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Original Paper

Clinical Evidence on the Interaction Between MLK4, KRAS and Microsatellite Instability to Determine the Prognosis of Early-Stage Colorectal Carcinoma

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Key Words
MLK4 • KRAS • MSI • MSS • Colorectal carcinomas • Prognosis

Abstract
Background/Aims: MLK4 (KIAA1804) is the second most frequently mutated kinase in microsatellite stable (MSS) colorectal carcinomas (CRC). This molecule is known to regulate different physiological cellular processes, including cell cycle, senescence and apoptosis, and mechanistic evidence has been provided that MLK4 plays a role in carcinogenesis. However, whether this kinase exerts a tumor suppressive role or an oncogenic function is still an object of debate. This study aims to elucidate the role of MLK4 in the pathogenesis of CRC by investigating human tumor specimens. ***Methods:*** This study assessed MLK4 expression levels by immunohistochemistry in surgical tumor samples from 204 early-stage CRC patients and their correlation with various clinical-pathological features and patients' outcomes. In addition, *MLK4* mRNA transcription was analysed in an independent cohort of 786 colon cancer samples. ***Results:*** Loss of MLK4 staining was associated with poor overall (OS) and progression free survival (PFS) in CRC patients during a univariate analysis (OS:101 vs 164 months, p=0.0002; PFS:85 vs 125 months, p=0.0001), as well as in multivariate analysis (OS:HR=1.70; p=0.001; PFS:HR=1,61; p=0.001). This was confirmed by analysis of *MLK4* mRNA in the second independent cohort. A subgroup analysis according to KRAS mutation status showed that MLK4 staining was associated with better OS and PFS in KRAS mutated cases (HR=2.77; p=0.0001 and HR=2.31; p=0.0003, respectively) and microsatellite stable tumors

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(HR=1.87; p=0.002 and HR=1.06; p=0.006) but not in KRAS wildtype and microsatellite unstable tumors. ***Conclusion:*** By providing the first report from clinical specimens on the prognostic significance of MLK4, we define an oncogenic loss-of-function of this kinase and suggest a possible role in the interaction with KRAS signaling in determining an aggressive phenotype of CRC. These findings warrant the further investigation of MLK4 in wider cohorts and various clinical settings.

Introduction

Colorectal cancer is a heterogeneous tumor. However, in recent years, efforts to establish a molecular classification of colorectal carcinomas (CRC) led to the definition of biological, prognostic and clinical relevance, of several signaling pathways and gene mutations including, among others, *APC*, *KRAS*, *BRAF,* and microsatellite instability (MSI) [1-3]. In particular, determining the *KRAS* and MSI status of tumors has acquired a specific clinical and therapeutic relevance.

Activating *KRAS* mutations are found in approximately 40% of colorectal carcinomas. Constitutive activation of KRAS is known to induce phosphorylation of β-catenin, which in turn, causes its dissociation from E-cadherin, thus enabling the transcriptional activity of β-catenin [4-7]. Determination of *KRAS* mutational status has become standard clinical practice as a predictor of response to the administration of EGFR-blocking compounds, such as cetuximab and panitumumab [8, 9].

MSI defines a subset of tumors frequently exhibiting a CpG island methylator phenotype (CIMP) and a hyper-mutation phenotype (for review see [4]); such tumors are characterized by a better outcome in comparison to microsatellite-stable (MSS) tumors, and it has most recently been shown that the higher mutation burden and tumor-specific neo-antigen formation typical of MSI tumors underlies the excellent response rates and outcomes observed during treatment with immune-checkpoint inhibitors [10, 11].

MLK4 (MAP3K21or KIAA1804), a member of the mixed-lineage serine/threonine kinase (MLK) family, activates c-Jun amino-terminal kinase (JNK) and p38 through its downstream targets MKK4/7 and MKK3/6 [12]. Within this signaling pathway, MLK4 is known to regulate several different cellular functions, including cell cycle, proliferation, senescence and apoptosis (for review see [13, 14]); thus, a role of this kinase in the development of CRC has been postulated. Specifically, mutations of *MLK4* have been reported in around 3% of CRC [15, 16], which makes this gene the second most frequently mutated kinase in MSS tumors [1, 2]. MKK4/7 and JNK have been reported to have a tumor suppressive function in different tumor types [17-21], and multiple loss-of-function (LOF) mutations for *MLK4* have been described in breast and pancreatic cancer [22, 23].

In spite of this putative function during cancer development, MLK4 is the least characterized member of the MLK family and its role in carcinogenesis is poorly understood. At present, the function of MLK4 in tumor formation has been addressed only by two preclinical studies providing contradictory results. The first characterized *MLK4* mutations as activating and promoting tumorigenesis in *KRAS* mutated colorectal tumors [15]; the second described the same mutations as loss-of-function mutations causing impairment of the enzymatic activity of MLK4 [24]. However, to our knowledge no study has yet assessed the relevance of MLK4 in determining the prognosis of colorectal cancer patients.

Due to these contradictory reports, we investigated the correlation between MLK4 protein levels in colorectal carcinomas and overall survival (OS) or progression free survival (PFS) in relation to KRAS and MSI status.

Materials and Methods

Clinical samples for survival analyses

Surgical specimens from patients with early-stage colorectal adenocarcinomas exhibiting moderate differentiation (G2 according to WHO, T-categories T2 and T3 and with no nodal or distant metastasis), undergoing surgery in curative intention at the hospital of the Ludwig-Maximilians-University Munich (LMU Munich) between 1994 and 2004 were considered. Follow up data were provided by the Munich Tumor Registry (TZM; Tumorregister München). To minimize biases related to the postoperative morbidity and comorbidity, patients who died within six months since surgical resection were not considered for analysis. The final case collection comprised tissue from 204 patients, 94 (46%) of whom died as a consequence of CRC within 5 years of diagnosis. The survival data of 158 cases (77%) was censored as case follow-up was discontinued or patients died due to other reasons than colorectal cancer. The characteristics of this collection are summarized in Table 1. The study was conducted in agreement with the requirements of the ethics committee of the University of Munich.

Table 1. Clinicopathological characteristics of the investigated CRC cases (n = 204)

Variable	Number of cases	%
Gender		
Male	112	55
Female	92	45
Age, y		
< 75	144	71
≥ 75	60	29
T-category		
T2	31	15
T3	173	85
Cancer specific survival, y		
< 5	94	46
≥ 5	110	54
Censored	158	77

Tissue microarray technique

As previously noted, tissue microarrays (TMA) from CRC were generated [25]. In brief, representative areas of viable carcinoma tissue were determined on 5 µm sections of formalin fixed, paraffin embedded carcinoma samples which were stained with hematoxylin-eosin. By using a tissue-arraying instrument (Beecher Instruments, Sun Prairie, WI, USA), 1 mm needle core-biopsies were taken from appropriate areas of the corresponding paraffin-embedded carcinoma blocks. They were then positioned in recipient paraffin array blocks at specified coordinates. To ensure representative sampling, six probes were taken from each tumor, three from central carcinoma areas and three from the invasive front. To enhance adherence between cores and paraffin, the recipient blocks were incubated for 30 min at 37°C.

Immunohistochemistry

Immunohistochemical staining was performed on 5 µm sections of TMA blocks. As the primary antibody, MLK4 polyclonal rabbit antibody (Acris, dilution 1:40, Herford, Germany) was used. Pre-Treatment for antigen retrieval was performed by microwaving for 2 x 15 min at 750 W in Enhancer (Linaris, Cat.No. E7000, Dossenheim, Germany). Detection was performed using SignalStain Boost IHC Detection Reagent HRP, Rabbit, (Cell Signaling, Cat.No. 8114). DAB+ (Dako, Cat.No. K3468, Hamburg, Germany) was used as a chromogen. Finally, slides were counterstained with hematoxylin Gill`s Formula (Vector Laboratories, Cat. No. H-3401, Eching, Germany). To verify staining specificity, system controls without primary antibodies, as well as immunoglobulin isotype control antibodies were employed.

Analyses of KRAS mutations

Analyses of *KRAS* exon 2 codon 12/13 were done as previously described [4, 6, 26]. Briefly, genomic DNA was extracted from micro-dissection carcinoma lesions using QIAamps DNA FFPE Tissue kit (Qiagen, Hilden, Germany). Pyro-sequencing was performed using the Pyro-Gold kit (Qiagen) and HotStar Taq-Polymerase (Qiagen). To identify anti-sense sequences, the PF2 primer (5'-tgt ggt agt tgg agc t-3') was used. For sequencing and sequence analyses, the PyroMark Q24 device (Qiagen) and the PyroMark™ Q24 software were applied [27, 28].

MSS analysis

As previously described, the status of MSS or high-grade microsatellite instability (MSI-H) was determined by analyzing the two-mononucleotide repeat markers BAT-25 and BAT-26 [29-32]. DNA was

amplified in a duplex PCR (Qiagen DNA Multiplex PCR kit, 100 nM BAT25 and 100 nM BAT26-specific primers) with the following cycle profile: denaturation at 95°C for 15 min, 34 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 90 sec and extension at 72°C for 60 sec, with a final extension step at 60°C for 30 min. One ml of the PCR product was mixed with 18.5 ml of highly deionized formamide (HiDi formamide) and 0.5 ml DNA Size Standard LIZ 500 / (2250) (both Applied Biosystems, Darmstadt, Germany). This mixture was denatured for 3 min at 94°C, instantly put on ice, and separated using an ABI 3130 Genetic Analyzer. Results were evaluated applying GeneMapper Software (Applied Biosystems).

Evaluation of MLK4 by immunohistochemistry

Sections were examined using light microscopy. As expected, because MLK4 stained positive in the cytoplasm of carcinoma cells, cytoplasmic staining was defined dichotomically according to the presence (score 1) or absence (score 0) of a staining signal (Fig. 1A, B). To exclude intraobserver variability, an observer who had no prior knowledge of prognosis or other clinicopathological variables, evaluated the specimens thrice.

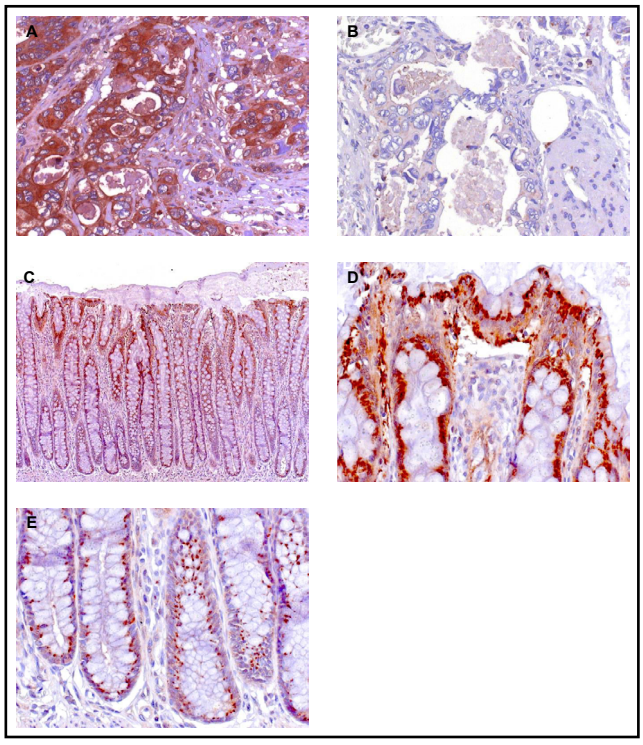

Fig. 1. MLK4 staining in human colorectal carcinomas (CRC) and normal colonic mucosa. Representative histological appearance of MLK4 staining with predominant cytoplasmic staining pattern (score 1 - A) or negative staining (score 0 - B) in different tumor specimens. Positive pattern of cytoplasmic staining of MLK4 in epithelial cell of normal colonic mucosa visible along the whole length of the crypts (C). Particularly high staining intensity in the apical regions of the crypts (D) and granular staining patter of MLK4 in cells of the crypt basis (E). Magnifications: × 100 (C) and × 400 (A, B, D, E).

Analysis of gene expression microarray data sets

Publicly available colorectal cancer gene expression datasets which matched tumor transcriptome and clinical data were available and retrieved from the Gene Expression Omnibus (GEO - accession codes GSE14333 and GSE39582). Both datasets were generated on Affymetrix HG-U133 Plus2.0 microarrays and normalized simultaneously in R (www.r-project.org) by Robust Multi-array Average (RMA) [33] using custom brainarray CDF (v19, ENTREZG) [34], which yielded one optimized probe set per gene [35, 36].

Statistical analyses

Cross-tabulations were calculated using Fisher's exact test. Kaplan-Meier analysis was employed to estimate cancer specific survival by the log-rank test. Optimal cutoffs for continuous variables were selected by receiver operating characteristic (ROC) curve analyses and Youden's index. Multivariate analysis was performed using the multivariate Cox regression model. P-values < 0.05 were considered statistically significant. Statistics were performed using SPSS statistical software (version 25.0; SPSS Inc., Chicago, IL).

Results

MLK4 protein levels in colorectal carcinomas

A first analysis was conducted to evaluate MLK4 expression and cellular localization by immunostaining in CRC and matched normal colonic mucosa samples. As expected, staining for MLK4 was observed only in the cytoplasm of cells. In CRC, as defined by a dichotomic assessment according to the presence or absence of staining signal, MLK stained positive in 146 cases (72%) and negative in 58 cases (28%) (Fig. 1A and 1B). In contrast, MLK4 staining was evident with no exception in all matched non-tumor colon mucosa tissue adjacent to tumor lesions. In particular, although a pattern of continuous expression of MLK4 could be seen along the whole length of the crypts, its staining intensity was accentuated on their apical portion (Fig. 1C-E). Therefore, these data suggest that the loss of MLK4 staining is a frequent feature of CRC.

Loss of MLK4 in colorectal carcinomas correlates with patient's survival

To assess the prognostic significance of MLK4 staining in determining the outcome of CRC patients, a Kaplan-Meier analysis according to the presence or absence of MLK4 staining was performed. Loss of MLK4 was associated with poorer OS and PFS in comparison to patients with positive MLK4 staining (p=0.0002 and p=0.0001, respectively; Fig. 2A and 2B). Age (p=0.019) but not gender (p=0.55) was significantly associated with patient outcomes. A multivariate Cox regression analysis including age, gender, T-category, *KRAS* mutational status and MSI status showed that loss of MLK4 staining was independently associated to a relative risk of 1.70 [confidence interval: 1.24 – 2.34] of poor overall survival and to a relative risk of 1.61 of disease progression [confidence interval: 1.22 – 2.11 – p = 0.001 each, Table 2 and 3).

To validate these findings, we tested for clinical correlations of MLK4 mRNA expression levels in a simultaneously normalized dataset comprising 786 CRC cases, which had follow-up data on tumor progression and in a subset of 562 patients within this collective with available data on OS. We identified ideal cutoff at the MLK4

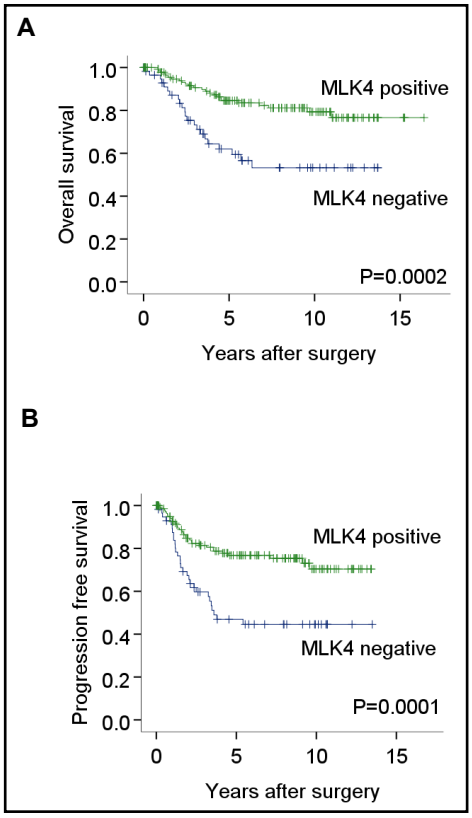

Fig. 2. Significance of MLK4 expression on overall survival (OS) and progression free survival (PFS). Kaplan-Meier plot of OS (A) and PFS (B) according to the presence or absence of MLK4 staining (n = 204; in this and the following figures, the log-rank test was used to estimate the indicated p values).

Table 2. Multivariate overall survival analysis including MLK4 and relevant clinic-pathological variables

Variable	Cases	Relative risk (95% confidence interval)	p
MLK4			
Positive	146/204 (72%)	1.00	
Negative	58/204 (28%)	1.70 (1.24 – 2.34)	0.001
Gender			
Male	112/204 (55%)	1.00	
Female	92/204 (45%)	1.14 (0.59 – 2.23)	0.698
Age, y			
< 70	144/204 (71%)	1.00	
≥ 70	60/204 (29%)	1.64 (0.84 – 3.18)	0.147
T-category			
T2	31/204 (15%)	1.00	
T3	173/204 (85%)	1.43 (0.58 – 3.55)	0.438
KRAS			
WT	118/191 (62%)		
mutated	73/191 (38%)	1.19 (0.62 - 2.27)	0.603
MSI-Status			
instable	64/183 (35%)		
stable	119 /183 (65%)	1.27 (0.66 - 2.44)	0.467

Table 3. Multivariate progression-free survival analysis, including MLK4 and relevant clinico-pathological variables

Variable	Cases	Relative risk (95% confidence interval)	p
MLK4			
Positive	146/204 (72%)	1.00	
Negative	58/204 (28%)	1.61 (1.22 – 2.11)	0.001
Gender			
Male	112/204 (55%)	1.00	
Female	92/204 (45%)	0.76 (0.42 – 1.36)	0.347
Age, y			
< 70	144/204 (71%)	1.00	
≥ 70	60/204 (29%)	1.19 (0.66 – 2.16)	0.561
T-category			
T2	31/204 (15%)	1.00	
T3	173/204 (85%)	1.35 (0.63 – 2.88)	0.445
KRAS			
WT	118/191 (62%)		
mutated	73/191 (38%)	1.47 (0.85 - 2.56)	0.172
MSI-Status			
instable	64/183 (35%)		
stable	119 /183 (65%)	0.96 (0.54 - 1.71)	0.894

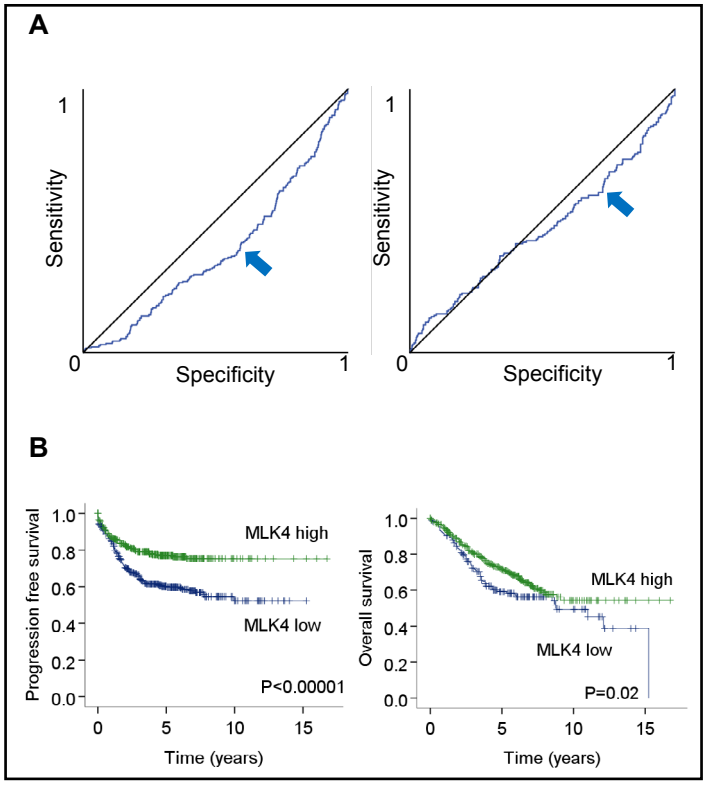

Fig. 3. Significance of MLK4 mRNA expression on progression-free (PFS) and overall survival (OS). (A) ROC curves for determining best discrimination thresholds for MLK4 expression. The arrow indicates the selected sensitivity and specificity cutoff value for binary classification for PFS (left panel) and OS (right panel). (B) Kaplan-Meier plots for PFS in this dataset for MLK4 (cutoff at the normalized expression intensity of 223, left panel) and OS (cutoff at the normalized expression intensity of 189, right panel).

expression intensity of 233 and 189 (natural scale) for PFS and OS respectively, using ROC curve analyses and Youden's index (Fig. 3A). Dichotomous categorization of cases by means of these cutoffs exhibited a highly significant positive correlation between MLK4 mRNA expression and PFS (p=0.000003) and OS (p=0.02) by using the Kaplan-Meier method (Fig. 3B). Applying a proportional hazards regression analysis, PFS was shown to be a prognostic factor independent of other key clinical and pathological variables (Table 4), whereas

Table 4. Multivariate analysis of MLK4 expression and clinical variables for disease free survival (PFS) and overall survival (OS). CI: confidence interval

Variables	HR	PFS (95% CI)	p	HR	OS (95% CI)	p
Age (≥ vs < median)	1.00	(0.77 - 1.13)	0.973	1.87	(1.39-2.53)	<0.0001
Gender (F vs M)	0.74	(0.57 - 0.96)	0.025	0.68	(0.50-0.91)	0.010
AJCC stage	2.62	(2.25 - 3.19)	<0.0001	2.09	(1.70-2.50)	<0.0001
MLK4	0.59	(0.45 - 0.77)	0.0001	0.75	(0.56-1.00)	0.053

OS showed a trend toward statistical significance (p=0.053). Collectively, these findings suggest that preserved expression of MLK4 is associated with a favorable outcome in patients with CRC.

Table 5. MLK4 expression in relation to KRAS mutational status

	KRAS wt	KRAS mut	Total
MLK4 neg	32 (27%)	21 (29%)	53 (28%)
MLK4 pos	86 (73%)	52 (71%)	138 (72%)
Total	118 (62%)	73 (38%)	191 (100%)

MLK4 staining correlates with patient outcomes in KRAS mutated but not in KRAS wild-type (WT) tumors

It has been recently proposed that MLK4 interacts with the RAS pathway to increase tumorigenicity in CRC [15]. To assess a possible interaction between MLK4 and *KRAS* mutation status in determining prognosis, a survival analysis was repeated by stratifying patients according to the presence or absence of *KRAS* mutations. In line with the expected incidence of *KRAS* mutations, 73 (38%) out of the 191 cases with available *KRAS* mutational status had an exon 2 codon 12 or codon 13 mutation. *KRAS* mutational status was not associated with age (p = 0.447), gender (p = 0.229), T-category (p = 0.228) or MSI Status (p = 0.223) and, as expected [37], did not correlate with OS (p = 0.57) or PFS (p = 0.07). Positive MLK4-staining was detected in 52 (71%) of *KRAS*-mutated and in 86 (73%) of *KRAS* WT patients (Table 5).

Analysis of survival showed that preserved MLK4 staining correlates with a better OS and PFS of patients with *KRAS* mutations (p = 0.0001 and p = 0.0003, respectively; Fig. 4A and 4B). Loss of MLK4 staining was associated with an independent relative risk of 2.77 [CI: 1.64–4.69] for OS and 2.31 [CI: 1.50– 3.56] for PFS in a multivariate Cox regression analysis including gender, age and T-category (p = 0.0001). In contrast, no correlation was found between MLK4 levels, OS and PFS in patients with *KRAS* WT (p = 0.10 and p = 0.17, respectively; Fig. 4C and 4D). The prognostic relevance of MLK4 in KRAS WT tumors points to a functional interaction between the loss of this kinase and KRAS mutations to determine an aggressive phenotype.

MLK4 staining in MSS colorectal carcinomas correlates with patient outcomes

In a subsequent analysis, MLK4 was assessed according to the microsatellite stability status of patients, which was available in 183 cases. Out of the 64 (35%) MSI tumors, 20 (31%) had no MLK4 staining. In patients with MSS tumors, absence of MLK4 staining was found in 32 (27%) cases (Table 6).

In MSS cases, no correlation was found between MLK4 and different clinical-pathological variables such as age, gender, T-category and *KRAS* mutation status (Fisher's exact test; data not shown). However, MLK4 positivity was associated with better OS and PFS (p = 0.002 and p = 0.006 respectively; Fig. 5A and 5B). This was confirmed by a multivariate Cox regression analysis including gender, age, T-category, *KRAS* mutational status and MLK4 staining, indicating an independent relative risk of 1.87 [CI: 1, 24 – 2, 82, p=0.003] for OS and of 1.60 [CI: 1, 14 – 2, 26, p=0.007] of tumor progression in this subgroup.

In MSI cases no significant correlation was found between cytoplasmic MLK4 levels and clinicopathological variables or patient survival (Fig. 5C). Although a significant correlation

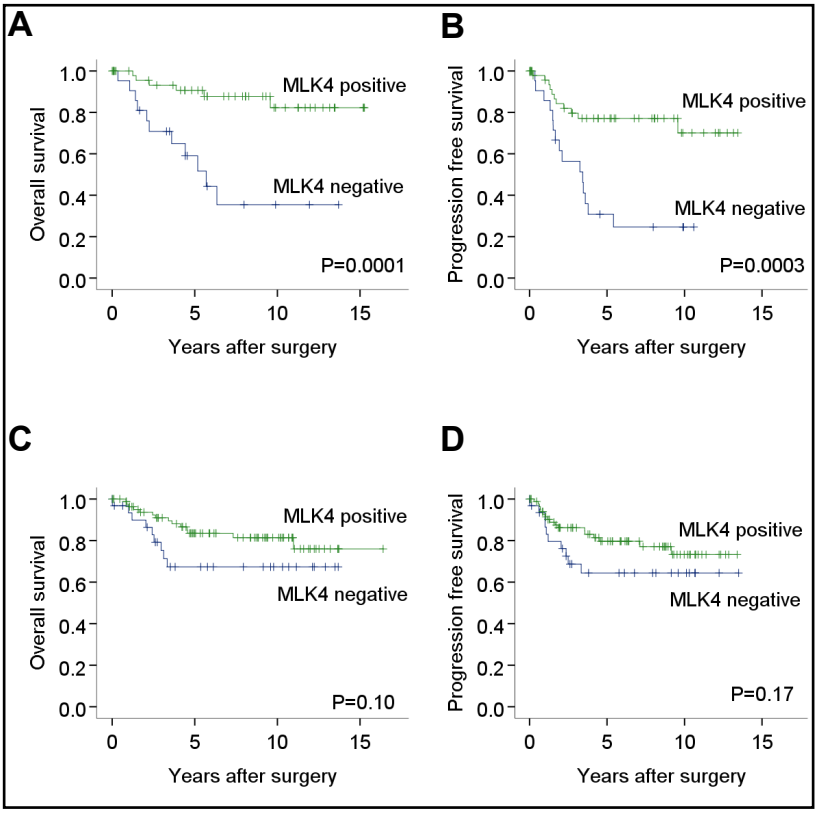

Fig. 4. Significance of MLK4 staining on overall survival (OS) and progression free survival (PFS) according to KRAS status. Kaplan-Meier plots of OS (A, C) and PFS (B, D) according to MLK4 expression in KRAS mutated (A,B) and KRAS wild-type cases (C,D).

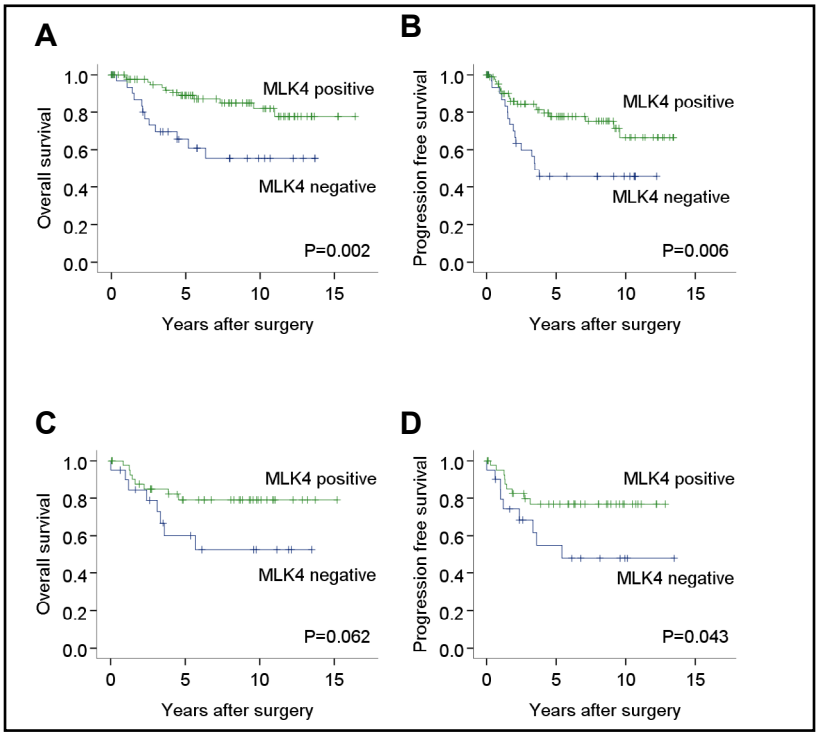

Fig. 5. Significance of MLK4 staining on overall survival (OS) and progression free survival (PFS) according to the presence or absence of microsatellite instability. Kaplan-Meier plots of OS (A, C) and PFS (B, D) according to MLK4 expression in microsatellite stable (MSS) or (A, B) microsatellite instable (MSI) tumors (C, D).

between MLK4 staining and PFS (p = 0.043; Fig. 5D) was observed in the univariate analysis, this could not be confirmed in a multivariate Cox regression analysis including age, gender, T-category and *KRAS* mutational status (p = 0.127).

Discussion

Mutations of *MLK4* have been reported to occur in 3% of CRC and in two out of 24 colorectal cancer cell lines [15, 16, 38]. In our assessment of human CRC, we found a positive correlation between the presence of MLK4 staining, OS and PFS, which was confirmed by a multivariate analysis. Our findings were validated by data on mRNA expression levels from a publicly available gene expression microarray cohort of 786 colon cancers, which confirmed the strong positive correlation between high *MLK4* levels expression and PFS (Fig. 3). Therefore, these data, which focus on patients with early-stage tumors, support the hypothesis that MLK4 has a tumor suppressive function in CRC. This is corroborated by the fact that, while MLK4 loss was found in 28% of samples, staining for MLK4 was invariably present in normal colonic mucosa, where it stained positive along the full length of the crypts. The higher prevalence of MLK4 loss in tumor samples vs. normal tissue, points to the fact that this kinase might play a more important and frequent role in the pathogenesis of CRC than suggested by the reported prevalence of its mutations (3%). This is likely due to the fact that epigenetic alterations, like aberrant methylation of the *KIAA1804* gene, might contribute to the loss of MLK4 together with less frequently observed mutations of this gene [39].

Our subgroup analysis showing that MLK4 has a prognostic significance in KRAS mutated and in MSS tumors, sheds light on how MLK4 mechanistically interferes with the biology of CRC in determining an aggressive phenotype. *KRAS* has been described to contribute to the pathogenesis of CRC by causing oncogene-induced senescence (OIS), a process which recent evidence has shown to be counteracted by JNK and p38 [40-42], both of which are downstream targets of MLK4. Consistently, preclinical investigation has shown that selectively restoring the function of MLK4 leads to activation of JNK and its downstream targets, cJUN and ATF in colon cancer cells [24]. Therefore, escape from OIS by preserved MLK4-JNK signaling might be one mechanism by which MLK4 counteracts the oncogenic function of *KRAS* in CRC.

Patients with MSS CRC have a poorer prognosis in comparison to patients with MSI tumors. This is thought to be due to several factors, comprising the higher immunogenicity of MSI tumors, which is responsible for a higher effectiveness of mechanisms of immuno-mediated elimination of cancer cells [43-48]. Our data showed that MLK4 positivity was associated with better OS and PFS in MSS cases, but not in MSI tumors (Fig. 5A). The lack of prognostic significance in MSI tumors might be due to the fact that a possible beneficial effect of MLK4 expression could be attenuated by the overall prognosis in MSI patients and may not be captured in our analysis due to the better small size of this patient subgroup in our collective. However, this may have a mechanistic cause: although no data are available on the significance of MLK4 in MSS tumors, MLK3, a closely related member of the MLK family was shown to function as a repressor of WNT signaling by reducing the transcriptional activity of the β-catenin/TCF complex. Since loss-of-function mutations of APC or stabilizing mutations of β-catenin are frequently found in MSS tumors, MLK4 might act to counteract the oncogenic effect of WNT signaling in these tumors. The favorable effect of MLK4-expression in MSS patients observed by us and the high mutation frequency reported for MLK4 in MSS tumors by The Cancer Genome Atlas (TCGA) consortium might reflect this function [2].

Conclusion

By analyzing human specimens, we provide the first evidence on the fact that loss of MLK4 is a determinant in the prognosis of CRC patients. We contribute to the debated question on the function of MLK4 in tumorigenesis by suggesting that MLK4 exerts a tumor suppressive function. Our results also shed light on possible mechanisms of action of MLK4 in CRC and other tumors by postulating an interaction with KRAS signaling in determining an aggressive phenotype. These findings warrant the further investigation of MLK4 in wider

cohorts and different clinical settings. In particular, we propose that MLK4 is assessed *in vitro* to detect a possible interaction with RAS-RAF-MEKK-ERK signaling and that its role in relation to β-catenin signaling is assessed in MSS tumors. In addition, MLK4 might play a role in determining whether and to what extent patients respond to treatment with EGF-receptor antagonists.

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The study protocol has been approved by the research institute's committee on human research.

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Disclosure Statement

No conflicts of interest exist.

References

1 Parsons MT, Buchanan DD, Thompson B, Young JP, Spurdle AB: Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. J Med Genet 2012;49:151-157.
2 Cancer Genome Atlas Network: Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330-337.
3 Popat S, Hubner R, Houlston RS: Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol 2005;23:609-618.
4 Fearon ER: Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479-507.
5 Herbst A, Jurinovic V, Krebs S, Thieme SE, Blum H, Goke B, Kolligs FT: Comprehensive analysis of beta-catenin target genes in colorectal carcinoma cell lines with deregulated Wnt/beta-catenin signaling. BMC Genomics 2014;15:74.
6 Kinch MS, Clark GJ, Der CJ, Burridge K: Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J Cell Biol 1995;130:461-471.
7 Zhou M, Yu P, Qu J, Chen Y, Zhou Y, Fu L, Zhang J: Efficacy of Bevacizumab in the First-Line Treatment of Patients with RAS Mutations Metastatic Colorectal Cancer: a Systematic Review and Network Meta-Analysis. Cell Physiol Biochem 2016;40:361-369.
8 Kishiki T, Ohnishi H, Masaki T, Ohtsuka K, Ohkura Y, Furuse J, Sugiyama M, Watanabe T: Impact of genetic profiles on the efficacy of anti-EGFR antibodies in metastatic colorectal cancer with KRAS mutation. Oncol Rep 2014;32:57-64.
9 Siddiqui AD, Piperdi B: KRAS mutation in colon cancer: a marker of resistance to EGFR-I therapy. Ann Surg Oncol 2010;17:1168-1176.
10 O'Neil BH, Wallmark JM, Lorente D, Elez E, Raimbourg J, Gomez-Roca C, Ejadi S, Piha-Paul SA, Stein MN, Abdul Razak AR, Dotti K, Santoro A, Cohen RB, Gould M, Saraf S, Stein K, Han SW: Safety and antitumor activity of the anti-PD-1 antibody pembrolizumab in patients with advanced colorectal carcinoma. PloS One 2017;12:e0189848.

11 Inderberg EM, Walchli S, Myhre MR, Trachsel S, Almasbak H, Kvalheim G, Gaudernack G: T cell therapy targeting a public neoantigen in microsatellite instable colon cancer reduces *in vivo* tumor growth. Oncoimmunology 2017;6:e1302631.
12 Gallo KA, Johnson GL: Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol 2002;3:663-672.
13 Handley ME, Rasaiyaah J, Chain BM, Katz DR: Mixed lineage kinases (MLKs): a role in dendritic cells, inflammation and immunity? Int J Exp Pathol 2007;88:111-126.
14 Craige SM, Reif MM, Kant S: Mixed - Lineage Protein kinases (MLKs) in inflammation, metabolism, and other disease states. Biochim Biophys Acta 2016;1862:1581-1586.
15 Martini M, Russo M, Lamba S, Vitiello E, Crowley EH, Sassi F, Romanelli D, Frattini M, Marchetti A, Bardelli A: Mixed lineage kinase MLK4 is activated in colorectal cancers where it synergistically cooperates with activated RAS signaling in driving tumorigenesis. Cancer Res 2013;73:1912-1921.
16 Shao RX, Kato N, Lin LJ, Muroyama R, Moriyama M, Ikenoue T, Watabe H, Otsuka M, Guleng B, Ohta M, Tanaka Y, Kondo S, Dharel N, Chang JH, Yoshida H, Kawabe T, Omata M: Absence of tyrosine kinase mutations in Japanese colorectal cancer patients. Oncogene 2007;26:2133-2135.
17 Ahn YH, Yang Y, Gibbons DL, Creighton CJ, Yang F, Wistuba, II, Lin W, Thilaganathan N, Alvarez CA, Roybal J, Goldsmith EJ, Tournier C, Kurie JM: Map2k4 functions as a tumor suppressor in lung adenocarcinoma and inhibits tumor cell invasion by decreasing peroxisome proliferator-activated receptor gamma2 expression. Mol Cell Biol 2011;31:4270-4285.
18 Teng DH, Perry WL 3rd, Hogan JK, Baumgard M, Bell R, Berry S, Davis T, Frank D, Frye C, Hattier T, Hu R, Jammulapati S, Janecki T, Leavitt A, Mitchell JT, Pero R, Sexton D, Schroeder M, Su PH, Swedlund B, et al.: Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor. Cancer Res 1997;57:4177-4182.
19 Yeasmin S, Nakayama K, Rahman MT, Rahman M, Ishikawa M, Katagiri A, Iida K, Nakayama N, Miyazaki K: MKK4 acts as a potential tumor suppressor in ovarian cancer. Tumour Biol 2011;32:661-670.
20 Yeasmin S, Nakayama K, Rahman MT, Rahman M, Ishikawa M, Katagiri A, Iida K, Nakayama N, Miyazaki K: Loss of MKK4 expression in ovarian cancer: a potential role for the epithelial to mesenchymal transition. Int J Cancer 2011;128:94-104.
21 Schramek D, Kotsinas A, Meixner A, Wada T, Elling U, Pospisilik JA, Neely GG, Zwick RH, Sigl V, Forni G, Serrano M, Gorgoulis VG, Penninger JM: The stress kinase MKK7 couples oncogenic stress to p53 stability and tumor suppression. Nat Genet 2011;43:212-219.
22 Su GH, Hilgers W, Shekher MC, Tang DJ, Yeo CJ, Hruban RH, Kern SE: Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res 1998;58:2339-2342.
23 Su GH, Song JJ, Repasky EA, Schutte M, Kern SE: Mutation rate of MAP2K4/MKK4 in breast carcinoma. Hum Mutat 2002;19:81.
24 Marusiak AA, Stephenson NL, Baik H, Trotter EW, Li Y, Blyth K, Mason S, Chapman P, Puto LA, Read JA, Brassington C, Pollard HK, Phillips C, Green I, Overman R, Collier M, Testoni E, Miller CJ, Hunter T, Sansom OJ, et al.: Recurrent MLK4 Loss-of-Function Mutations Suppress JNK Signaling to Promote Colon Tumorigenesis. Cancer Res 2016;76:724-735.
25 Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998;4:844-847.
26 Neumann J, Zeindl-Eberhart E, Kirchner T, Jung A: Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. Pathol Res Pract 2009;205:858-862.
27 Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M, Fuchs CS: Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 2005;7:413-421.
28 Poehlmann A, Kuester D, Meyer F, Lippert H, Roessner A, Schneider-Stock R: K-ras mutation detection in colorectal cancer using the Pyrosequencing technique. Pathol Res Pract 2007;203:489-497.
29 Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248-5257.

30 Deschoolmeester V, Baay M, Wuyts W, Van Marck E, Van Damme N, Vermeulen P, Lukaszuk K, Lardon F, Vermorken JB: Detection of microsatellite instability in colorectal cancer using an alternative multiplex assay of quasi-monomorphic mononucleotide markers. J Mol Diagn 2008;10:154-159.
31 Kriegl L, Jung A, Horst D, Rizzani A, Jackstadt R, Hermeking H, Gallmeier E, Gerbes AL, Kirchner T, Goke B, De Toni EN: Microsatellite instability, KRAS mutations and cellular distribution of TRAIL-receptors in early stage colorectal cancer. PloS One 2012;7:e51654.
32 Walther A, Houlston R, Tomlinson I: Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. Gut 2008;57:941-950.
33 Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249-264.
34 Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F: Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res 2005;33:e175.
35 Grunewald TG, Bernard V, Gilardi-Hebenstreit P, Raynal V, Surdez D, Aynaud MM, Mirabeau O, Cidre-Aranaz F, Tirode F, Zaidi S, Perot G, Jonker AH, Lucchesi C, Le Deley MC, Oberlin O, Marec-Berard P, Veron AS, Reynaud S, Lapouble E, Boeva V, et al.: Chimeric EWSR1-FLI1 regulates the Ewing sarcoma susceptibility gene EGR2 via a GGAA microsatellite. Nat Genet 2015;47:1073-1078.
36 Sahay D, Leblanc R, Grunewald TG, Ambatipudi S, Ribeiro J, Clezardin P, Peyruchaud O: The LPA1/ZEB1/miR-21-activation pathway regulates metastasis in basal breast cancer. Oncotarget 2015;6:20604-20620.
37 Andreyev HJ, Norman AR, Cunningham D, Oates J, Dix BR, Iacopetta BJ, Young J, Walsh T, Ward R, Hawkins N, Beranek M, Jandik P, Benamouzig R, Jullian E, Laurent-Puig P, Olschwang S, Muller O, Hoffmann I, Rabes HM, Zietz C, et al.: Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. Br J Cancer 2001;85:692-696.
38 Bardelli A, Parsons DW, Silliman N, Ptak J, Szabo S, Saha S, Markowitz S, Willson JK, Parmigiani G, Kinzler KW, Vogelstein B, Velculescu VE: Mutational analysis of the tyrosine kinome in colorectal cancers. Science 2003;300:949.
39 Farkas SA, Vymetalkova V, Vodickova L, Vodicka P, Nilsson TK: DNA methylation changes in genes frequently mutated in sporadic colorectal cancer and in the DNA repair and Wnt/beta-catenin signaling pathway genes. Epigenomics 2014;6:179-191.
40 Spallarossa P, Altieri P, Barisione C, Passalacqua M, Aloi C, Fugazza G, Frassoni F, Podestà M, Canepa M, Ghigliotti G, Brunelli C: p38 MAPK and JNK Antagonistically Control Senescence and Cytoplasmic p16INK4A Expression in Doxorubicin-Treated Endothelial Progenitor Cells. PloS One 2010;5:e15583.
41 Lee JJ, Lee JH, Ko YG, Hong SI, Lee JS: Prevention of premature senescence requires JNK regulation of Bcl-2 and reactive oxygen species. Oncogene 2009;29:561.
42 Jia H, Wang Z: Telomere Length as a Prognostic Factor for Overall Survival in Colorectal Cancer Patients. Cell Physiol Biochem 2016;38:122-128.
43 Jorissen RN, Christie M, Mouradov D, Sakthianandeswaren A, Li S, Love C, Xu ZZ, Molloy PL, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruszkiewicz AR, Moore J, Burgess AW, Busam D, Zhao Q, Strausberg RL, Lipton L, Desai J, et al.: Wild-type APC predicts poor prognosis in microsatellite-stable proximal colon cancer. Br J Cancer 2015;113:979-988.
44 Phipps AI, Limburg PJ, Baron JA, Burnett-Hartman AN, Weisenberger DJ, Laird PW, Sinicrope FA, Rosty C, Buchanan DD, Potter JD, Newcomb PA: Association between molecular subtypes of colorectal cancer and patient survival. Gastroenterology 2015;148:77-87.e2.
45 Lin CC, Lin JK, Lin TC, Chen WS, Yang SH, Wang HS, Lan YT, Jiang JK, Yang MH, Chang SC: The prognostic role of microsatellite instability, codon-specific KRAS, and BRAF mutations in colon cancer. J Surg Oncol 2014;110:451-457.
46 Xiao H, Yoon YS, Hong SM, Roh SA, Cho DH, Yu CS, Kim JC: Poorly differentiated colorectal cancers: correlation of microsatellite instability with clinicopathologic features and survival. Am J Clin Pathol 2013;140:341-347.
47 Lochhead P, Kuchiba A, Imamura Y, Liao X, Yamauchi M, Nishihara R, Qian ZR, Morikawa T, Shen J, Meyerhardt JA, Fuchs CS, Ogino S: Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication.J Natl Cancer Inst 2013;105:1151-1156.
48 Zhu Y, Yang SR, Wang PP, Savas S, Wish T, Zhao J, Green R, Woods M, Sun Z, Roebothan B, Squires J, Buehler S, Dicks E, McLaughlin JR, Parfrey PS, Campbell PT: Influence of pre-diagnostic cigarette smoking on colorectal cancer survival: overall and by tumour molecular phenotype. Br J Cancer 2014;110:1359-1366.