CHAPTER 4

Specific activity of cyclin-dependent kinase I is a new potential predictor of tumor recurrence in stage II colon cancer

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British Journal of Cancer. 2012: 106 133-140

ABSTRACT

Background: There are no established biomarkers to identify tumor recurrence in stage II colon cancer. As shown previously, the enzymatic activity of the cyclin dependent kinases 1 and 2 (CDK1 and CDK2) predicts outcome in breast cancer. Therefore, we investigated whether CDK activity identifies tumor recurrence in colon cancer.

Methods: In all, 254 patients with completely resected (R0) UICC stage II colon cancer were analyzed retrospectively from two independent cohorts from Munich (Germany), and Leiden (Netherlands). None of the patients received adjuvant treatment. Development of distant metastasis was observed in 27 patients (median follow-up: 86 months). Protein expression and activity of CDKs were measured on fresh-frozen tumor samples.

Results: Specific activity of CDK1 (CDK1SA), but not CDK2, significantly predicted distant metastasis (concordance index = 0.69, 95%CI: 0.55–0.79, p=0.036). Cut-off derivation by maximum log-rank statistics yielded a threshold of CDK1SA at 11 (specific activity units, p=0.029). Accordingly, 59% of patients were classified as high-risk (CDK1SA ≥11). Cox proportional hazard analysis revealed CDK1SA as independent prognostic variable (hazard ratio = 6.2, 95% CI: 1.44–26.9, p=0.012). Moreover, CKD1SA was significantly elevated in microsatellite stable tumors.

Conclusion: Specific activity of CDK1 is a promising biomarker for metastasis risk in stage II colon cancer.

INTRODUCTION

Each year >1 million individuals worldwide develop colon cancer with a disease specific mortality rate of almost 33% (1-3). Approximately 40% of resected colon cancers are from stage II (T3-4N0M0). The 5 year survival rates vary between 88% in T3N0 patients, and 75% in T4N0 patients. Chemotherapy is widely accepted as adjuvant treatment for stage III patients, whose 5 year survival (stage III A and B) is higher than 75% (4). Use of chemotherapy for stage II, T4 patients remains controversial despite their worse survival rates. This indicates that the allocation of treatment based solitary on conventional staging methods is not optimal (5-9). Over the last decade there have been important developments towards the discovery of new prognostic and predictive markers that might improve staging methods. The American Society of Clinical Oncology's Tumor Markers Expert Panel (ASCO TEMP-2006) and its European counterpart, the European Group on Tumor Markers (EGTM-2007) have recently reviewed the literature on these biomarkers. However, all biomarkers reviewed lacked the significant, discriminative value that is required to become implemented into clinical practice (10-12). There is a stringent need for new assays that are able to identify stage II colon cancer patients who might benefit from adjuvant therapy. Genomic instability and altered cell proliferation are major contributors to tumor growth and aggressiveness. Measuring these hallmarks of colon cancer in a quantitative fashion could be a suitable option for risk stratification. The proliferation rate of tumor cells has so far been studied with methods such as ^{3}H-thymidine/BrdU incorporation, mitotic index, or Ki-67/PCNA immunohistochemistry, but none of these tests have reached clinical application (13;14). Therefore, analysis of the highly conserved drivers of the cell cycle, the cyclin-dependent kinases (CDKs) 1 and 2, may be a more promising approach (15). CDK expression is constitutive in tumors but their enzymatic activity changes markedly according to the specific cell cycle phase. On the molecular level, the activity of CDK is regulated by subunits known as cyclins, and by phosphorylation of conserved tyrosine and threonine residues. Over-expression of cyclins, as well as inactivation of CDK inhibitors, are well documented as prognostic markers for esophageal, gastric, colorectal, breast and lung cancer (16-22)However, expression analysis of cyclins and other factors may not necessarily indicate the enzymatic activity of CDKs, which is crucial for the cell cycle status of the cancer cells. We have recently reported an assay that measures the specific activity of CDK 1 and CDK2 (23-25), based on a well standardized biochemical assay that requires only small amounts of fresh frozen tissue and is (23). The hallmark of this approach is the extraction of functional CDK enzyme from tumor tissue, followed by determination of its kinase activity. We hypothesize that intratumoral kinase activity of CDKs predicts the prognosis of tumor patients with great fidelity, because it directly represents a quantifiable readout for two hallmarks of tumors: increased proliferation and genomic instability. Two large, independent cohorts of breast cancer patients demonstrated that this assay had prognostic value (24;25). A CDK-based risk score validated in these studies was a significant and independent prognostic factor, especially for distant

recurrence. The aim of this study was to determine the ability of CDK-based analysis to predict recurrence in patients with locally restricted colon cancer. The study was carried out retrospectively on two independent patient cohorts derived from large surgical oncology centers in the Netherlands and Germany. Our results demonstrate that the specific activity of CDK1 identifies stage II colon cancer patients with a high risk of distant disease recurrence. This patient group may benefit from adjuvant chemotherapy, which would not be recommended according to standard criteria.

MATERIALS & METHODS

Patients

The study was approved by the local ethics committees at LUMC and TUM. Informed, written consent had been obtained prior to the study. Fresh frozen samples of 271 of stage II colon carcinomas were analyzed, collected at Leiden University Medical Center (LUMC, 1985 - 2005), and at Klinikum rechts der Isar (TUM, 1987 to 2006). All patients had curative (R0) tumor resection, and none of them received adjuvant or neoadjuvant therapy. Tumor tissue was dissected immediately after resection by a pathologist, snap frozen in liquid nitrogen and stored at -80°C. Development of distant metastasis was observed in 27 patients (11%) after a follow-up of 7.2 years (median). Five samples (1.8%) were excluded due to tumor cell content of less than 10 percent. All remaining tissue samples underwent C2P-analysis, 12 cases were excluded due to assay failure, or CDK expression level below detection threshold (n=3). Of note, all 12 excluded cases were free of tumor recurrence. Hence, 254 samples were available for further analysis (n=217 from TUM, and n=37 from LUMC).

Determination of CDK-specific activities

Ten to 20 sections of 100 µm thickness were cut with a cryostat and subjected to CDK analysis. One section of 7 µm thickness was cut from the middle of each block and evaluated by a pathologist after standard H&E staining. Cases with tumor cell content <10% were excluded. The system to measure the CDK specific activity (CDKSA) is called “C2P” (for “Cell Cycle Profiling”; Sysmex, Kobe, Japan; Ishihara *et al*, 2005; Kim *et al*, 2008). In brief, lysates of frozen material were applied to a well of 96-well PVDF filter plate (Millipore, MA, USA). Expression of CDKs was detected quantitatively by sequential reactions with primary anti-CDK antibodies, biotinylated anti-rabbit antibodies, and fluorescein-labeled streptavidin. To measure the kinase activity, CDK molecules were immunoprecipitated from the lysate using protein beads, as reported in detail earlier (Ishihara *et al*, 2005; Kim *et al*, 2008). CDKSA was calculated as CDK kinase activity units (aU/µL lysate) divided by its corresponding CDK expression units (eU/µL lysate). Both, aU (CDK *activity unit),* and eU (CDK *expression unit*) were defined as the expression and activity equivalent to 1 ng of recombinant CDK1, and CDK2, respectively. The distribution

of the CDK1SA and CDK2SA within the LUMC and the TUM cohort can be found in supplementary Figure 1. Further details regarding the quality controls for this assay can be found in the supplementary data.

Immunofluorescence analysis

Tissue specimens (7 µm) from 207 samples were available for evaluation by immunofluorescence microscopy (Axiovert 200, Zeiss, Göttingen, Germany). After fixation with 3% PFA and antigen retrieval (10 min boiling, sodium citrate buffer, pH=6.0), slides were incubated with anti-Ki-67 antibody (clone MIB-1, M 7240, DAKO) and/or anti-cytokeratin-20 antibody (rabbit monoclonal, 2039-1, Epitomics, Burlingame, CA) diluted 1:200, followed by incubation with secondary antibodies (Molecular Probes, Darmstadt, Germany; Dianova, Hamburg, Germany), and counterstaining with 4', 6'-diamidino-2-phenylindole (DAPI, Invitrogen, Darmstadt, Germany). Ki-67 positive nuclei from CK20 positive cells were regarded as *bona fide* tumor cells and were counted in a semi-automated manner using ImageJ freeware (http://rsb.info.nih.gov/ij/).

MSI (Microsatellite instability) determination

Tissue from 200 patients of the Munich cohort and all 37 patients of the LUMC was available for DNA isolation with the QIAampDNA™Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentration and quality was checked with an ND-1000 NanoDrop Spectrophotometer (Thermo Fisher, Schwerte, Germany). Subsequently, microsatellite instability was tested with the MSI Analysis System, Version 1.2 (Promega, Mannheim, Germany). This assay co-amplifies five mononucleotide repeat markers; BAT-25, BAT-26, NR-21, NR-24, and MONO-27 to determine MSI status. It includes two pentanucleotide repeats, Penta C and D, to make sure that normal and tumor samples are derived from the same patient. The results of this assay have been previously compared with the Bethesda panel markers and proven highly sensitive for MSI determination (26). The MSI status was determined for 32 of the 37 LUMC cases, and for 191 of 200 TUM patients. In 6% of the cases (14 of 237 available DNA samples), MSI status could not be determined based on evaluation of the PCR array data by an experienced pathologist due to ambiguous results.

BRAF

The mutational status of the oncogene BRAF (V600E, GTG>GAG substitution in exon 15) was assessed by high resolution melting analysis of genomic DNA on a Lightcycler 480 II platform (Roche, Mannheim; SYBR Green I /HRM Dye Protocol), in accordance to published protocols (23). Briefly, 20 ng of genomic DNA (10 ng/µL) were amplified in total volume of 20 µL with 10 µL High Resolution Master Mix, 2 mM $MgCl_2$, and 1 mM each of oligonucleotide primers, 2µL template DNA and 4.8 µL dH_2O. Primer sequences were: BRAF Exon 15 For:5'-TGA AGA CCT CAC AGT AAA AAT AGG-3', BRAF Exon 15 Rev: 5'-TCC AGA CAA CTG TTC AAA CTG AT-3'. After pre-incubation (95°C, 10 min), amplification of a 147 Bp product was carried

out in 40 cycles (95°C, 15 sec / 61°C, 15 sec / 72°C, 15 sec.), followed by melting point analysis with an inital phase: 95°C, 5 sec, and 72 °C, 90 sec, followed by a melting profile ranging from 72°C to 95°C in 25 min. As positive control, genomic DNA from the BRAF-mutated colon cancer cell line HT29 was used.

Statistical analysis

Statistical analyses were conducted using R Software version 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria). In order to derive optimal cut off values of quantitative CDK measurements for recurrence risk stratification, maximally selected log-rank statistics have been used. To consider multiple test issue within these analyses, the R-function 'maxstat.test' was employed (27). To internally validate the derived cut-off, the entire data set was randomly divided in a training and test set (ratio: 70:30). Furthermore, bootstrap re-sampling analysis was conducted to estimate distribution of derived cut-off values and 95% confidence intervals respectively. Multivariable Cox-regression was performed to assess recurrence risk differences between derived sub-groups in simultaneous consideration of potential confounding factors. Due to the low number of critical events, multivariable regression analyses had to be performed consecutively (one by one inclusion of potential confounding factors) to avoid over-adjustment. By the use of survival-ROC analysis, predictive capability of recurrence risk stratification was assessed cumulatively over the course of the follow-up. In this term, area under the time dependent ROC-curve (concordance index) was reported with 95% bootstrap confidence interval. The Kaplan-Meier Methods was used for survival plotting and log-rank test for comparison of survival curves. All statistical tests were conducted two-sided and a p-value <0.05 was considered significant.

RESULTS

We have determined the specific activity of CDK1 and CDK2 (CDK1SA and CDK2SA) in a study population comprised of samples from two independent cohorts of stage II colon cancer patients originating from the Leiden University Medical Centre (LUMC, The Netherlands) and the Klinikum Rechts der Isar, of the Technical University in Munich (TUM, Germany). Five samples (1.8%) were excluded due to tumor cell content of less than 10 percent. Twelve cases were excluded due to assay failure, and in three cases the CDK expression levels were below the detection threshold. Of note, all excluded cases were free of tumor recurrence. Altogether, the expression and kinase assay ("C2P", in short for "Cell cycle profiling") yielded results in 96% of patients (254 out of 266; n=217 from TUM, and n=37 from LUMC). There were no statistically significant differences in clinico-pathological characteristics between both cohorts (table 1). The specific activity (SA) was calculated and indicated as kinase activity in relation to its corresponding mass concentration. The CDK activity unit and CDK expression unit were defined as the equivalent of 1 ng recombinant CDK protein. The distribution of the CDK1SA did not vary significantly

Table 1
patient characteristics

Category	Subcategory	Total collective(%)	Patients from TUM (%)	Patients from LUMC (%)
Total		254 (100%)	217 (100%)	37 (100%)
Sex	Male	141 (56)	124 (57)	17 (46)
	Female	113 (44)	93(43)	20 (54)
Age		65 (median)	65 (median)	69 (median)
		15-91 (range)	15-91 (range)	26-82 (range)
Open surgery		254 (100)	217 (100)	37 (100)
Location	Caecum	40(16)	31 (14)	9 (24)
	Ascending colon	66 (26)	59 (27)	7 (19)
	Transverse colon	26 (10)	23 (11)	3 (8)
	Descending colon	30 (12)	28 (13)	2 (5)
	Sigmoid	92 (36)	76 (35)	16 (43)
Tumor size		6 (median)	6 (median)	5 (median)
		2-15 (range)	2-15 (range)	3-14 (range)
PT	T3	221 (87)	188 (87)	33 (89)
	T4	33 (13)	29 (13)	4 (11)
Lymph nodes total		19 (median)	20 (median)	10 (median)
		1-72 (range)	7-72 (range)	1-26 (range)
Grading	G1,G2	170 (67)	149 (69)	21 (57)
	G3,G4	77 (30)	65 (30)	12 (32)
	Missing	7 (3)	3 (1)	4 (11)
Recurrence	None	220 (87)	191 (88)	29 (78)
	Distant	27 (11)	22 (10)	5 (14)
	Local	7 (3)	4 (2)	3 (8)
Survival information	Alive	172 (68)	155 (71)	17 (46)
	Tumor-related death	25 (10)	19 (9)	6 (16)
	Non-tumorrelated death	58 (23)	44 (20)	14 (38)

Abbreviation: pT= tumour stage

between the two study cohorts (Mann-Whitney U test, p=0.35), whereas the average of CDK2SA was higher in samples from the Netherlands (p=0.012).

Predictive performance and cut-off derivation of CDK specific activity for distant recurrence

The distribution of clinical samples was plotted on a scatter diagram according to CDK1SA and CDK2SA (figure 1A). Cases with distant metastasis clustered in the region with high CDK1 activity, suggesting that mainly CDK1SA could have prognostic power. In order to evaluate the prognostic performance of CDK activity for distant metastasis risk, the true positive rates of distant disease recurrence (sensitivity) and corresponding false positive rates (100-specificity) were summarized in a time-dependent receiver operating characteristic (ROC) curve. The average area under the ROC curve (concordance index or *AUC*) was 0.69 for CDK1SA (95%CI:

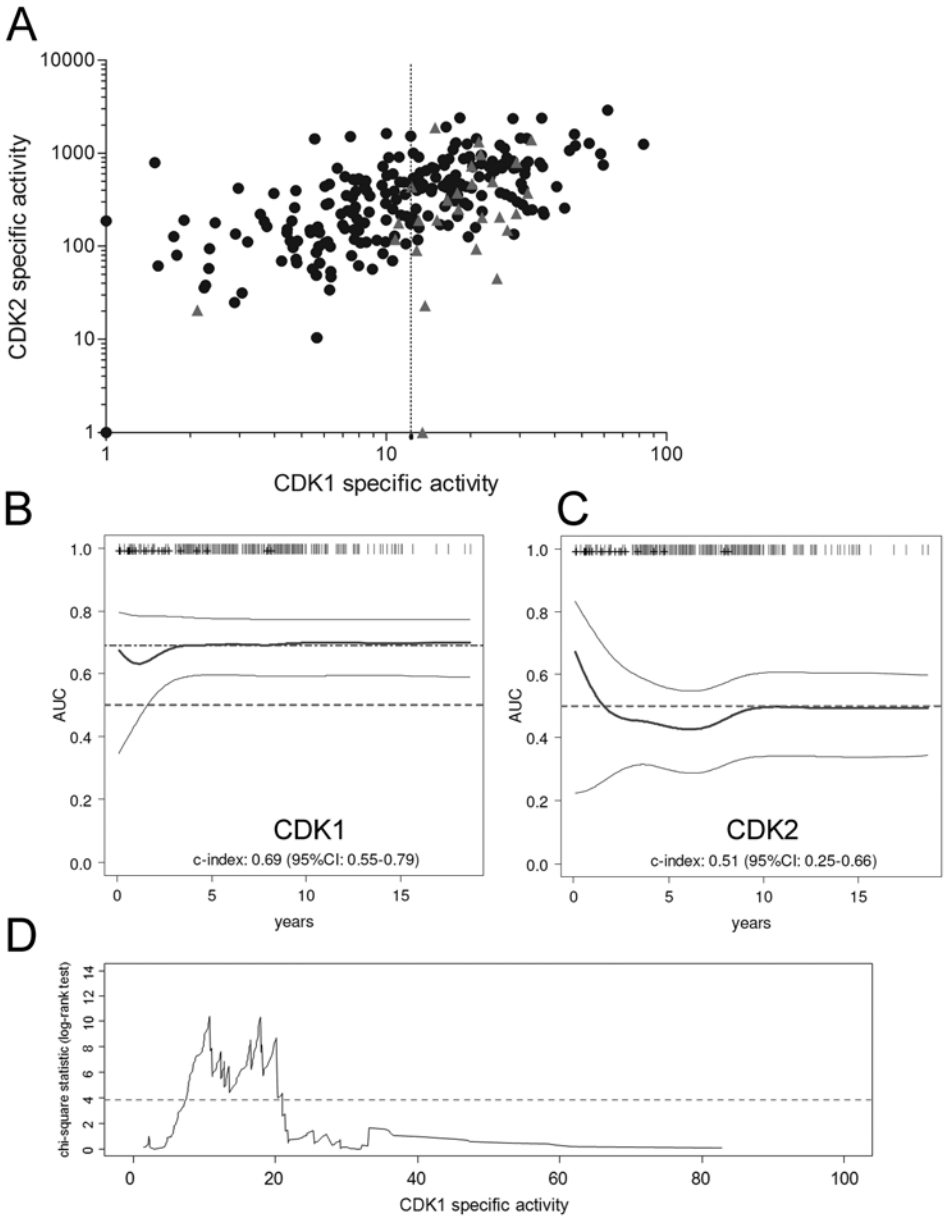

Figure 1

Prognostic performance of the specific activities of CDK1 and CDK2. (A) All cases (n=254) spotted on a scatter diagram with logarithmic scales according to CDK1 SA and CDK2 SA, respectively. Grey triangel: patient with distant metastasis, black dot: no metastasis. (B and C) Time-dependent ROC analysis against CDK1 SA (B) or CDK 2SA (C). Thick line: concordance index, thin line: 95% CI. Concordance index was 0.69 for CDK1 SA (95% CI: 0.55-0.79, P=0.036), and 0.51 for CDK2 SA (95% CI: 0.25-0.66, P=0.57). (D) Derivation of an optimal CDK1 SA cutoff value. The maximum log-rank test statistic was obtained when CDK1 SA was 11 or 18 (maUeU^{-1}).

0.55 to 0.79, p =0.024), and 0.51 for CDK2SA, respectively (95%CI: 0.29 to 0.66; p=0.57) (figure 1B, C). Combined, these results suggested that CDK1SA, but not CDK2SA, is valuable for long-term distant recurrence prediction. Therefore, we focused on CDK1SA and derived the statistically best discriminating cut-off value for CDK1SA, as indicated by maximum log-rank test. For 254 cases, two local maxima of log-rank test statistic were obtained, one for CDK1SA=11 (milli-activity unit per expression unit, maU/eU), and one for CDK1SA=18 (maU/eU) (figure 1D). In order to test the robustness of the selected cut-off values, a second cut-off derivation was performed using the subset of samples with CDK1SA>11 (maU/eU) (n=150). In this analysis, the previously proposed cut-off value of 18 (maU/eU) neither showed a significant maximum peak, nor was considerably elevated compared to the other candidate cut-off values. This result suggested that the optimal cut-off value for CDK1SA was indeed at 11 (maU/eU). The final bootstrap analysis confirmed a cut-off value for CDK1 SA of 11 (maU/eU) to be of sufficient discriminant value for further analysis. In conclusion, patients with CDK1 activity level > 11 (maU/eU) were classified in the high-risk group (n=104, 40% of the patients), and the remaining patients as low-risk (n=150, 60%).

CDK1-based risk prediction for distant metastasis-free survival and cause-specific survival

Univariable "time to event" analysis showed that patients from the CDK1SA-based low risk group had significantly longer distant metastasis-free intervals than patients in the high risk group (HR = 6.2; 95%CI: 1.45 to 26.9; p=0.0049) (figure 2A). Importantly, this finding was retained to be statistically significant after adjusting for the multiple log-rank testing which had been performed in order to obtain the optimal cut-off value of 11 (maU/eU) (exact conditional Monte-Carlo p-value = 0.029). The independence of prognostic ability of CDK1SA-based recurrence risk stratification was further evaluated and finally confirmed by multivariable analyses (table 2). Hazard ratio estimates remained nearly unchanged after consecutive adjustment for the most important clinical-pathological variables which are currently used for risk evaluation in stage II colon cancer: T4, poor differentiation, presence of obstruction or perforation, lymphatic and vessel invasion, high CEA level, and ≤12 regional lymph nodes examined (24;25)(table 2). Next, a putative confounding influence of mutations in the BRAF oncogene were analyzed. In 217 patients, tissue was available for high resolution melting analysis of mutations in exon 15 of BRAF. In 32 cases (14.8%), $BRAF^{V600E}$ mutations were detected, 183 patients had BRAF wild-type status, and 2 cases were not informative. In Kaplan-Meier analysis, the BRAF mutation status was not significantly associated with metastasis-free survival (p=0,337), nor with cause-specific survival (p=0,253; not shown), and it was excluded as confounding factor for CDK1SA-based risk prediction (table 2).
However, when considering stroma content as adjustment variable, a lack of statistical significance was apparent for the effect of dichotomized CDK1SA. The apparent absence of significance may be explained by the reduced statistical power for this parameter, since about 30% of the cases lacked available stroma content

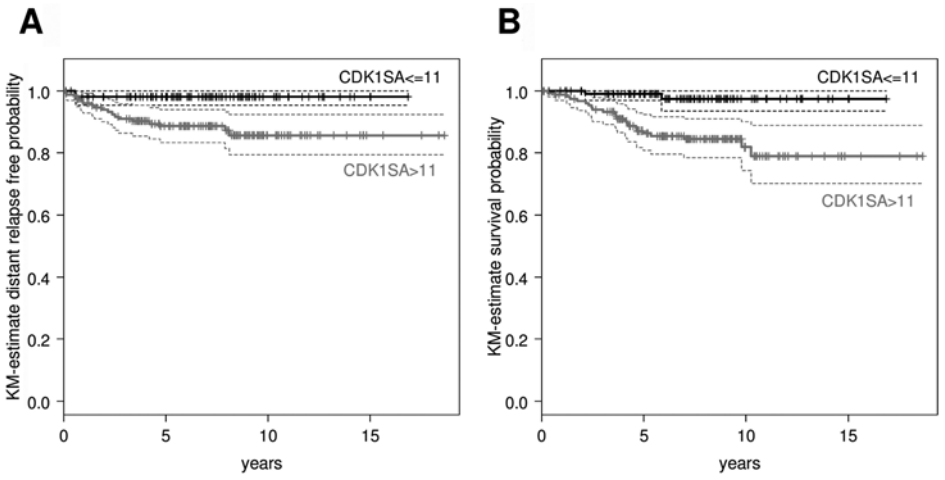

Figure 2

Analysis of distant metastasis-free survival and cause-specific survival. (A) Patients classified in the high-risk group (based on CDK1 SA > 11 maUeU^{-1}) had a significantly worse distant metastasis event rate as compared with the low-risk group (HR= 6.2, 95% CI: 1.45-26.9, P = 0.0049; exact conditional Monte-Carlo P-value = 0.029). (B) Patients classified in the CDK1 SA-based high-risk group had a significantly lower cause-specific survival (HR=7.62, 95% CI: 1.80-32.2, P= 0.001).

data. Twenty-five patients (10%) died during the follow-up, among them were all 20 patients with distant metastases, and only five patients with no evidence for distant metastases, but with local tumor recurrence. Due to this strong association of distant relapse and death, CDK1SA categorization was found to be a significant predictor for cause-specific survival (HR high-risk vs. low-risk group: 7.62; 95%CI: 1.80-32.2; p=0.001) (figure 2B). This result was thoroughly confirmed in the multivariable analyses. All adjusted estimates of the hazard ratio showed values of >7.75, with lower 95% confidence limits >1.80, and p-values <0.01. However, a non-significant hazard ratio was estimated after adjustment for stroma content (HR high-risk vs. low-risk group: 5.22; 95%CI: 0.65-41.5; p=0.12).

Correlation between CDK specific activity, cell proliferation and microsatellite status

Based on the knowledge of the process of tumorigenesis, high CDK1SA levels could be a reflection of strongly elevated tumor cell proliferation rates. Therefore, we have analyzed tumor cell proliferation with the established proliferation marker Ki-67. The Ki-67 labeling index, defined as the percentage of Cytokeratin20-positive cancer cells with Ki-67-positive nuclei, was determined for n=207 cases. The median of the Ki-67 index was 21.4%, but it was not retained by Cox regression analysis as significant prognostic factor for distant metastasis (HR = 0.69; 95%CI: 0.02 to 24.0; p=0.84). Next, a putative correlation between CDKSAs and the Ki-67 index was examined. However, no significant correlation was found between CDK1SA and Ki-67 index (Spearman's rho=+0.04; p=0.54) (figure 3).

Table 2

Consecutive (one-by-one) adjustment for confounding factors.

Pairwise comparison	Category	Subcategory	HR	95% CI for HR Lower	Upper	P-value
1	CDK1 SA	>11 vs ≤11	4.23	0.52	34.11	**0.180a**
	Stroma content	(%)	1.02	0.99	1.04	0.230
2	CDK1SA	>11 vs ≤11	6.24	1.44	26.93	**0.014**
	Histol. grade	>2 vs ≤ 2	0.99	0.13	7.49	>0.99
3	CDK1 SA	>11 vs ≤11	6.29	1.46	27.10	**0.014**
	pT stage	4 vs 3	1.29	0.38	4.40	0.69
4	CDK1 SA	>11 vs ≤11	6,52	1.51	28.14	**0.012**
	Sex	Female/male	0.48	0.18	1.25	0.130
5	CDK1 SA	>11 vs ≤11	6.57	1.51	28.54	**0.012**
	Age	(years)	1.01	0.98	1.05	0.470
6	CDK1 SA	>11 vs ≤11	5.6	1.287	24.42	**0.022**
	LN resected	>12 vs ≤ 12	0.52	0.21	1.31	0.165
7	CDK1 SA	>11 vs ≤11	8.23	107	63.54	**0.043**
	CEA	(serumlevel)	0.99	0.89	1.10	0.828
8	CDK1 SA	>11 vs ≤11	11.08	1.46	83.93	**0.020**
	Obstruction	Yes/No	0.68	0.19	2.39	0.545
9	CDK1 SA	>11 vs ≤11	9.85	1.29	75.32	**0.028**
	Perforation	Yes/No	0.00	0.00		0.988
10	CDK1 SA	>11 vs ≤11	11.43	1.51	86.73	**0.018**
	Lymphinvasion	Yes/No	0.22	0.63	7.73	0.219
11	CDK1 SA	>11 vs ≤11	11.44	1.51	86.73	**0.018**
	Angioinvasion	Yes/No	4.75	0.62	36.69	0.135
12	CDK1 SA	>11 vs ≤11	11/17	1.48	84.57	**0.019**
	BRAF	Mutated/WT	0.39	0.05	2.92	0.356

Abbrevations: CI = confidence interval; HR = hazard ratio; pT = tumour stage; WT = wild type. [a]82 cases (32%) with missing value for stroma content. CDK1SA is not significant (P= 0.180), however; in all other test against confounding factors, CDK1 SA achieved significance.

Lastly, a putative correlation between genomic instability and CDK1 activity was tested, since CDKs have been shown to be implicated in cellular responses to genetic instability. Microsatellite instability (MSI), caused by defects in the cellular mismatch repair system, has been suggested for colorectal cancer as a favorable prognostic marker. The microsatellite instability status was determined with standard methods for 223 cases, and a high level of instability was detected in 59 tumors (26.5%, MSI-High), whereas 164 samples showed stable microsatellite repeats (73%, MSS). Cox regression analysis indicated an estimated five-fold risk-difference regarding distant metastasis free survival for microsatellite stable patients, but the results did not attain significance (HR 5.898; CI95% 0.782-44.481; p=0.085). A significant association of microsatellite instability and CDK1SA-based risk stratification was apparent, based on the cut-off for CDK1SA of 11 (maU/eU). In the patient group with stable microsatellites, significantly more cases with

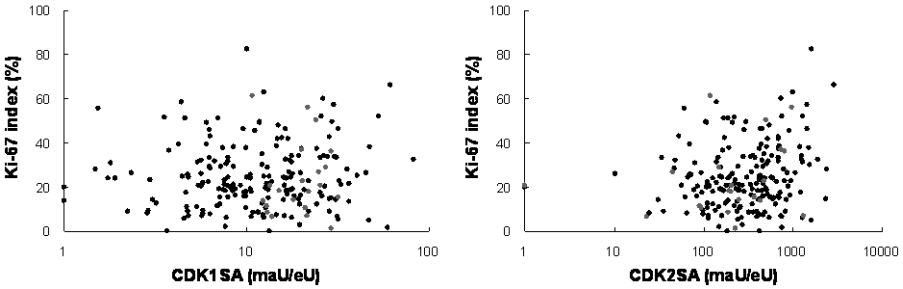

Figure 3

Correlation between CDK SAs and Ki-67 index (percent of Ki-67 positive cells of all CDK20-positive tumour cells). Cases were plotted on a scatter diagram according to Ki-67 index against CDK1 SA (left), or CDK2 SA (right). Grey circle: tumor with distant metastasis. Ki-67 showed a weak but significant positive correlation with CDK2 SA (Spearman's ρ = 0.17, P=0.016), but not with CD1 SA (Spearman's ρ= 0.54).

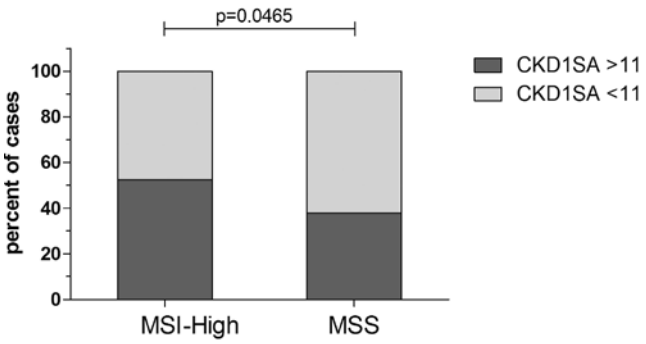

Figure 4

Association of CDK1 SA-based risk stratification with microsatellite-stable phenotype. Among the patients with a stable microsatellite phenotype (MSS), 62% (102 out of 164) were classified in the high-risk group based on CDK1 SA. On the other hand, 47.5% (28 out of 59) of the patients with high MSI (MSI-H) were classified as high-risk, based on the CDK1 SA threshold (χ^2 –test, P=0.0465).

elevated CDK1 specific activity were observed (chi-square test: p=0.0465, figure 4). However, a direct comparison of CD1SA between patients with stable or unstable microsatellites did not attain significance.

DISCUSSION

This study is the first report demonstrating the specific activity of CDK1 (CKD1SA) as prognostic biomarker for stage II colon cancer in a blinded and retrospective manner. Two patient cohorts from Germany and the Netherlands were included in this study. Essentially, no differences were observed between these cohorts regarding clinical parameters or CDK1 activity, indicating that the patients were recruited in an unbiased manner. However, the average of CDK2SA was slightly but significantly higher in the samples from the Netherlands. This may be due to differences in sample embedding and preparation between the study centers, and to technical variations between the assay systems for CDK1SA and CDK2SA. Previously, CDK1SA- and CDK2SA-based risk was shown to be a clinically useful prognostic marker of early breast cancer of Caucasian and Asian cohorts (23-25).

To identify patients with unfavorable prognosis who might benefit from adjuvant chemotherapy, several types of staging systems have been developed (4;7;28;29). The current staging systems, however, do not provide accurate risk assessment for stage II patients (29). Moreover, a number of molecular markers have been proposed, such as mutations in KRAS and TP53, loss of heterozygosity of chromosome 18, and microsatellite instability (12;30;31). However, none of these candidate biomarkers has yet clearly proven to be useful for diagnosis or staging of patients with stage II colorectal cancer, except for mutations in the BRAF oncogene, which were found to be prognostic for overall survival, particularly in patients with microsatellite stable tumors (32;33). Comprehensive approaches using "omics" technologies have been applied to find biomarkers for colorectal cancer, and we and many others have proposed prognostic transcriptome profile sets so far (34-38). However, inter-patient and even intra-tumoral heterogeneity, as well as cost factors have precluded wide-scale clinical application. A promising strategy to circumvent tumor heterogeneity is to focus on the central hallmarks of cancer, which are present in almost all tumors irrespective of the underlying molecular changes. Altered cell proliferation and genomic instability are central hallmarks in the case of colon cancer (15;39). Therefore, we focused on the enzymatic activities and protein expression of cyclin dependent kinases (CDKs), the main drivers of cell cycle progression. Moreover, CDK regulators have been well documented as prognostic indicators in many solid tumors (16-22).

Indeed, CDK1SA was a substantial and constant marker for long-term event prediction of distant metastasis in the present study. A robust cut-off value for CDK1SA was derived by choosing a threshold with maximum log-rank statistics (27). Importantly, the cut-off value of 11 (maU/eU) was verified by the adjusted multiple log-rank test. Multivariate analysis retained CDK1 specific activity as independent predictor of distant recurrence. None of the currently accepted clinical risk factors, e.g., T4 stage, poor differentiation, obstruction or tumor perforation (40), was identified as confounding factor (table 2). Moreover, CDK1SA was independent of the mutation status in the BRAF oncogene. Therefore, we conclude that CDK1SA-based risk stratification is a reliable prognostic marker for distant metastasis in

stage II colon cancer. Two hypotheses, which are not mutually exclusive, may explain the increased intratumoral CDK1SA level in patients with worse prognosis. First, specific activity of CDK1 may directly reflect higher cancer cell proliferation. To address this question, we have examined a putative correlation between CDK1SA and proliferation. The index of proliferating cancer cells did not significantly correlate with CDK1SA. Moreover, the Ki-67 proliferation index itself was not significant for prognosis, in accordance with earlier findings (41). Second, CDK1 activity may be elevated due to chromosomal instability, a factor already associated with worse prognosis (40). Indeed, high CDK1SA levels were significantly correlated with a stable microsatellite phenotype (chi-square test: $p = 0.0465$). To the best of our knowledge, no reports exist that provide a cause-and-effect link between CDK1 activity and microsatellite instability. However, colorectal tumors with stable microsatellites are thought to present chromosomal instability (CIN), associated with worse prognosis. Thus, microsatellite-stable tumors with high CDK1SA levels in our collective are likely to display chromosomal instability. On the molecular level, regulation of CDK1 activity is orchestrated by cellular checkpoints. Altered expression and activity of the DNA damage and spindle-checkpoint proteins are frequently observed in cancer cells, and contribute to chromosomal instability (15). Thus, deregulated checkpoint pathways could cause an aberrant activation of CDK1. Indeed, over-expression of both cyclinB1 and CDC25, important regulators of CDK1 activity, are prognostic markers in colorectal and other cancers (18;21;42). In conclusion, CDK1SA-based analysis is a robust and useful assay to identify patients with a high risk of distant recurrence, who could benefit from adjuvant chemotherapy.

ACKNOWLEDGEMENTS

The authors wish to thank Alexandra Gnann, Sabine Leis, Gabi van Pelt and Inès Goossens-Beumer for their excellent technical assistance and for helpful discussion. The study was supported in part by Sysmex Corporation, Hyogo, Japan.

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