Mutations in the Ras-Raf axis underlie the prognostic value of CD133 in colorectal cancer

Kristel Kemper¹, Miranda Versloot¹, Katherine Cameron¹, Selçuk Colak¹, Felipe de Sousa e Melo¹, Joan H. de Jong¹, Joanne Bleackley¹, Louis Vermeulen¹, Rogier Versteeg², Jan Koster² and Jan Paul Medema¹

¹ Laboratory for Experimental Oncology and Radiobiology, Center for Experimental Molecular Medicine, Academic Medical Center, Amsterdam, the Netherlands
² Department of Human Genetics, Academic Medical Center, Amsterdam, the Netherlands

Running head: Prognostic value of CD133 caused by mutant K-Ras and B-Raf

Keywords: CD133, Ras-Raf pathway, prognosis

Research Support: KK was supported by a scholarship of the AMC Graduate School, JPM was supported by an NWO VICI grant and by a Dutch Cancer Society grant (UvA2009-4416).

Corresponding author:
Jan Paul Medema
Meibergdreef 9
1105 AZ Amsterdam
j.p.medema@amc.uva.nl
tel# +31 20 5667777
fax# +31 20 6977192

Total number of figures and tables: 6
Translational relevance:
Reliable markers for predicting which stage II colorectal cancer (CRC) patients have a higher change of relapse and should receive adjuvant therapy after surgery, are needed. Because cancer stem cells (CSCs) are identified as the driving force behind tumor initiation, growth and metastasis, the expression of CSC markers are studied as predictors 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.
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 downregulated 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 qPCR analysis. Additionally, several common mutations were analyzed to determine the relation with CD133 expression.

Results

CD133 mRNA expression predicted relapse-free survival in our patient series, while 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 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 datasets 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 with 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.
INTRODUCTION

In order to develop a better strategy for defining which colorectal cancer (CRC) patients should receive adjuvant chemotherapy after surgery, reliable markers for predicting relapse have been studied extensively (reviewed in [1]). Mutations in signaling pathways are used, 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 is indeed identified as an independent prognostic marker for CRC by showing that either high expression of CD133 mRNA or intense CD133 staining in immunohistochemistry (IHC) relates to poor relapse-free/overall survival [2-7, 13, 15] (Suppl. Table 1). 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.

Other factors are shown to correlate to poor prognosis in CRC, like mutations in tumor suppressor gene p53[29, 30] or proto-oncogenes like 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 CRC, while 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 5-10% of all CRC cases. The latter two 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 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 is indeed regulated by the RAS-RAF-MEK-ERK pathway.
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 AJCC stage II CRC patients (AMC-AJCCII-90) as described previously in ref[38]. The study was approved for by the medical ethical board of the AMC and in accordance with the rules and legislation on human experimentation in the Netherlands. Shortly, these stage II CRC patients received surgery with curative intent in the Academic Medical Center in Amsterdam, the Netherlands, between 1997 and 2006 (AMC-AHCCII-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 performed in accordance with the manufacturer's protocol (Affymetrix). Microarray data can be viewed online (http://www.ncbi.nlm.nih.gov/geo/index.html) under GEO accession number (to be determined). 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 Bild et al.[39] The following datasets were used: neuroblastoma[40], glioma (GSE4290)[41], breast (GSE12276)[42] and ovarian cancer (GSE12172)[43].

qPCR

cDNA was prepared with reverse transcriptase III (Invitrogen) according to manufacturer’s protocol. qPCR was performed on the LC480 II (Roche) with intron-spanning primers for CD133 and GAPDH (Suppl. Table 2)

Mutation analysis

MSI analysis was performed with the MSI analysis System, Version 1.2 (Promega) according to manufacturer’s protocol. Sequencing of K-Ras, B-Raf and p53 was performed with Big Dye Terminator (BDT, Applied Biosystems). Primers are described in Suppl. Table 2.

Statistical analysis, K-means clustering and gene set enrichment analysis
The association between CD133 and clinicopathological features was analyzed by Chi-square test. Survival curves were drawn by the Kaplan-Meier method while 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 performed in SPSS and Graphpad Prism 5. K-means clustering was performed by in-house-developed software. Clusters were identified by GSEA (http://www.broadinstitute.org/gsea/index.jsp).

**Cell culture and MEK inhibition**

HCT116, HKH2, Dld1 and DKO4 were kindly provided by Onno Kranenburg (Department of Surgery, University Medical Center Utrecht, the Netherlands), and cultured as previously described[44]. CSC lines, approved by the medical ethical committee of the Academic Medical Center and the University of Palermo, were derived and cultured as described previously[22]. MEK inhibition was performed with 10 μM U0126 (#9903, Bioke) for 24h. Methylation was inhibited by 2 μM 5-aza-deoxycytidine for 24h. After treatment with U0126, cells were analyzed for ERK phosphorylation either by western blot. The antibodies used for Western blot were p44/42MAPK (ERK1/2) and phospho-p44/42MAPK (ERK1/2) (Thr202/Tyr204) (BIOKE, Leiden, NL).

**FACS analysis**

Cells were stained with CD133-PE (AC133, Miltenyi) or mIgG1-PE (Miltenyi) as isotype control for 20min 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, Leiden, NL) was used. Briefly, cells were washed with cold PBS and fixed with 4% paraformaldehyde at 37°C for 10min. After fixation, cells were permeabilized in 90% ice-cold methanol for 30min. Next, cells were stained for 1hr in PBA at a dilution of 1:50, washed and subsequently analyzed. FACS analysis was performed on the FACSCanto and analysis was performed 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 IHC, can be used to predict prognosis in CRC [2-7, 13, 15] (Suppl. Table 1). 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 was performed to determine the expression level of CD133. The median expression level of CD133 was used to separate the cohort in two groups. In five 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 to 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). Chi-square analysis of clinicopathological 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 (Suppl. Table 3).

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, like 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 was used, while all other CSC markers failed to show any prognostic value (Fig.2A). Only when the highest quartile was used to divide the groups, also CD44 showed some prognostic value (Suppl. Fig.1). Strikingly, some of the CSC markers, like CD24 and Lgr5, even displayed an opposite trend, with high expression of the marker relating to good prognosis. This is a counter-intuitive 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 showed no significant difference in CSC marker expression was found between the CD133_{high} and CD133_{low} group (Fig.2B). Furthermore, the expression of CSC markers did not correlate to each other (Suppl. Fig.2). 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 if changes in CD133 levels reflect expression differences and not differences in number of CSCs, we performed immunofluorescence 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 CRC.

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 microsatellite instability (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$^{\text{high}}$ and CD133$^{\text{low}}$ group, as analyzed by chi-square 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 to K-Ras/B-Raf wild-type patients. More than half of the tumors in the CD133$^{\text{high}}$ group had a mutation in either K-Ras or B-Raf, whereas only a fourth of the tumors in the CD133$^{\text{low}}$ group had such a mutation (p=6.4 $\times$ 10$^{-5}$, Table 1). Then, we analyzed if CD133 was an independent prognostic marker by comparing univariate and multivariate Cox regression analyses and found that CD133 was independent of any of the clinicopathological 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 contested for K-Ras mutations [45]. 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 [46].
Therefore, mutations in K-Ras/B-Raf could result in increased CD133 expression. To investigate this, two CRC cell lines HCT116 and Dld1 were used, which both 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 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 [47](Suppl.Fig.3A-B). 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-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.

Since these isogenic lines were kept in culture for a large number of passages they may have acquired more difference 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 (Suppl.Fig.3C-D). Moreover, MEK inhibition reduced the CD133 mRNA and protein expression in HCT116 and LS411N (Fig.3B-C). In addition, treatment of two 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, Supp.Fig.3E). 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 perform k-means clustering in our patient set. The group with the activated K-Ras signature was identified by gene set
enrichment analysis (GSEA) (Suppl. Fig.4A). 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 compared to the control cluster (Fig.4A). Additionally, the activated K-Ras signature was able to predict the relapse-free survival in our patient series (p=0.0016, Fig.4B). So, in CRC, 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 were analyzed between these two clusters, some markers, like CD29 and CD44 were indeed upregulated in the activated K-Ras cluster, whereas others, like 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 association 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 datasets from neuroblastoma, glioma, breast carcinoma and ovarian cancer patients [40] [41] [42] [43]. These sets represent sets of tumours with a variation in genetic background and histopathology. For instance, the glioma set contains astrocytomas, oligodendrogliomas and glioblastomas, while the breast cancer set contains both basal and luminal subtypes. However, such datasets have previously been used to determine whether CD133 is a prognostic marker mostly without regarding a potential association with a specific genetic background or histopathological subtype. K-means clustering using the K-Ras signature was performed with these datasets. Intriguingly, in all four datasets, the activated K-Ras cluster had higher average expression of CD133 compared to the normal K-Ras cluster, 1.5-fold, 2-fold, 8-fold and 3-fold respectively for neuroblastoma, glioma, breast and ovarian carcinoma (Fig.4D, Suppl.Fig.4B-E), indicating that CD133 is also associated with an active Ras-Raf-MEK-ERK pathway in other tumor types. This does not necessarily mean Ras or Raf are mutated in these tumours, but that the pathway is hyperactive. It does however indicate that stratification based on CD133 expression does not stratify patients based on CSC, but rather serves as a pathway read-out and could potentially point to different subsets. In agreement, CD133 is clearly differentially expressed between glioblastomas, astrocytomas and oligodendrogliomas (Suppl.Fig.4F),
confirming this hypothesis. Finally, when the datasets were divided into two 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 (Suppl.Fig.5). So, CD133 expression reflects the hyperactivation status of the K-Ras and its downstream signaling pathway, providing an alternative reason for the prognostic value of CD133 in CRC.
DISCUSSION

Using microarray and qPCR, we here confirm that CD133 mRNA expression can predict the relapse-free survival in stage II CRC patients. 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 to more differentiated tumor cells and as such CD133 mRNA cannot be used to identify CSCs. Secondly, CD133 expression did not correlate with the expression of any of the other frequently used colon CSC markers. Thirdly, 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 activation of CD133 promoter 5 via its nuclear target Ets, thereby inducing CD133 expression [48]. 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<sup>high</sup> 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. Since mutations in B-Raf and K-Ras are known to correlate with a worse prognosis in CRC [31-33], but also in other tumor types [49-52], 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 performed by FACS analysis, whereas the prognostic value is determined using either mRNA expression or IHC staining. We have shown previously that differentiation of colon CSCs does not reduce total CD133 protein or mRNA expression, while detection by the AC133 antibody by FACS is decreased. Additionally, different IHC 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 CSC.

Recently, we have used high Wnt activity to define the CRC CSC population [53] and used this to 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 performed by selection for marker expression using either FACS or MACS sorting. Firstly, the marker expression level in these tumors is not determined as an on- or off-state, but as a high versus low expression difference. Secondly, 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. Thirdly, the CSC number does not reflect the mutation status of these cells. For instance, CSCs with a B-Raf mutation are 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. Analysing 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.

Acknowledgements
We would like to thank Onno Kranenburg for kindly providing the HCT116, HKH2, Dld1 and DKO4 cells.
REFERENCE LIST


FIGURE LEGENDS

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 cut-off 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.

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 cut-off, 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 three patients expressing low, intermediate and high CD133 mRNA. Right panel shows microarray expression levels of CD133.

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 panel), and protein, as shown by FACS analysis (middle panel). 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 panel). After treatment with 5-aza-deoxycytidine for 24h, Dld-1 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 24h 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. K-Ras mutant CSC line C002.

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 Figure 2). The CD133 expression was analyzed between the different clusters. B. Kaplan-Meier curve of the two clusters obtained with K-means clustering with the activated K-Ras gene expression signature. C. The expression of other CSC markers, like 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 performed on a neuroblastoma, glioma, breast and ovarian tumor collection. The clusters were correlated to the gene signature by GSEA (Supplementary Figure 2). CD133 expression was higher expressed in the activated K-Ras cluster compared to the control K-Ras cluster. The asterisks indicate the p-value: * = p<0.05, ** = p<0.01, *** = p<0.005

Table 1. Mutation correlated to CD133 expression in stage II colon tumors. Chi-square test of different mutations compared to CD133 expression levels.

Table 2. Univariate and multivariate Cox analyses for survival of CRC patients. Univariate proportional hazard COX regression analysis was performed for all variables, whereas two sets of multivariate analysis were performed. Analysis #1 includes only CD133, K-Ras and B-Raf as variables, while analysis #2 includes CD133 and all the other variables, showing that CD133 is independent of p53 mutations, MSI, age, gender, differentiation status and localization, while it is dependent on K-Ras and B-Raf mutations. HZ: Hazard Ratio; CI: Confidence Interval; P: p-value; ND: not done
Figure 1: CD133 as prognostic marker for relapse-free survival in colorectal tumor patients

A

Follow up (days)

Relapse free survival probability

CD133 high (43)

CD133 low (42)

B

Relative CD133 expression by qPCR

CD133 expression by microarray

R² = 16.22

P < 0.0001
Figure 2: Other CSC markers can not be used as prognostic marker

**A**

- **CD24**
  - CD24 high (43) vs CD24 low (42): 0.1371
  - Follow up (days) vs Relapse free survival probability

- **CD29**
  - CD29 high (43) vs CD29 low (42): 0.0960
  - Follow up (days) vs Relapse free survival probability

- **CD44**
  - CD44 high (43) vs CD44 low (42): 0.4314
  - Follow up (days) vs Relapse free survival probability

- **CD166**
  - CD166 high (43) vs CD166 low (42): 0.2942
  - Follow up (days) vs Relapse free survival probability

- **ALDH1**
  - ALDH1 high (43) vs ALDH1 low (42): 0.5015
  - Follow up (days) vs Relapse free survival probability

- **Lgr5**
  - Lgr5 high (43) vs Lgr5 low (42): 0.5015
  - Follow up (days) vs Relapse free survival probability

**B**

- **2log of CD24**
  - CD133 high (43) vs CD133 low (42): 0.8752

- **2log of CD29**
  - CD133 high (43) vs CD133 low (42): 0.6877

- **2log of CD44**
  - CD133 high (43) vs CD133 low (42): 0.1743

- **2log of CD166**
  - CD133 high (43) vs CD133 low (42): 0.4314

- **2log of Lgr5**
  - CD133 high (43) vs CD133 low (42): 0.5590

- **2log of ALDH1**
  - CD133 high (43) vs CD133 low (42): 0.5000

**C**

- **CD133 expression**
  - mRNA rel. exp
  - Col078: CD133 High
  - Col094: CD133 intermediate
  - Col056: CD133 low/negative
Figure 3: Disruption of mutant K-ras allele or downstream inhibition downregulates CD133

A

B

C

D

Research.
**Figure 4: Mutant K-Ras gene expression signatures correlate with CD133**

A. 2log of CD133

B. Relapse free survival probability

C. 2log of CD24, CD29, CD44, Lgr5

D. 2log of CD133 for Neuroblastoma, Glioma, Breast, Ovarian

Activated K-Ras cluster (41) vs. control K-Ras cluster (44)
Table 1: Mutations correlated to CD133 expression in stage II colon tumors

<table>
<thead>
<tr>
<th></th>
<th>CD133 high</th>
<th>CD133 low</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p53 mutation</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutated</td>
<td>11/43</td>
<td>13/40</td>
<td>0.487</td>
</tr>
<tr>
<td>WT</td>
<td>32/43</td>
<td>27/40</td>
<td></td>
</tr>
<tr>
<td><em>MSI status</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI</td>
<td>15/43</td>
<td>9/42</td>
<td>0.190</td>
</tr>
<tr>
<td>MSS</td>
<td>28/43</td>
<td>32/42</td>
<td></td>
</tr>
<tr>
<td><em>Ras</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>12/43</td>
<td>8/42</td>
<td>0.336</td>
</tr>
<tr>
<td>WT</td>
<td>31/43</td>
<td>34/42</td>
<td></td>
</tr>
<tr>
<td><em>Raf</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>14/43</td>
<td>3/42</td>
<td>0.003</td>
</tr>
<tr>
<td>WT</td>
<td>29/43</td>
<td>39/42</td>
<td></td>
</tr>
<tr>
<td><em>Ras/Raf</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ras/Raf mutated</td>
<td>26/43</td>
<td>11/42</td>
<td>0.000064</td>
</tr>
<tr>
<td>WT</td>
<td>17/43</td>
<td>31/42</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Univariate and multivariate Cox analyses for survival of CRC patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate analysis</th>
<th>Multivariate analysis #1</th>
<th>Multivariate analysis #2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>CD133</td>
<td>3,546</td>
<td>1,165-10,885</td>
<td>0.027</td>
</tr>
<tr>
<td>K-Ras mutation</td>
<td>1,422</td>
<td>0,501-4,036</td>
<td>0.509</td>
</tr>
<tr>
<td>B-Raf mutation</td>
<td>2,233</td>
<td>0,826-6,041</td>
<td>0.114</td>
</tr>
<tr>
<td>p53 mutation</td>
<td>1,098</td>
<td>0,386-3,123</td>
<td>0.860</td>
</tr>
<tr>
<td>MSI status</td>
<td>0,765</td>
<td>0,249-2,347</td>
<td>0.640</td>
</tr>
<tr>
<td>Differentiation grade</td>
<td>0,806</td>
<td>0,298-2,180</td>
<td>0.671</td>
</tr>
<tr>
<td>Gender</td>
<td>1,387</td>
<td>0,513-3,751</td>
<td>0.520</td>
</tr>
<tr>
<td>Age at resection</td>
<td>0,434</td>
<td>0,160-1,173</td>
<td>0.100</td>
</tr>
<tr>
<td>Localization</td>
<td>1,149</td>
<td>0,425-3,109</td>
<td>0.784</td>
</tr>
</tbody>
</table>
Mutations in the Ras-Raf axis underlie the prognostic value of CD133 in colorectal cancer

Kristel Kemper, Miranda Versloot, Kate Cameron, et al.

*Clin Cancer Res* Published OnlineFirst April 10, 2012.

Updated version: Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-3066

Supplementary Material: Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/04/10/1078-0432.CCR-11-3066.DC1

Author Manuscript: Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.