

Mutations in the Ras–Raf Axis Underlie the Prognostic Value of CD133 in Colorectal Cancer

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Abstract

Purpose: High expression of cancer stem cell (CSC) marker CD133 has been used as a predictor for prognosis in colorectal cancer (CRC), suggesting that enumeration of CSCs, using CD133, is predictive for disease progression. However, we showed recently that both CD133 mRNA and protein are not down-regulated during differentiation of colon CSCs, pointing to an alternative reason for the prognostic value of CD133. We therefore set out to delineate the relation between CD133 expression and prognosis.

Experimental Design: A CRC patient series was studied for expression of CD133 and other CSC markers by microarray and quantitative PCR analysis. In addition, several common mutations were analyzed to determine the relation with CD133 expression.

Results: CD133 mRNA expression predicted relapse-free survival in our patient series, whereas several other CSC markers could not. Moreover, no correlation was found between expression of other CSC markers and CD133. Interestingly, high CD133 expression was related to mutations in K-Ras and B-Raf, and inhibition of mutant K-Ras or downstream mitogen-activated protein kinase kinase (MEK) signaling decreases CD133 expression. In addition, an activated *K-Ras* gene expression signature could predict CD133 expression in our patient set as well as data sets of other tumor types.

Conclusion: CD133 expression is upregulated in CRC tumors that have a hyperactivated Ras–Raf–MEK–ERK pathway and is therefore related to mutations in *K-Ras* or *B-Raf*. As mutations in either gene have been related to poor prognosis, we conclude that CD133 expression is not indicative for CSC numbers but rather related to the mutation or activity status of the Ras–Raf pathway. *Clin Cancer Res*; 18(11); 3132–41. ©2012 AACR.

Introduction

To develop a better strategy for defining which patient with colorectal cancer (CRC) should receive adjuvant chemotherapy after surgery, reliable markers for predicting relapse have been studied extensively (reviewed in ref. 1). Mutations in signaling pathways are used as markers for predicting prognosis, but also expression levels of proteins that are suggested to identify the cancer stem cell (CSC) fraction of the tumor (2–15). CSCs, the driving force behind tumor initiation, growth, and metastasis, are hypothesized to be crucial for patient prognosis. This current model

hypothesizes that the expression of CSC markers correlates to the number of CSCs within a tumor and thereby to the patient prognosis. The following markers are used to identify CRC CSCs: CD24 (16), CD29 (16), CD44 (17–19), CD133 (16, 20–22), CD166 (18), Lgr5 (23), and ALDH1A1 (24). Previously, CD133 was indeed identified as an independent prognostic marker for CRC by showing that either high expression of CD133 mRNA or intense CD133 staining in immunohistochemistry relates to poor relapse-free/overall survival (refs. 2–7, 13, 15; Supplementary Table S1). As CD133 marks CSCs, it was postulated that high CD133 expression correlates to more CSCs and thus more tumorigenic and/or metastatic capacity. However, CD133 is not only expressed on CSCs, but also on differentiated cells, both at the mRNA (25) and protein level (25, 26). Previously, we found that the use of CD133 as a CSC marker depends on the availability of the AC133 epitope and is not determined by CD133 mRNA or protein expression, which remain unchanged during *in vitro* and *in vivo* differentiation of colon CSCs (27). Recently, it was confirmed that *N*-glycosylation of CD133 affects the cell surface recognition of AC133 (28). Altogether, this suggests that the prognostic value of CD133 in CRC is not due to an enumeration of CSCs.

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Translational Relevance

Reliable markers for predicting which patients with stage II colorectal cancer (CRC) have a higher change of relapse and should receive adjuvant therapy after surgery are needed. Because cancer stem cells (CSC) are identified as the driving force behind tumor initiation, growth, and metastasis, the expression of CSC markers is studied as a predictor for prognosis. We show here that high mRNA expression of CSC marker CD133 can identify patients with higher change of relapse in a stage II CRC cohort. Interestingly, high CD133 expression was related to mutations in *K-Ras* and *B-Raf*. The Ras–Raf–MEK–ERK pathway can regulate CD133 expression, explaining how activating mutations in *K-Ras* or *B-Raf* can correlate to higher CD133 expression. We found that a *K-Ras* gene expression profile can even better predict prognosis, suggesting that the activity status of the Ras–Raf pathway might be a better predictor for relapse than CD133 expression or mutations in *K-Ras* and *B-Raf*.

Other factors are shown to correlate to poor prognosis in CRC, such as mutations in tumor suppressor gene *p53* (29, 30) or proto-oncogenes such as *K-Ras* (31, 32) and *B-Raf* (33). The prognostic value of *K-Ras* although was not identified in large stage II–III cohort of CRC (34). *p53* is frequently mutated or lost (60%–70%) in CRCs, whereas *K-Ras* is mutated in approximately 40% of all CRCs (35). *B-Raf* is directly downstream of Ras and is mutated at position V600 in 5% to 10% of all CRC cases. The latter 2 mutations both lead to aberrant activation of the Ras–Raf–MEK–ERK pathway (35, 36) and are mutually exclusive in tumors (37).

To understand the role of CD133 expression levels in CRC prognosis, we analyzed a stage II CRC patient set for specific mutations and expression of CSC markers by microarray and quantitative PCR (qPCR). We found that high CD133 expression, in contrast to other CSC markers, can predict relapse-free survival. However, high CD133 expression was related to mutations in *K-Ras* or *B-Raf*. We confirmed that CD133 was indeed regulated by the Ras–Raf–MEK–ERK pathway. Altogether, we conclude that CSC marker CD133 is prognostic in CRC because its expression reflects the mutation or activation status of the Ras–Raf–MEK–ERK pathway.

Materials and Methods

Clinical samples, microarray, K-Ras profile, and patient databases

Tumor samples were collected of 90 patients with American Joint Committee on Cancer (AJCC) stage II CRC (AMC-AJCCII-90) as described previously in the work of de Sousa e Melo and colleagues (38). The study was approved for by the medical ethical board of the Academic Medical Center

(AMC; Amsterdam, Netherlands) and in accordance with the rules and legislation on human experimentation in the Netherlands. Shortly, these patients with stage II CRC received surgery with curative intent in the AMC in Amsterdam, the Netherlands, between 1997 and 2006 (AMC-AJCCII-90). Extensive medical records and long-term follow-up are available for these patients. Fresh-frozen tissue was used to isolate total RNA from by TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA was analyzed by NanoDrop ND-1000 and RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Affymetrix microarray analysis, fragmentation of RNA, labeling, hybridization to Human Genome U133 Plus 2.0 microarrays, and scanning were conducted in accordance with the manufacturer's protocol (Affymetrix). Microarray data can be viewed online (<http://www.ncbi.nlm.nih.gov/geo/index.html>) under Gene Expression Omnibus (GEO) accession number (GSE33113). Five patients were excluded for further analysis because CD133 expression levels were below detection threshold. The following probe sets, recognizing all isoforms of these proteins, were selected to avoid effects caused by differential splicing: 204304_s_at (CD133), 209771_x_at (CD24), 1553530_a_at (CD29), 210916_s_at (CD44), 201952_at (CD166), 213880_at (Lgr5), and 212224_at (ALDH1A1). The *K-Ras* profile was extracted from the study of Bild and colleagues (39). The following data sets were used: neuroblastoma (40), glioma (GSE4290; ref. 41), breast (GSE12276; ref. 42), and ovarian cancer (GSE12172; ref. 43).

qPCR

cDNA was prepared with reverse transcriptase III (Invitrogen) according to manufacturer's protocol. qPCR was conducted on the LC480 II (Roche) with intron-spanning primers for CD133 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Supplementary Table S2).

Mutation analysis

Microsatellite instability (MSI) analysis was conducted with the MSI Analysis System, Version 1.2 (Promega) according to manufacturer's protocol. Sequencing of *K-Ras*, *B-Raf*, and *p53* was conducted with Big Dye Terminator (BDT, Applied Biosystems). Primers are described in Supplementary Table S2.

Statistical analysis, k-means clustering, and gene set enrichment analysis

The association between CD133 and clinicopathologic features was analyzed by the χ^2 test. Survival curves were drawn by the Kaplan–Meier method whereas *P* values were calculated by log-rank test. Cox proportional hazard model was used for uni- and multivariate analysis. All *P* values are two-sided. Statistical analysis was conducted in SPSS and Graphpad Prism 5. The *k*-means clustering was conducted by in-house developed software. Clusters were identified by gene set enrichment analysis (GSEA; <http://www.broadinstitute.org/gsea/index.jsp>).

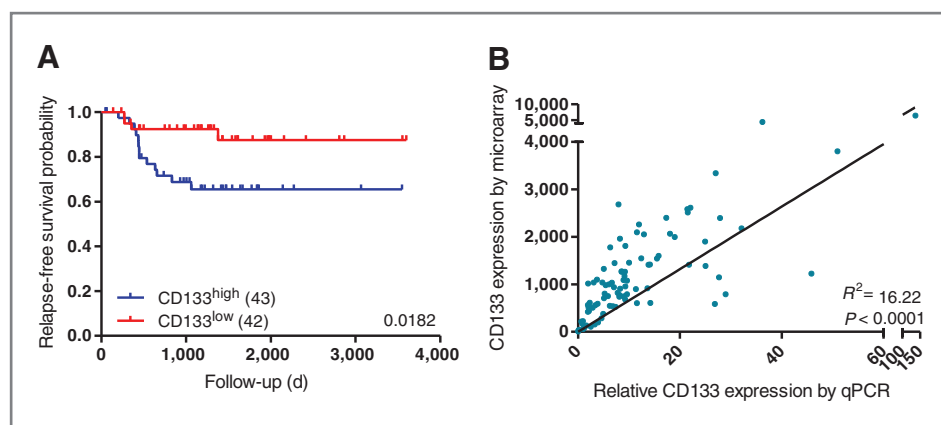


Figure 1. CD133 is a predictor for poor prognosis in relapse-free survival. A, high CD133 expression can be used as a prognostic marker for relapse-free survival. As cutoff value between the groups, the median expression level is used. B, the microarray data were validated by qPCR. Values are normalized to GAPDH. Graph represents qPCR data plotted against microarray data. Linear regression indicates a very significant correlation between the qPCR and the microarray data.

Cell culture and MEK inhibition

HCT116, HKH2, Dld1, and DKO4 were kindly provided by Onno Kranenburg (Department of Surgery, University Medical Center Utrecht, Utrecht, the Netherlands) and cultured as previously described (44). CSC lines, approved by the medical ethical committee of the AMC and the University of Palermo (Buenos Aires, Argentina), were derived and cultured as described previously (22). Mitogen-activated protein kinase (MEK) inhibition was conducted with 10 $\mu\text{mol/L}$ U0126 (#9903, Bioke) for 24 hours. Methylation was inhibited by 2 $\mu\text{mol/L}$ 5-aza-deoxycytidine for 24 hours. After treatment with U0126, cells were analyzed for ERK phosphorylation by Western blotting. The antibodies used for Western blotting were p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; Bioke).

Fluorescence-activated cell-sorting analysis

Cells were stained with CD133-PE (phycoerythrin; AC133, Miltenyi) or mlgG1-PE (Miltenyi) as isotype control for 20 minutes at 4°C in PBA (PBS containing 1% bovine serum albumin and 0.02% sodium azide). For intracellular detection of pERK of LM5, phospho-p44/42 MAPK (ERK1/2; Thr202/Tyr204; Alexa Fluor488 conjugate) or rabbit IgG isotype control (Alexa Fluor488 conjugate; Bioke) was used. Briefly, cells were washed with cold PBS and fixed with 4% paraformaldehyde at 37°C for 10 minutes. After fixation, cells were permeabilized in 90% ice-cold methanol for 30 minutes. Next, cells were stained for 1 hour in PBA at a dilution of 1:50, washed, and subsequently analyzed. Fluorescence-activated cell-sorting (FACS) analysis was conducted on the FACSCanto and analysis was conducted with FlowJo software.

Results

CD133 is a predictor for poor prognosis in relapse-free survival

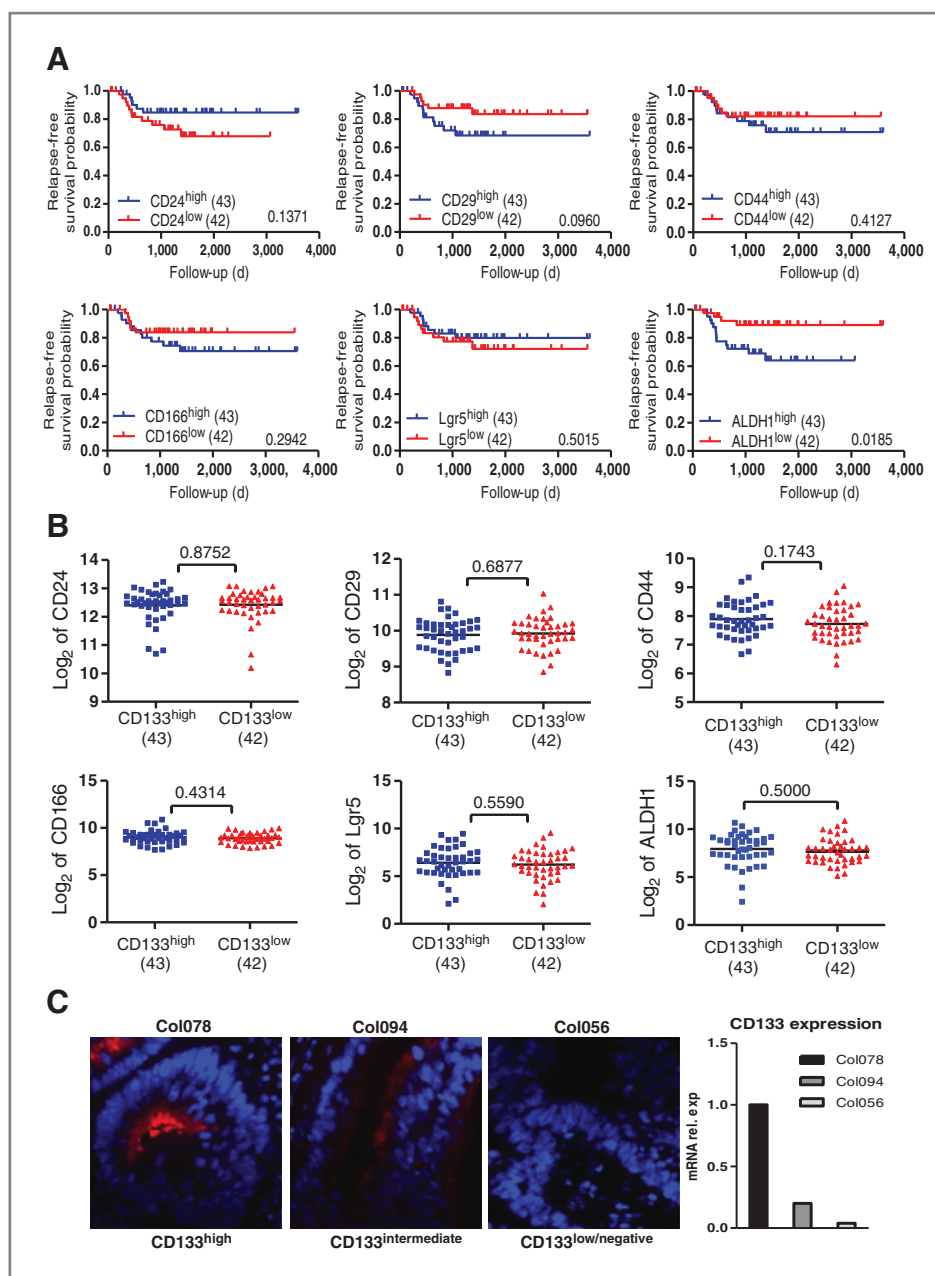
Previously, several groups showed that CD133 expression, analyzed by mRNA expression or immunohistochemistry, can be used to predict prognosis in CRCs (refs. 2–7, 13, 15; Supplementary Table S1). We used our

AMC-AJCCII-90 CRC patient set to study CD133 as a prognostic marker. Isolation of RNA from these tumor samples and subsequent microarray analysis were conducted to determine the expression level of CD133. The median expression level of CD133 was used to separate the cohort in 2 groups. In 5 cases, CD133 expression levels were not above background values and these tumors were excluded from further analyses. Using this approach, we observed that tumors in the CD133^{high} group expressed on average 3.7-fold more CD133 compared with the CD133^{low} group. Validation of the microarray CD133 expression data with qPCR showed a very significant correlation between the microarray and the qPCR data (Fig. 1B). In agreement with previous studies, high CD133 expression predicted poor relapse-free survival with a significance of $P = 0.0182$ (Fig. 1A). The χ^2 analysis of clinicopathologic features showed that elevated CD133 expression was associated with metastasis and local recurrence ($P = 0.026$ and $P = 0.017$, respectively) but not with gender, differentiation grade, location of the tumor, or age of the patient at surgery (Supplementary Table S3).

Other CSC markers do not predict prognosis

CD133 expression levels have been suggested to predict prognosis because they identify the CSC fraction of the tumor (2–7, 13, 15). We therefore hypothesized that expression of other CSC markers should also be able to predict patient outcome. Several colon CSC markers, such as CD24, CD29, CD44, CD166, Lgr5, and ALDH1A1, were analyzed for their prognostic value. As reported before (24), of these, only ALDH1A1 showed an association with prognosis when using the median value of expression, whereas all other CSC markers failed to show any prognostic value (Fig. 2A). Only when the highest quartile was used to divide the groups, CD44 showed some prognostic value (Supplementary Fig. S1). Strikingly, some of the CSC markers, such as CD24 and Lgr5, even displayed an opposite trend, with high expression of the marker relating to good prognosis. This is a counterintuitive finding as these CSC markers should all be highly expressed in the same cell population (the CSCs). However, closer evaluation of the relation between CD133 expression and the other CSC markers

Figure 2. Other CSC markers can not be used as prognostic markers. A, colon CSC markers CD24, CD29, CD44, CD166, Lgr5, and ALDH1A1 were tested for their prognostic value. As cutoff value, the median expression level was used. B, the expression levels of CSC markers CD24, CD29, CD44, CD166, Lgr5, and ALDH1A1 were analyzed in the CD133^{high} and CD133^{low} group. C, immunofluorescence of CD133 in 3 patients expressing low, intermediate, and high CD133 mRNA. Right, microarray expression levels of CD133. rel, relative.



showed no significant difference in CSC marker expression found between the CD133^{high} and CD133^{low} group (Fig. 2B). Furthermore, the expression of CSC markers did not correlate to each other (Supplementary Fig. S2). This suggests that even though isolated CSC markers may predict prognosis, their expression does not appear to enumerate CSCs as this would have resulted in a consistent correlation between the markers. To directly determine whether changes in CD133 levels reflect expression differences and not differences in number of CSCs, we conducted immunofluorescent stainings on patient sections with low, intermediate, and high CD133 expression as determined by the microarray. In agreement with our earlier observations

(27), these sections reveal that CD133 expression is detected in a large part of the tumor cells when using paraformaldehyde-fixed frozen sections and that the level of expression correlates with the mRNA expression data (Fig. 2C). We therefore conclude that CD133 levels do not correlate to CSC numbers and that an alternative explanation must exist as to why the CSC marker CD133 is related to poor prognosis in CRCs.

High expression of CD133 associates with *K-Ras* and *B-Raf* mutations

We hypothesized that a mutation could be responsible for the prognostic potential of CD133. Therefore, all tumors

were analyzed for MSI status and mutations in *p53*, *K-Ras*, and *B-Raf*. Around 28% of the tumors were MSI, 29% carried a *p53* mutation, whereas *K-Ras* and *B-Raf* mutations were found in, respectively, 24% and 20% of the tumor set. There was no significant difference in MSI and mutations in *p53* and *K-Ras* between the CD133^{high} and CD133^{low} group, as analyzed by χ^2 tests. Interestingly, tumors with a *B-Raf* mutation clustered significantly in the CD133^{high} group ($P = 0.003$). As *B-Raf* and *K-Ras* both activate the same pathway, we combined patients having either a *K-Ras* or *B-Raf* mutation and compared them with *K-Ras/B-Raf* wild-type patients. More than half of the tumors in the CD133^{high} group had a mutation in either *K-Ras* or *B-Raf*, whereas only a fourth of the tumors in the CD133^{low} group had such a mutation ($P = 6.4 \times 10^{-5}$; Table 1). Then, we analyzed whether CD133 was an independent prognostic marker by comparing uni- and multivariate Cox regression analyses and found that CD133 was independent of any of the clinicopathologic features but was dependent on *K-Ras* and *B-Raf* mutations (Table 2). Patients with a *K-Ras* or *B-Raf* mutated CRC have been shown to have a worse prognosis (31–33), although this has recently been challenged for *K-Ras* mutations (34). A link between CD133 expression and activation of the Ras–Raf–MEK–ERK pathway, specifically by *B-Raf* mutations could therefore explain the prognostic value of CD133.

Disruption of mutant *K-Ras* or downstream signaling decreases CD133 expression

Previously, it has been shown that activation of the Ras–Raf–MEK–ERK pathway results in activation of promoter 5 of the *CD133* gene, thereby inducing CD133 expression (45). Therefore, mutations in *K-Ras/B-Raf* could result in increased CD133 expression. To investi-

Table 1. Mutation correlated to CD133 expression in stage II colon tumors

	CD133 ^{high}	CD133 ^{low}	P
p53 mutation			
Mutated	11/43	13/40	0.487
WT	32/43	27/40	
MSI status			
MSI	15/43	9/42	0.190
MSS	28/43	32/42	
Ras			
Mutated	12/43	8/42	0.336
WT	31/43	34/42	
Raf			
Mutated	14/43	3/42	0.003
WT	29/43	39/42	
Ras/Raf			
Ras/Raf mutated	26/43	11/42	0.000064
WT	17/43	31/42	

NOTE: χ^2 test of different mutations compared with CD133 expression levels.

Abbreviations: MSS, microsatellite stable; WT, wild-type.

gate this, 2 CRC cell lines HCT116 and Dld1 were used, both of which harbor an activating mutation in one allele of the *K-Ras* gene. By gene targeting of the mutant *K-Ras* allele, derivative lines that only contain wild-type *K-Ras* were produced (44). These wild-type *K-Ras* HCT116 and Dld1 lines display reduced growth *in vitro* and *in vivo* (44). We used the parental lines (HCT116 and Dld1) and their targeted offspring (called HKH2 and DKO4,

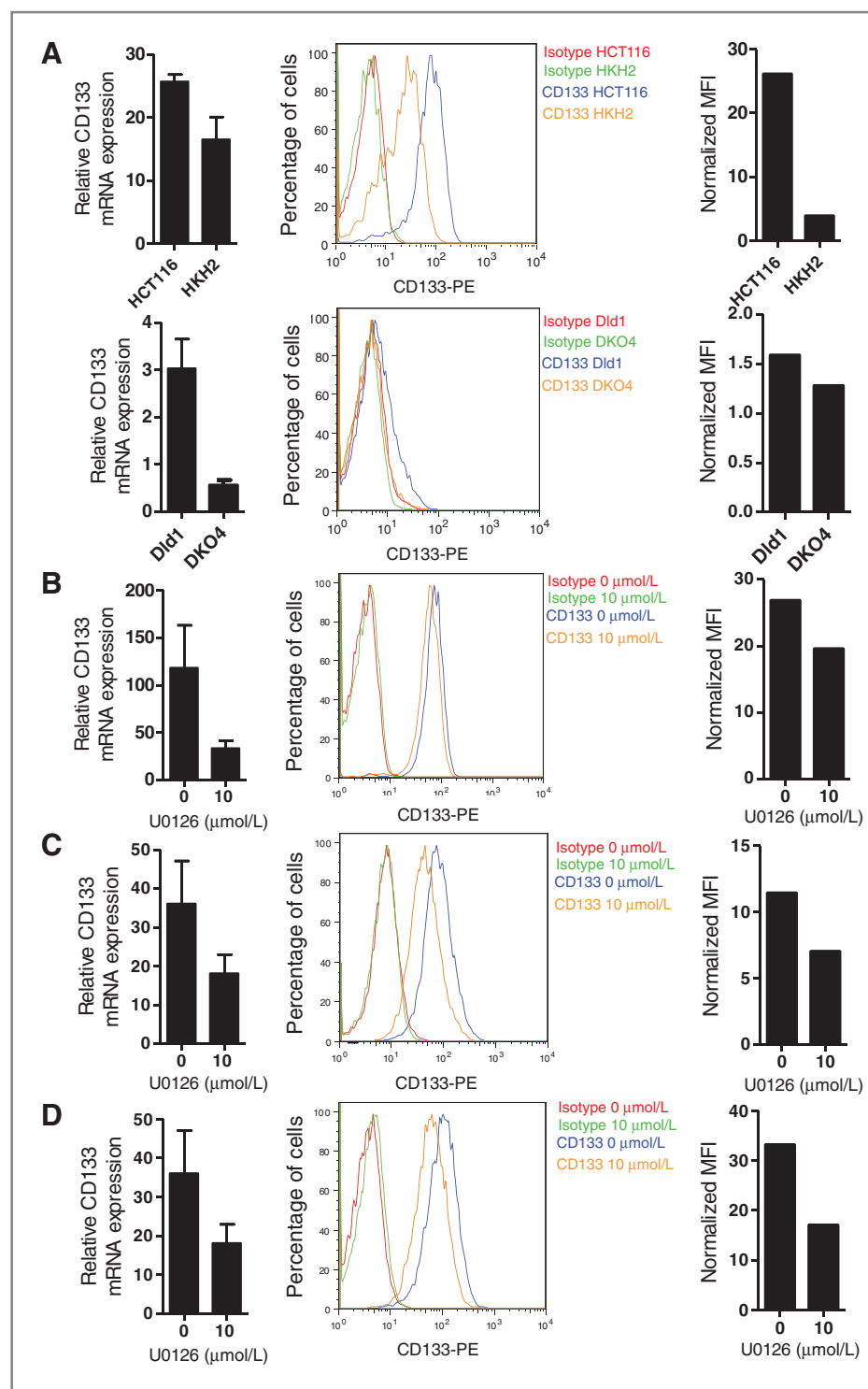
Table 2. Uni- and multivariate Cox analyses for survival of patient with CRCs

Variable	Univariate analysis		Multivariate analysis #1		Multivariate analysis #2	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
CD133	3.546 (1.155–10.885)	0.027	2.720 (0.805–9.189)	0.107	3.933 (1.250–12.374)	0.019
K-Ras mutation	1.422 (0.501–4.036)	0.509	1.489 (0.402–5.278)	0.537	ND	
B-Raf mutation	2.233 (0.826–6.041)	0.114	2.035 (0.611–6.772)	0.247	ND	
p53 mutation	1.098 (0.386–3.123)	0.860	ND		1.038 (0.331–3.249)	0.950
MSI status	0.765 (0.249–2.347)	0.640	ND		0.517 (0.137–1.948)	0.329
Differentiation grade	0.806 (0.298–2.180)	0.671	ND		0.672 (0.255–2.004)	0.476
Gender	1.387 (0.513–3.751)	0.520	ND		1.662 (0.516–5.351)	0.394
Age at resection	0.434 (0.160–1.173)	0.100	ND		0.312 (0.109–0.892)	0.030
Localization	1.149 (0.425–3.109)	0.784	ND		1.239 (0.363–4.231)	0.732

NOTE: Univariate proportional hazard Cox regression analysis was conducted for all variables, whereas 2 sets of multivariate analysis were conducted. Analysis #1 includes only CD133, *K-Ras*, and *B-Raf* as variables, whereas analysis #2 includes CD133 and all the other variables, showing that CD133 is independent of *p53* mutations, MSI, age, gender, differentiation status, and localization, whereas it is dependent on *K-Ras* and *B-Raf* mutations.

Abbreviations: CI, confidence interval; ND, not done.

Figure 3. Disruption of mutant *K-Ras* or downstream signaling decreases CD133 expression. **A**, targeted disruption of the mutant *K-Ras* allele in HCT116 reduces CD133 expression both on mRNA, normalized for GAPDH expression (left), and protein, as shown by FACS analysis (middle). The mean fluorescence intensity (MFI) of the specific CD133 staining was divided by the background isotype staining to determine the ratio of specific staining (right). After treatment with 5-aza-deoxycytidine for 24 hours, Dld1 cells show expression of CD133 whereas DKO4, wherein the mutant *K-Ras* is disrupted, do not. **B–D**, inhibition of downstream MEK by U0126 inhibitor for 24 hours reduces CD133 expression in CRC lines on mRNA as well as protein shown by FACS analysis. **B**, *K-Ras* mutant HCT116. **C**, *B-Raf* mutant LS411N D. **D**, *K-Ras* mutant CSC line C002.



respectively) to study the CD133 expression in the presence or absence of mutant *K-Ras*. HCT116 cells had high expression of CD133 shown by FACS and qPCR. In contrast, expression of CD133 was significantly lower in the wild-type *K-Ras* derivative HKH2 (Fig. 3A). In contrast to HCT116, Dld1 and DKO4 do not express CD133,

which is due to specific promoter methylation (ref. 46; Supplementary Fig. S3A and S3B). To determine whether *K-Ras* mutation also enhances CD133 expression in this cell line, we first treated Dld1 and DKO4 cells with 5-aza-deoxycytidine, an inhibitor of DNA methylation, to alleviate this repression. Treatment of Dld1 with 5-aza-

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deoxycytidine resulted in a small shift in the FACS profiles pointing to a low expression of CD133. However, in the wild-type *K-Ras* derivative DKO4, this CD133 was lost again, which was also reflected on the mRNA level (Fig. 3A). So, disruption of mutant *K-Ras* is decreasing the expression of CD133 both on the level of mRNA and protein.

Because these isogenic lines were kept in culture for a large number of passages, they may have acquired more differences than only their Ras status. We therefore decided to directly test the effect of Ras pathway inhibition by treatment of CRC lines with a potent MEK inhibitor U0126. Inhibition of MEK resulted in a strong reduction of ERK phosphorylation (Supplementary Fig. S3C and S3D). Moreover, MEK inhibition reduced the CD133 mRNA and protein expression in HCT116 and LS411N (Fig. 3B and C). In addition, treatment of 2 established colon spheroid cultures derived from a primary CRC (16), having high CD133 expression, with U0126 resulted in reduced CD133 mRNA and protein expression (Fig. 3D; Supplementary Fig. S3E). These data show that CD133 expression is dependent on the activity of the Ras–Raf–MEK–ERK pathway.

Activated *K-Ras* gene expression signature correlates to CD133 expression

Although *K-Ras* and *B-Raf* mutations both lead to activation of the Ras–Raf–MEK–ERK pathway, alternative means may lead to pathway hyperactivity in cancers. We therefore studied whether CD133 expression directly correlated with pathway activity. We made use of an activated *K-Ras* gene expression signature (39) to conduct *k*-means clustering in our patient set. The group with the activated *K-Ras* signature was identified by GSEA (Supplementary Fig. S4A). The activated *K-Ras* cluster contained 26 patients with mutations in *K-Ras* or *B-Raf*, whereas only 9 patients with *K-Ras/B-Raf* mutations were found in the control group, confirming that this signature identifies activated Ras–Raf–MEK–ERK signaling but also suggesting that not all *K-Ras/B-Raf* mutant CRCs display a hyperactive pathway. Tumors in the activated *K-Ras* cluster had a higher CD133 expression than the control cluster (Fig. 4A). In addition, the activated *K-Ras* signature was able to predict the relapse-free survival in our patient series ($P = 0.0016$; Fig. 4B). So, in CRCs, we can define a relation between the hyperactivation of the Ras–Raf–MEK–ERK pathway, CD133, and prognosis.

When the expression of other CSC markers was analyzed between these 2 clusters, some markers, such as CD29 and CD44, were indeed upregulated in the activated *K-Ras* cluster, whereas others, such as CD24 and *Lgr5*, were downregulated (Fig. 4C), indicating that activation of the Ras–Raf–MEK–ERK pathway does not simply increase the numbers of CSCs in these tumors but apparently regulates, directly or indirectly, the expression of these proteins.

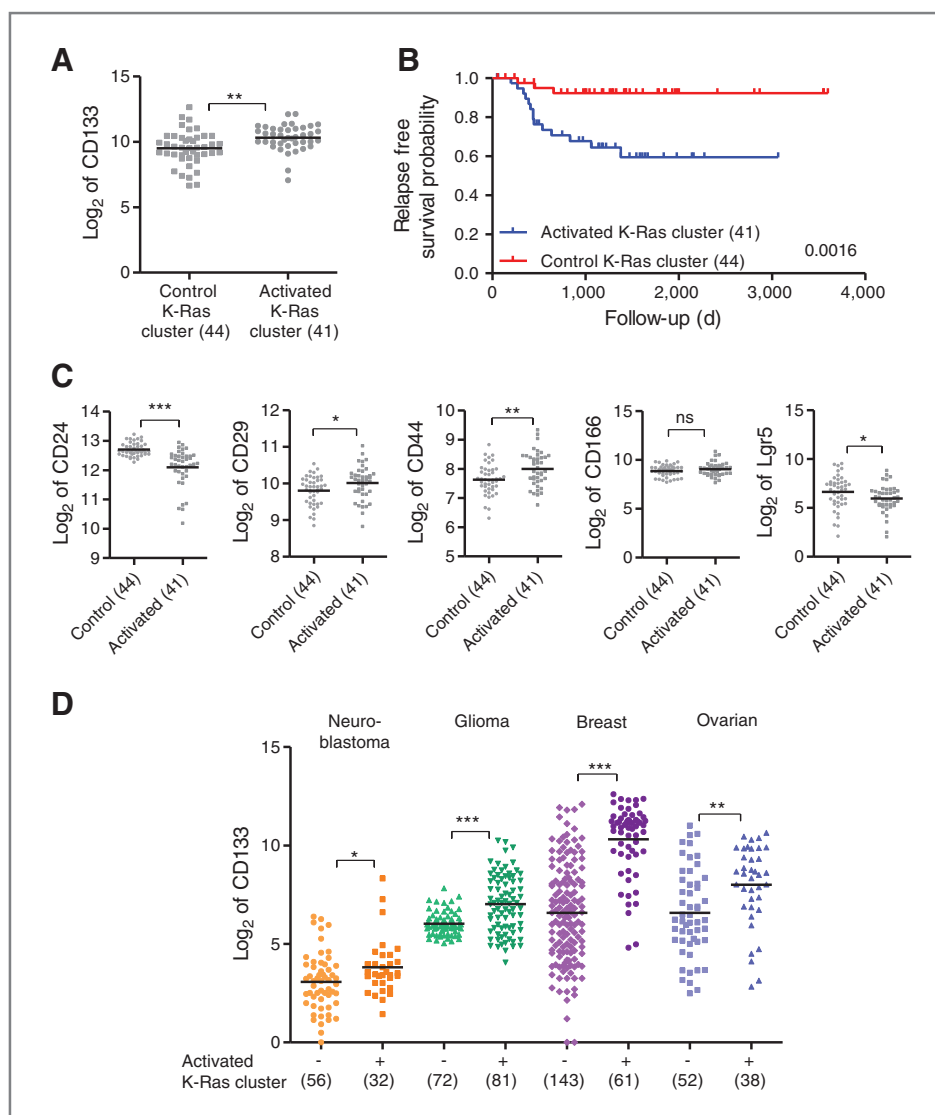
As CD133 has been shown to be prognostic in multiple different malignancies, we wondered whether the associ-

ation with Ras pathway activation would be observed in other malignancies as well. Therefore, to extend our findings to different malignancies, we made use of several publically available data sets from patient with neuroblastoma, glioma, breast carcinoma, and ovarian cancer (40–43). These cohorts represent sets of tumors with a variation in genetic background and histopathology. For instance, the glioma set contains astrocytomas, oligodendrogliomas, and glioblastomas, whereas the breast cancer set contains both basal and luminal subtypes. However, such data sets have previously been used to determine whether CD133 is a prognostic marker mostly without regarding a potential association with a specific genetic background or histopathologic subtype. The *k*-means clustering using the *K-Ras* signature was conducted with these data sets. Intriguingly, in all 4 data sets, the activated *K-Ras* cluster had higher average expression of CD133 than in the normal *K-Ras* cluster, 1.5-, 2-, 8-, and 3-fold, respectively, for neuroblastoma, glioma, breast, and ovarian carcinoma (Fig. 4D; Supplementary Fig. S4B–S4E), indicating that CD133 is also associated with an active Ras–Raf–MEK–ERK pathway in other tumor types. This does not necessarily mean that Ras or Raf are mutated in these tumors but that the pathway is hyperactive. It does however indicate that stratification based on CD133 expression does not stratify patients based on CSCs but rather serves as a pathway readout and could potentially point to different subsets. In agreement, CD133 is clearly differentially expressed between glioblastomas, astrocytomas, and oligodendrogliomas (Supplementary Fig. S4F), confirming this hypothesis. Finally, when the data sets were divided into 2 groups based on their median CD133 expression and the presence of the mutant *K-Ras* gene expression signature was analyzed by GSEA, a similar association was observed (Supplementary Fig. S5). So, CD133 expression reflects the hyperactivation status of *K-Ras* and its downstream signaling pathway, providing an alternative reason for the prognostic value of CD133 in CRCs.

Discussion

Using microarray and qPCR, we here confirm that CD133 mRNA expression can predict the relapse-free survival in patients with stage II CRC. These observations are in accordance with previous data (2–7, 13, 15). However, we show that the prognostic value of CD133 is independent of its use as a CSC marker. First of all, we previously reported that CD133 mRNA expression is not different in CSCs as compared with more differentiated tumor cells and as such CD133 mRNA cannot be used to identify CSCs. Second, CD133 expression did not correlate with the expression of any of the other frequently used colon CSC markers. Third, neither of the other CSC markers showed any prognostic value in our cohort. Mutation analysis indicated that high CD133 expression was related to mutations in *K-Ras* or *B-Raf*. Interestingly, it was previously shown that activation of the Ras–Raf–MEK–ERK pathway can induce the

Figure 4. Mutant *K-Ras* gene expression signature correlates to CD133. **A**, an activated *K-Ras* gene expression signature (39) was used for *k*-means clustering of our CRC patient database. The gene signature was correlated to the correct cluster by GSEA (Supplementary Fig. S2). The CD133 expression was analyzed between the different clusters. **B**, Kaplan–Meier curve of the 2 clusters obtained with *k*-means clustering with the activated *K-Ras* gene expression signature. **C**, the expression of other CSC markers, such as CD24, CD29, CD44, CD166, and Lgr5, were analyzed between the control and the activated *K-Ras* cluster. **D**, *k*-means clustering with the mutant *K-Ras* gene expression signature was conducted on a neuroblastoma, glioma, breast, and ovarian tumor collection. The clusters were correlated to the gene signature by GSEA (Supplementary Fig. S2). CD133 expression was higher in the activated *K-Ras* cluster than in the control *K-Ras* cluster. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. ns, not significant.



activation of CD133 promoter 5 via its nuclear target Ets, thereby inducing CD133 expression (47). We confirmed this Ras dependency of CD133 mRNA and protein expression either by disrupting the mutant *K-Ras* allele in CRC cell lines or by inhibiting Ras downstream signaling. In addition, an activated *K-Ras* gene expression signature (39) could identify the CD133^{high} cluster in our patient cohort as well as in patient sets of other tumor types, emphasizing the connection between hyperactivation of the Ras–Raf–MEK–ERK pathway and CD133 expression. Because mutations in *B-Raf* and *K-Ras* are known to correlate with a worse prognosis in CRCs (31–33), but also in other tumor types (48–51), we concluded that CD133 can be used as a prognostic marker because it relates to hyperactivation of the Ras–Raf–MEK–ERK pathway.

The prognostic value of CD133 is thus not related to its function as CSC marker. Interestingly, CSC isolations using CD133 are conducted by FACS analysis, whereas the prog-

nostic value is determined using either mRNA expression or immunohistochemical staining. We have shown previously that differentiation of colon CSCs does not reduce total CD133 protein or mRNA expression, whereas detection by the AC133 antibody by FACS is decreased. In addition, different immunohistochemical protocols resulted in completely different CD133 stainings. We hypothesized that this is due to differential folding of CD133 on the surface of CSCs, which may be influenced by glycosylation that affects accessibility of the AC133 epitope (27). A recent report confirmed that *N*-glycosylation of CD133 determines the recognition of the AC133 epitope (28). We therefore conclude that the level of CD133 mRNA or protein is not a reliable marker for the presence and enumeration of CSCs.

Recently, we have used high Wnt activity to define the CRC CSC population (52) and determine a CSC gene expression profile (38). When this profile was used to

predict prognosis in the AMC-AJCC-90 cohort, we observed that all typical Wnt target genes, including the CSC markers *Lgr5* and *ASCL2*, were rather inversely correlated with prognosis. Although this was due to promoter methylation of these Wnt target genes, it also points out that simple CSC marker analysis does not reflect the actual CSC numbers. Indeed, a direct comparison of CSC numbers and *Lgr5* and *ASCL2* expression showed no correlation at all. Moreover, the data indicated that even when analyzing complete CSC gene expression signatures, the observed association does not correlate to the amount of CSCs in a tumor but rather reflects a more immature status of the tumor (38).

Our data do not exclude the use of CSC markers to identify and select CSCs. As mentioned above, CSC isolation is conducted by selection for marker expression using either FACS or magnetic activated cell sorting (MACS). First, the marker expression level in these tumors is not determined as an on- or off-state, but as a high versus low expression difference. Second, difference in CSC marker expression levels between tumors is not considered. A tumor may have an overall lower marker expression level, but the marker^{high} cells can still identify the CSCs within the tumor. Third, the CSC number does not reflect the mutation status of these cells. For instance, CSCs with a *B-Raf* mutation are more prone to escape therapy and relapse than *B-Raf* wild-type CSCs. Clonal variation within a tumor could therefore be more determinative for patient prognosis than the CSC amount. Last, CSCs are thought to be a small tumor population, whereas the tumor bulk consists of differentiated cells. Analyzing CD133 expression in the total

tumor by qPCR or microarray to enumerate CSCs might therefore be problematic. We therefore conclude that the association between CD133 expression levels and patient prognosis is not reflecting the presence of CSCs but rather points to differential Ras–Raf pathway activity.

Disclosure of Potential Conflicts of Interest

J.P. Medema has honoraria from Speakers' Bureau of Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Cameron, F. de Sousa e Melo, R. Versteeg
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Colak, J. Bleackley, L. Vermeulen, F. de Sousa e Melo, R. Versteeg, J. Koster, K. Kemper
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Kemper, M. Versloot, S. Colak, J. Bleackley, L. Vermeulen, J.H. de Jong

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