCR) amplification and by reverse transcription PCR using allelic discrimination method, respectively, as previously described as previously reported [11,12]. Analysis was performed using the SDS 2.3 software [13].

4. MSI status

The MSI analysis was carried out with the use of Promega MSI Analysis System (Promega, Madison, WI) according to the manufacturer's instructions. Amplicons for MSI detection was performed by capillary electrophoresis on an ABI 3130xl Genetic Analyzer following PCR amplification and analyzed using GeneMapper Software, ver. 3.7 (Applied Biosystems/Life Technologies, Grand Island, NY) [14].

5. mRNA expression analysis

Synthesis of cDNA was done as described previously [15]. Primers and probes were designed using the Primer Express 2.0 Software (Applied Biosystems) according to the Ref Seq NM_002467.4 for *MYC-ERCC1-NEDD9-TS*. The sets of primers probes are provided in S1 Table, while those for the housekeeping genes, *β-actin* and *PGK* have been previously published [13]. The quantification of mRNA expression was carried out using the $2^{-(\Delta CT\ sample - \Delta CT\ calibrator)}$ method, as previously described [13]. Only triplicates with a standard deviation less than 0.25 were accepted.

6. Immunohistochemistry of LKB1

We performed immunostain using Thermo Scientific UltraVision Quanto Detection System HRP and polyclonal antibody for LKB1 (1:100 dilution, Thermo Scientific, Waltham, MA). Adult seminiferous tubules of the testis were used as a positive control as it shows the highest levels of LKB1 expression [16]. Negative control was obtained by omitting the primary antibody. Immunohistochemistry (IHC) staining intensity was measured using a scaling system of 0 (no expression), 1, 2, and 3 (highest expression) blinded by a pathologist. A weighted index (WI) was applied in both the nucleus and the cytoplasm using the equation WI=% tumor stain X intensity score as used in another study [16]. Cyto-

plasmic, nuclear expression or both were considered a positive stain. The staining of LKB1 was interpreted without knowledge of its genetic status.

7. Study design and statistical analysis

DFS was estimated as the interval between the date of colectomy to the first documented disease progression, second primary CC or death. OS was calculated from the date of surgery to date of death. The Kaplan-Meier survival curves were used for survival analysis and correlations with the studied parameters, while Cox proportional hazards model was applied to weigh the validity of considered factors on study defined events. The statistically significant factors were then incorporated in a multivariate Cox proportional hazards regression model in order to estimate their unbiased significance on progression or survival. The only p-value of < 0.05 was considered as significant.

The median values of mRNA expression were used as cut-off points, with samples above or equal to the median characterized as high expressing, while those with value below the median as low expression. The laboratory research was completed blinded to the clinical parameters. Correlations between the studied biomarker with baseline characteristics were calculating by Fisher exact test for categorical variables or logistic regression for continuous ones.

8. Ethical statement

The study has been approved by the Ethics and Scientific Committees of the University General Hospital of Heraklion (number of approval: 2058) and written informed consent for the use of their tissue for translational research was obtained from all patients. All authors, declare no competing interest regarding this study.

Table 1. Stage II or III patients: clinical characteristics and pathological features

Feature	No. (%) (n=262)
Age, median (range, yr)	67 (33-75)
≤ 70	162 (62.0)
> 70	100 (38.0)
Sex	
Male	152 (58.0)
Female	110 (42.0)
Performance status (ECOG)	
0	196 (75.0)
1	66 (25.0)
Stage	
IIa	90 (34.0)
IIb	14 (6.0)
IIIa	19 (7.0)
IIIb	81 (31.0)
IIIc	58 (22.0)
Tumor grade	
Low	160 (61.0)
High	102 (39.0)
Primary tumor (T)	
T2	27 (10.3)
T3	216 (82.4)
T4	19 (7.3)
Mucinous	
Yes	59 (23.0)
No	203 (77.0)
Obstruction	27 (10.0)
Perforation	40 (15.0)
Location	
Right sided	91 (35.0)
Left sided	171 (65.0)
Regimen	
CAPOX	171 (65.0)
FOLFOX	91 (35.0)
No. of retrieved lymph nodes, median (min-max)	15 (6-108)
No. of positive lymph nodes, median (min-max)	1 (0-18)

Results

1. Patients' characteristics and clinicopathological features

The main demographic and clinical characteristics of the study population are summarized in Table 1. Briefly, patients with CC were predominately males (58%), with a median age of 67 years and the majority of them with a good performance status of 0-1. In addition, 60% of the patients were diagnosed with stage III colon cancer, 65% had primary tumor located in the left colon and 61% low-grade tumors (Table 1). Almost two-thirds of the patients (65%) received adjuvant treatment with CAPOX and the rest one third with FOLFOX. At the time of analysis and after a median follow-up of 120.7 months (min-max, 11.3 to 161.1 months); 71 (27%) disease relapses and 48 (18%) deaths have been recorded.

2. Laboratory analysis and correlations

The results of the laboratory analysis are presented in Table 2. Analysis for LKB1 protein expression was successfully performed in all 254 specimens (96.9%), whereas *KRAS*

Table 2. Laboratory analysis

Feature	No. (%) (n=262)
LKB1 protein expression	
Negative	117 (44.7)
Positive	137 (52.3)
Failed	8 (3.1)
***ERCC1* mRNA expression**	
Low	124 (47.3)
High	123 (46.9)
Failed	15 (5.7)
***MYC* mRNA expression**	
Low	125 (47.7)
High	125 (47.7)
Failed	12 (4.6)
***NEDD9* mRNA expression**	
Low	125 (47.7)
High	124 (47.3)
Failed	13 (5.0)
***TS* mRNA expression**	
Low	113 (43.1)
High	113 (43.1)
Failed	36 (13.8)
***BRAF*^{V600E} status**	
WT	233 (88.9)
Mutant	13 (5.0)
Failed	16 (6.1)
***KRAS* exon 2 mutation**	
WT	169 (64.5)
Mutant	82 (31.3)
Failed	11 (4.2)
MMR status	
Proficient	200 (76.3)
Deficient	35 (13.4)
Failed	27 (10.3)

MMR, mismatch repair.

exon 2 and *BRAF* exon 15 mutation analysis was performed in 251 (95.8%) and 246 (93.9%) specimens, respectively (Table 2, S2-S5 Figs.). Mismatch repair (MMR) system status was successfully analyzed in 235 (89.7%) specimens, while mRNA expression of *ERCC1*, *MYC*, *NEDD9*, and *TS* was successfully done in 247 (94.3%), 250 (95.4%), 249 (95%), and 226 (86.2%) specimens, respectively (S6 Fig.).

Loss of LKB1 protein expression was observed in 117 (44.7%) tumors and correlated significantly with pericolic lymph node involvement (p=0.003) and primary tumors located in the right colon (p=0.032), whereas no significant correlation of LKB1 protein expression with age, sex, and grade were found (Table 3). In addition, LKB1 protein expression loss was significantly correlated with *BRAF*^{V600E} mutation (p=0.024), and *TS* mRNA (p=0.041). In contrast, LKB1 protein expression loss was not significantly associated with *KRAS* exon 2 mutations, MMR status or *ERCC1*, *MYC*, *NEDD9* mRNA expression (all p > 0.05).

3. Laboratory analysis and patients' outcome

The correlations of analyzed markers and clinic-pathological features with DFS and OS are presented in Tables 4 and 5, respectively. Patients with tumors with LKB1 expression loss showed significantly lower DFS compared with those with LKB1 positive tumors (hazard ratio [HR], 1.287; 95% confidence interval [CI], 1.093 to 1.654; p=0.021) (Table 4, Fig. 1). In addition, patients with *BRAF*^{V600E} mutations in their primary tumors presented higher probability for relapse compared to patients with *BRAF*^{V600E} wild type tumors (HR, 1.976; 95% CI, 1.793 to 2.495; p=0.001) (Table 4). Likewise, patients with *KRAS* exon 2 mutations in their primary tumors presented higher probability for relapse compared to patients with *KRAS* exon 2 wild type tumors, but the difference was marginally significant (HR, 1.757; 95% CI, 1.000 to 3.090; p=0.05). Furthermore, patients with proficient MMR (pMMR) tumor had a significantly higher risk of relapse in comparison with those with deficient MMR (dMMR) tumors (HR, 1.726; 95% CI, 1.289 to 3.514; p=0.025). Finally, stage III disease at diagnosis is correlated with increased risk of progression compared with stage II (HR, 1.803; 95% CI, 1.605 to 2.055; p=0.023). All other comparisons between the mRNA expression of the *ERCC1*, *MYC*, *NEDD9*, and *TS* or several clinicopathological features, such as age, sex, tumor location and grade, did not reveal any significant correlations with DFS (all long-rank p > 0.05).

Regarding OS, patients with tumors with LKB1 expression loss showed significantly lower OS compared with those with LKB1 positive tumors (HR, 1.541; 95% CI, 1.197 to 1.932; p=0.002) (Table 4, Fig. 2). Similarly, patients with *BRAF*^{V600E} mutations in their primary tumors presented higher probability for death compared to patients with *BRAF*^{V600E} mutations wild type tumors (HR, 1.624; 95% CI, 1.143 to 2.309; p=0.007) (Table 4). In addition, patients with pMMR tumor had a significantly higher risk of death in comparison with those with dMMR tumors (HR, 1.375; 95% CI, 1.043 to 2.711; p=0.036). Also, stage III disease at diagnosis is correlated with increased risk of progression compared with stage II (HR, 1.636; 95% CI, 1.487 to 2.011; p=0.03). All other comparison did not reveal any significant correlations between the mRNA expression of the *ERCC1*, *MYC*, *NEDD9*, and *TS* or the detection of *KRAS* exon 2 mutation as well as clinicpathological features, such as age, sex, tumor location and grade, with DFS (all long-rank p > 0.05).

Multivariate analysis for DFS, revealed that LKB1 expression loss (HR, 1.217; 95% CI, 1.074 to 1.812; p=0.034) (Table 5),

Table 3. Correlation of LKB1 expression with patients' characteristics and tumor's features and DNA markers

LKB1 protein expression (n=254)	Negative (n=117)	Positive (n=137)	p-value
Age, median (min-max, yr)	65 (33-75)	67 (37-75)	0.147[a]
Age group (yr)			
≤ 70	72 (61.5)	86 (62.8)	0.897[b]
> 70	45 (38.5)	51 (37.2)	
Sex			
Male	72 (61.5)	74 (54.0)	0.253[c]
Female	45 (38.5)	63 (46.0)	
Lymph node status			
N0	33 (28.2)	68 (49.6)	0.003[b]
N1-2	84 (71.8)	69 (50.4)	
Tumor location			
Right	49 (41.9)	41 (29.9)	0.032[b]
Left	68 (58.1)	96 (70.1)	
Primary tumor (T)			
T2-T3	74 (36.8)	114 (56.7)	0.008[b]
T4	10 (5.0)	3 (1.5)	
Grade			
Low grade	70 (59.8)	90 (65.7)	0.880[b]
High grade	47 (38.5)	47 (34.3)	
***BRAF*^{V600E} status (n=236)**	ND 7	ND 11	
Wild type (n=223)	100 (90.9)	123 (97.6)	0.024
Mutant (n=13)	10 (9.1)	3 (2.4)	
***KRAS* exon 2 (n=246)**	ND 6	ND 5	
Wild type (n=224)	70 (62.7)	91 (70.1)	0.328
Mutant (n=22)	41 (37.3)	41 (29.9)	
MMR status (n=235)	ND 9	ND 16	
Proficient	96 (88.9)	99 (81.8)	0.102
Deficient	12 (11.1)	22 (18.2)	
***ERCC1* mRNA expression (n=246)**	ND 4	ND 4	
High	59 (52.2)	69 (51.9)	0.609
Low	54 (47.8)	64 (48.1)	
***MYC* mRNA expression (n=249)**	ND 2	ND 3	
High	55 (47.8)	69 (51.5)	0.612
Low	60 (52.2)	65 (48.5)	
***NEDD9* mRNA expression (n=247)**	ND 3	ND 4	
High	58 (50.9)	64 (48.1)	0.703
Low	56 (49.1)	69 (51.9)	
***TS* mRNA expression (n=226)**	ND 15	ND 13	
High	58 (56.9)	49 (39.5)	0.041
Low	44 (43.1)	75 (60.5)	

Values are presented as number (%). MMR, mismatch repair. [a]Mann-Whitney test, [b]Pearson chi-square, [c]Fisher exact test.

BRAF^{V600E} mutations (HR, 1.696; 95% CI, 1.365 to 2.294; p=0.011), pMMR status (HR, 1.775; 95% CI, 1.343 to 3.011; p=0.007) and stage III disease (HR, 1.784; 95% CI, 1.335 to 2.762; p=0.006) as independent factors for increased risk of relapse. Similarly, LKB1 expression loss (HR, 1.467; 95% CI, 1.226 to 2.122; p=0.019), *BRAF*^{V600E} mutations (HR, 1.961; 95% CI, 1.656 to 2.949; p=0.001), pMMR status (HR, 1.575; 95% CI, 1.243 to 3.001; p=0.018), and stage III disease (HR, 1.843; 95% CI, 1.356 to 2.623; p=0.009) was statistically associated with risk of death.

Table 4. Univariate analysis for median disease-free and overall survival

	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
LKB1 protein expression (negative vs. positive)	1.287	1.093-1.654	0.021	1.541	1.197-1.932	0.002
KRAS exon2 mutations (mutant vs. wild type)	1.757	1.000-3.090	0.050	1.190	0.607-2.335	0.113
BRAF^{V600E} mutation (mutant vs. wild type)	1.976	1.793-2.495	0.001	1.624	1.143-2.309	0.007
MMR status (proficient vs. deficient)	1.726	1.289-3.514	0.025	1.375	1.043-2.711	0.036
ERCC1 mRNA expression (high vs. low)	1.164	0.610-1.570	0.930	1.003	0.558-1.802	0.993
MYC mRNA expression (high vs. low)	1.179	0.735-1.892	0.494	1.170	0.856-1.598	0.324
NEDD9 mRNA expression (high vs. low)	1.070	0.634-1.219	0.343	1.068	0.601-1.895	0.823
TS mRNA expression (high vs. low)	1.701	0.804-2.598	0.165	1.113	0.472-2.625	0.807
Stage III vs. II	1.803	1.605-2.055	0.023	1.636	1.487-2.011	0.030
Tumor location	1.131	0.683-1.873	0.632	1.016	0.557-1.851	0.906
Age (> 70 yr vs. ≤ 70 yr)	1.035	0.641-1.673	0.887	1.207	0.938-1.843	0.106
Sex (men vs. women)	1.176	0.915-1.804	0.112	1.011	0.981-1041	0.471
Grade (high vs. low)	1.061	0.872-1.201	0.722	1.108	0.536-2.209	0.781

CI, confidence interval; MMR, mismatch repair.

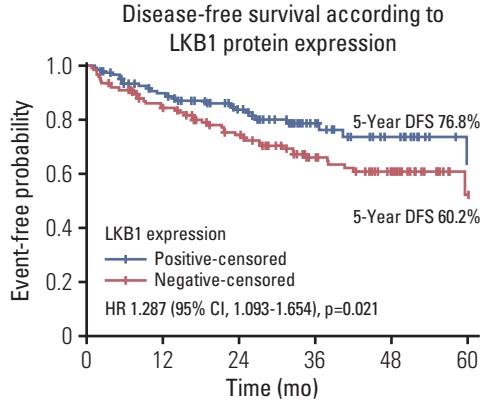

Fig. 1. Disease-free survival according to LKB1 protein expression loss by immunohistochemistry. DFS, disease-free survival; HR, hazard ratio; CI, confidence interval.

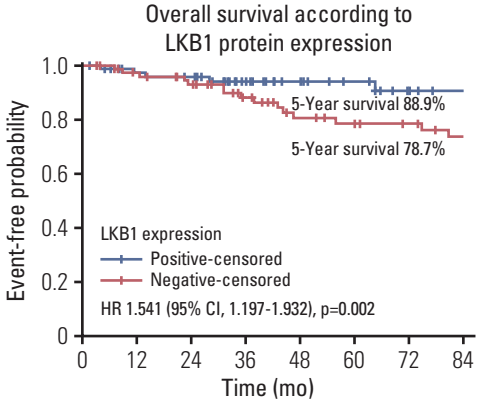

Fig. 2. Overall survival according to LKB1 protein expression loss by immunohistochemistry. HR, hazard ratio; CI, confidence interval.

Table 5. Multivariate analysis for median disease-free and overall survival

	Disease-free survival			Overall survival		
	Hazard ratio	95% CI	p-value	Hazard ratio	95% CI	p-value
LKB1 protein expression (negative vs. positive)	1.217	1.074-1.812	0.034	1.467	1.226-2.122	0.019
KRAS exon2 mutations (mutant vs. wild type)	1.054	0.816-1.806	0.317	-	-	-
BRAF^{V600E} mutation (mutant vs. wild type)	1.696	1.365-2.294	0.011	1.961	1.656-2.949	0.001
MMR status (proficient vs. deficient)	1.775	1.343-3.011	0.007	1.575	1.243-3.001	0.018
Stage III vs. II	1.784	1.335-2.762	0.006	1.843	1.356-2.623	0.009

CI, confidence interval; MMR, mismatch repair.

Discussion

The data presented in the current study, demonstrated for the first time in literature, the impact of testing LKB1 protein expression in stage II/III CC patients that underwent resection and subsequently received CAPOX or FOLFOX adjuvant chemotherapy. Additionally, this retrospective study aims to identify correlations of LKB1 expression loss with known clinic-pathological features and common mutations (*KRAS* exon 2 and $BRAF^{V600E}$) mutation in operable CC. Moreover, we analyzed the predictive significance of these biomarkers in conjunction with *ERCC1*, *MYC*, *NEDD9*, and *TS* mRNA expression. Based on the literature, this is the first study that correlates a combination of all these parameters, hence; the results of our analysis could potentially serve as an advantageous guide for every day clinical practice.

AMPK is greatly regulated by LKB1 activity and is vital for cell metabolism through the maintenance of energy homeostasis. LKB1 growth-suppressing effect is operated through activation of twelve AMPK-related kinases. This AMPK-related kinases activation by LKB1 is crucial for the regulation of (1) cell metabolism, (2) polarity, and (3) aberrant proliferation inhibition in malignant cells [17], indicating the role of LKB1 as a tumor suppressor gene [18]. Therefore, LKB1 loss promotes cancer evolution and is considered a negative factor in cancer patients [19]. The results of the present investigation indicate, that stage II-III CC patients with loss of LKB1 protein expression exhibited significantly lower DFS (p=0.021) and lower OS (p=0.002) compared to those with LKB1 positive tumors. Thus, the principal finding emerged from the current multivariate analysis, is the prognostic value of LKB1 in adjuvant CRC patients.

It is previously well described, that *LKB1* loss confers poor clinical outcome in human gastric cancer, breast cancer and hepatocellular carcinoma [18,20,21]. Furthermore, in 14 eligible studies that met the inclusion criteria, a first comprehensive meta-analysis demonstrated that the decreased LKB1 expression was significantly associated with a poorer OS in solid tumor patients, based on a random effect model [10]. Previous research has demonstrated that *LKB1* loss at the transcriptional level, promotes tumor malignancy, not only in lung adenocarcinoma but also in CRC [22]. Another study has revealed that reduced *LKB1* expression in patients with gastric cancer is correlated with higher clinical stage, T-stage, lymph-node metastasis and vascular invasion [21]. Similarly, in the present study, a significant correlation of LKB1 loss with certain pathological and clinical parameters of CRC was observed, such as pericolic lymph node involvement (p=0.003) and primary tumors located in the right colon (p=0.032). In contrast, no significant correlation of LKB1 protein expression associated with age, gender and grade was revealed.

The present study failed to demonstrate any statistically significant correlation between LKB1 protein expression and *KRAS* exon 2 mutation, as has been previously reported in lung adenocarcinomas [23]. On the other hand, our analysis demonstrated significant correlations of LKB1 loss with $BRAF^{V600E}$ mutation and high *TS* mRNA expression.

In support to previous data that have correlated factors such as *KRAS* and $BRAF^{V600E}$ mutations, pMMR positive tumors and stage III disease status, with a higher probability of relapse, our results clearly demonstrate that $BRAF^{V600E}$ mutations, pMMR tumors and patients with stage III disease at diagnosis are associated with lower DFS and OS. Although the expression of *ERCC1* and *TS* genes has been shown to be involved in the metabolism of the two main drug categories used in the adjuvant CRC setting, such as oxaliplatin and 5-fluorouracil, respectively [24,25], the current study did not reveal any significant correlation between the *ERCC1* and *TS* mRNA expression and the patients' outcome. Despite the previously identified prognostic value of *NEDD9* and *MYC* in CC patients [26,27], the current analysis failed to reveal any significant correlation between the *NEDD9* and *MYC* mRNA expression with either DFS or OS in patients with CC receiving adjuvant chemotherapy.

The prognostic or predictive value of LKB1 expression loss in patients with CC is currently unknown. As reported in a previous study, this could be due to the wide range of *LKB1* genomic alterations observed in sporadic cancers, emerging the challenge of developing a single assay capable of the detection of all these alterations combined [28]. Moreover, Sanchez-Cespedes [29] reported that many different types of *LKB1* somatic mutations in sporadic cancers have been identified including insertions, deletions, nonsense, and frameshift and missense mutations. However, most of the *LKB1* genomic alterations can result in either a truncated and therefore inactive form of the protein [28,29] or the complete absence of the protein. To overcome such a limitation, we considered the *in-situ* IHC assay as a potentially trustworthy, simple, and cost-effective method for evaluating the expression status of LKB1.

Besides, the robust results for LKB1 expression loss and the large patients' number of the current study, the finding should be interpreted with caution and mainly as hypothesis generated results. One of the main limitations is the lack of validation sets of patients treated or not treated with adjuvant chemotherapy, in order to elucidate the potential prognostic or predictive role of LKB1 expression loss in CC. Consequently, the design of an independent prospective validation trial is one of the future perspectives of our laboratory, where the prognostic power of LKB1 expression loss would be tested and validated prospectively. In summary, the results of the presented study indicate that loss of LKB1

protein expression is clearly associated with poor outcomes of patients with stage III or high-risk stage II CC and merits further evaluation in larger prospective patients' cohorts.

Electronic Supplementary Material

Supplementary materials are available at Cancer Research and Treatment website (https://www.e-crt.org).

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin. 2015;65:5-29.
2. Boland GM, Chang GJ, Haynes AB, Chiang YJ, Chagpar R, Xing Y, et al. Association between adherence to National Comprehensive Cancer Network treatment guidelines and improved survival in patients with colon cancer. Cancer. 2013;119:1593-601.
3. Schmoll HJ, Twelves C, Sun W, O'Connell MJ, Cartwright T, McKenna E, et al. Effect of adjuvant capecitabine or fluorouracil, with or without oxaliplatin, on survival outcomes in stage III colon cancer and the effect of oxaliplatin on post-relapse survival: a pooled analysis of individual patient data from four randomised controlled trials. Lancet Oncol. 2014;15:1481-92.
4. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372:2509-20.
5. Shorning BY, Clarke AR. LKB1 loss of function studied in vivo. FEBS Lett. 2011;585:958-66.
6. Forster LF, Defres S, Goudie DR, Baty DU, Carey FA. An investigation of the Peutz-Jeghers gene (LKB1) in sporadic breast and colon cancers. J Clin Pathol. 2000;53:791-3.
7. Lee SW, Lin HK. A new mechanism for LKB1 activation. Mol Cell Oncol. 2018;5:e1035691.
8. Lee SM, Choi JE, Na YK, Lee EJ, Lee WK, Choi YY, et al. Genetic and epigenetic alterations of the LKB1 gene and their associations with mutations in TP53 and EGFR pathway genes in Korean non-small cell lung cancers. Lung Cancer. 2013;81:194-9.
9. Carretero J, Medina PP, Pio R, Montuenga LM, Sanchez-Cespedes M. Novel and natural knockout lung cancer cell lines for the LKB1/STK11 tumor suppressor gene. Oncogene. 2004;23:4037-40.
10. Xiao J, Zou Y, Chen X, Gao Y, Xie M, Lu X, et al. The prognostic value of decreased LKB1 in solid tumors: a meta-analysis. PLoS One. 2016;11:e0152674.
11. Souglakos J, Philips J, Wang R, Marwah S, Silver M, Tzardi M, et al. Prognostic and predictive value of common mutations for treatment response and survival in patients with metastatic colorectal cancer. Br J Cancer. 2009;101:465-72.
12. Benlloch S, Paya A, Alenda C, Bessa X, Andreu M, Jover R, et al. Detection of BRAF V600E mutation in colorectal cancer: comparison of automatic sequencing and real-time chemistry methodology. J Mol Diagn. 2006;8:540-3.
13. Saridaki Z, Tzardi M, Papadaki C, Sfakianaki M, Pega F, Kalikaki A, et al. Impact of KRAS, BRAF, PIK3CA mutations, PTEN, AREG, EREG expression and skin rash in ≥2 line cetuximab-based therapy of colorectal cancer patients. PLoS One. 2011;6:e15980.
14. Cushman-Vokoun AM, Stover DG, Zhao Z, Koehler EA, Berlin JD, Vnencak-Jones CL. Clinical utility of KRAS and BRAF mutations in a cohort of patients with colorectal neoplasms submitted for microsatellite instability testing. Clin Colorectal Cancer. 2013;12:168-78.
15. Papadaki C, Mavroudis D, Trypaki M, Koutsopoulos A, Stathopoulos E, Hatzidaki D, et al. Tumoral expression of TXR1 and TSP1 predicts overall survival of patients with lung adenocarcinoma treated with first-line docetaxel-gemcitabine regimen. Clin Cancer Res. 2009;15:3827-33.
16. Rowan A, Churchman M, Jefferey R, Hanby A, Poulsom R, Tomlinson I. In situ analysis of LKB1/STK11 mRNA expression in human normal tissues and tumours. J Pathol. 2000;192:203-6.
17. Gan RY, Li HB. Recent progress on liver kinase B1 (LKB1): expression, regulation, downstream signaling and cancer suppressive function. Int J Mol Sci. 2014;15:16698-718.
18. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat Rev Cancer. 2009;9:563-75.
19. Yang JY, Jiang SH, Liu DJ, Yang XM, Huo YM, Li J, et al. Decreased LKB1 predicts poor prognosis in pancreatic ductal adenocarcinoma. Sci Rep. 2015;5:10575.
20. Shen Z, Wen XF, Lan F, Shen ZZ, Shao ZM. The tumor suppressor gene LKB1 is associated with prognosis in human breast carcinoma. Clin Cancer Res. 2002;8:2085-90.
21. Sun J, Ling B, Xu X, Ma R, Li G, Cao X, et al. Decreased expression of tumor-suppressor gene LKB1 correlates with poor prognosis in human gastric cancer. Anticancer Res. 2016;36:869-75.
22. He TY, Tsai LH, Huang CC, Chou MC, Lee H. LKB1 loss at transcriptional level promotes tumor malignancy and poor patient outcomes in colorectal cancer. Ann Surg Oncol. 2014;21

Suppl 4:S703-10.
23. Koivunen JP, Kim J, Lee J, Rogers AM, Park JO, Zhao X, et al. Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients. Br J Cancer. 2008;99:245-52.
24. Donada M, Bonin S, Nardon E, De Pellegrin A, Decorti G, Stanta G. Thymidilate synthase expression predicts longer survival in patients with stage II colon cancer treated with 5-flurouracil independently of microsatellite instability. J Cancer Res Clin Oncol. 2011;137:201-10.
25. Liang J, Jiang T, Yao RY, Liu ZM, Lv HY, Qi WW. The combination of ERCC1 and XRCC1 gene polymorphisms better predicts clinical outcome to oxaliplatin-based chemotherapy in metastatic colorectal cancer. Cancer Chemother Pharmacol. 2010;66:493-500.
26. Lee KS, Kwak Y, Nam KH, Kim DW, Kang SB, Choe G, et al. Favorable prognosis in colorectal cancer patients with co-expression of c-MYC and ss-catenin. BMC Cancer. 2016;16:730.
27. Li P, Zhou H, Zhu X, Ma G, Liu C, Lin B, et al. High expression of NEDD9 predicts adverse outcomes of colorectal cancer patients. Int J Clin Exp Pathol. 2014;7:2565-70.
28. Calles A, Sholl LM, Rodig SJ, Pelton AK, Hornick JL, Butaney M, et al. Immunohistochemical loss of LKB1 is a biomarker for more aggressive biology in KRAS-mutant lung adenocarcinoma. Clin Cancer Res. 2015;21:2851-60.
29. Sanchez-Cespedes M. A role for LKB1 gene in human cancer beyond the Peutz-Jeghers syndrome. Oncogene. 2007;26:7825-32.