Molecular identification and targeting of colorectal cancer stem cells

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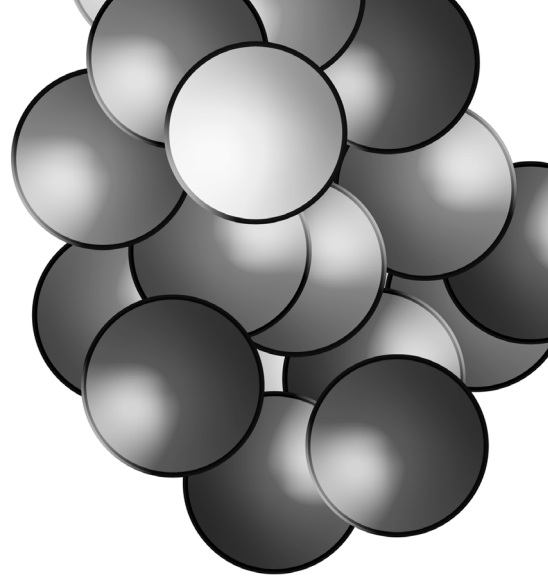*Mutations in the Ras-Raf axis underlie the prognostic value of CD133 in colorectal cancer*

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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 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.

Methods

A CRC patient series was studied for expression of CD133 and other CSC markers by microarray analysis. In addition, several common mutations were analyzed in these tumors 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 describes the idea that the expression of CSC markers correlates to the number of CSCs within a tumor and thereby to the prognosis of the patient. For CRC, the following markers are used to identify CSCs: CD24[16], CD29[16], CD44[17-19], CD133[16,20-22], CD166[18] and Lgr5[23]. Previously, CD133 is indeed identified as an independent prognostic marker for CRC by showing that either high expression of CD133 mRNA or intense staining for CD133 in immunohistochemistry (IHC) relates to poor relapse-free/overall survival[2-7,13,15] (Supplementary Table 1). As CD133 marks CSCs, it was postulated that high CD133 expression correlates with 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[24] and protein level[24,25]. In addition, we have previously shown 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[26]. 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[27,28] or proto-oncogenes K-Ras[29,30] and B-Raf[31]. p53, frequently mutated or lost (60-70%) in CRC, plays an essential role in responding to DNA damage by inducing cell-cycle arrest and apoptosis. K-Ras, mutated in approximately 40% of all CRCs, encodes for a small GTPase, which is regulating the response to various external growth factors[32]. B-Raf is directly downstream of Ras and encodes for a serine/threonine protein kinase, which is mutated (V600E) in 5-10% of all CRC cases. Both mutations lead to aberrant activation of the Ras-Raf-MEK-ERK pathway[32,33] and are mutually exclusive in tumors[34].

To understand the role of CD133 expression levels in CRC prognosis, we analyzed a stage II CRC patient set for expression of CSC markers by microarray. We found that high CD133 expression, in contrast to other CSC markers, can predict relapse-free survival in our patient set. High CD133 expression was related to mutations in K-Ras or B-Raf. Also, we showed that CD133 is indeed regulated by the RAS-RAF-MEK-ERK pathway. Altogether, we concluded that CSC marker CD133

is prognostic in CRC because its expression is regulated by aberrant activation 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). Total RNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer's protocol, 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). Five patients were excluded for further analysis because CD133 expression levels could not be determined. The following probe sets, that recognize 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) and 213880_at (Lgr5). The K-Ras profile was extracted from Bild et al.[35] The following datasets were used: neuroblastoma[36], glioma (GSE4290), breast (GSE12276) and ovarian cancer (GSE12172).

qPCR
Total RNA was isolated as described above. 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 (Supplementary Table 2)

Mutation analysis
MSI analysis was performed with the MSI analysis System, Version 1.2 (Promega) according to the manufacturer's protocol. Sequencing of K-Ras, B-Raf and p53, was performed with Big Dye Terminator (BDT, Applied Biosystems, Foster City, CA). Primers are described in Supplementary 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 O. Kranenburg (Laboratory of Experimental Oncology, University Medical Centre, Utrecht, the Netherlands), and cultured as previously described[37]. CSC lines, obtained from a primary carcinoma and a liver metastasis, 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 done with 10uM of U0126 (#9903, Bioke) for 24h. Methylation was inhibited by 2 uM 5-aza-deoxycytidine for 24h.

FACS analysis
Cells were stained with directly labeled CD133-PE (AC133, Miltenyi) or mIgG1-PE (Miltenyi) as isotype control for 20 min at 4°C in PBS containing 1% bovine serum albumin and 0.02% sodium azide. 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] (Supplementary Table 1). We used our CRC patient set to study CD133 as a prognostic marker by collecting RNA from these tumor samples and perform microarray analysis. To identify the prognostic value of CD133, the median expression level was used to separate the cohort in two groups. Tumors in the CD133^{high} group expressed 3.7-fold more CD133 compared to the CD133^{low} group. High CD133 expression could predict poor relapse-free survival with a significance of p=0.0182 (Figure 1A). The microarray CD133 expression data was validated by qPCR, showing a very significant correlation between the microarray and the qPCR data (Figure 1C). Chi-square analysis of clinicopathological features showed that elevated CD133 expression was associated with metastasis and recurrence (p=0.026 and p=0.017, respectively), but not with

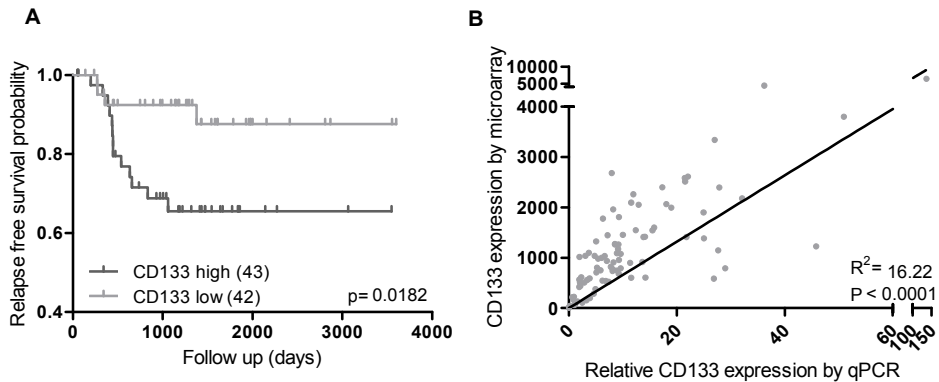gender, differentiation grade, location of the tumor or age of the patient at surgery (Supplementary Table 3).

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.

Other CSC markers do not to predict prognosis

It has been suggested that CD133 expression levels predict prognosis, because it identifies 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 and Lgr5, were analyzed for their prognostic value. None of these CSC markers had any prognostic value when the median expression level was used to separate the groups (Figure 2A-E). When the highest quartile was used to divide the groups, only CD44 showed some prognostic value (Supplementary Figure 1). Others, like CD24 and Lgr5, even

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Figure 2. Other CSC markers can not be used as prognostic markers. B-E. Colon CSC markers CD24 (**A**), CD29 (**B**), CD44 (**C**), CD166 (**D**) and Lgr5 (**E**) were tested for their prognostic value. As cut-off, the median expression level was used. **F-J** The expression levels of CSC markers CD24 (**F**), CD29 (**G**), CD44 (**H**), CD166 (**I**) and Lgr5 (**J**) were analysed in the CD133 high and CD133 low group.

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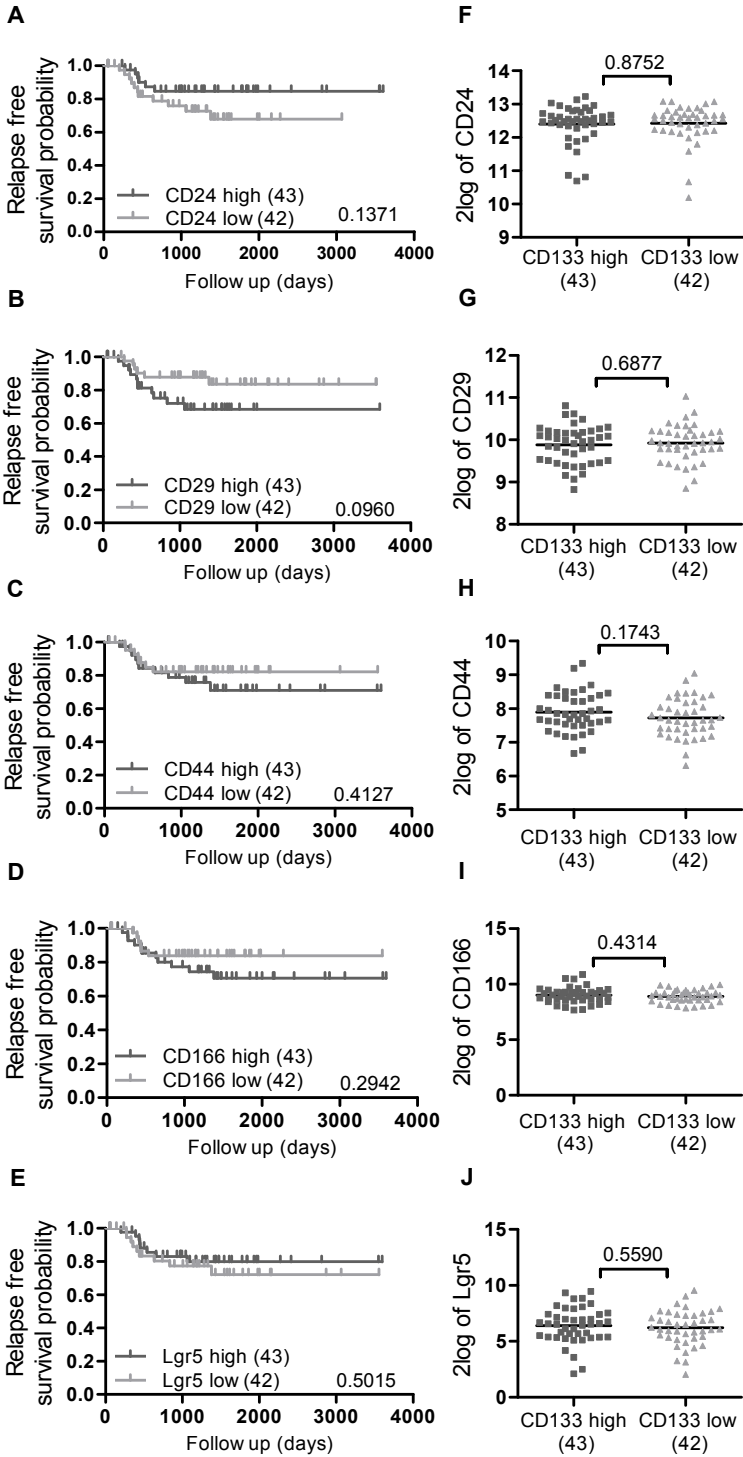
displayed an opposite trend, having high expression of the marker relating to good prognosis. As these CSC markers should all be highly expressed in the same cell population, namely the CSCs, we assumed that their expression should be correlated to CD133 expression. We therefore compared the expression level of these markers in the CD133^{high} versus CD133^{low} group. Interestingly, no significant difference in CSC marker expression was found between the CD133^{high} and CD133^{low} group (Figure 2F-J). Furthermore, the expression of CSC markers did not even correlate to each other (Supplementary Figure 2), indicating that the enumeration of CSCs is apparently not reflected in the mRNA expression level of CSC markers. These findings suggest that there must be an alternative reason why CD133 expression levels are 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 tumors. 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 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 the patients having either a K-Ras or B-Raf mutation and compared them to the 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=$6.4\cdot10^{-5}$, 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). As patients with a K-Ras or B-Raf mutated CRC are known to have a worse prognosis[29-31], a link between CD133 expression and activation of the Ras-Raf-MEK-ERK pathway could explain the prognostic value of CD133.

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

	CD133 high	CD133 low	p-value
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	

Table 2. Univariate and multivariate Cox analyses for survival of CRC patients.

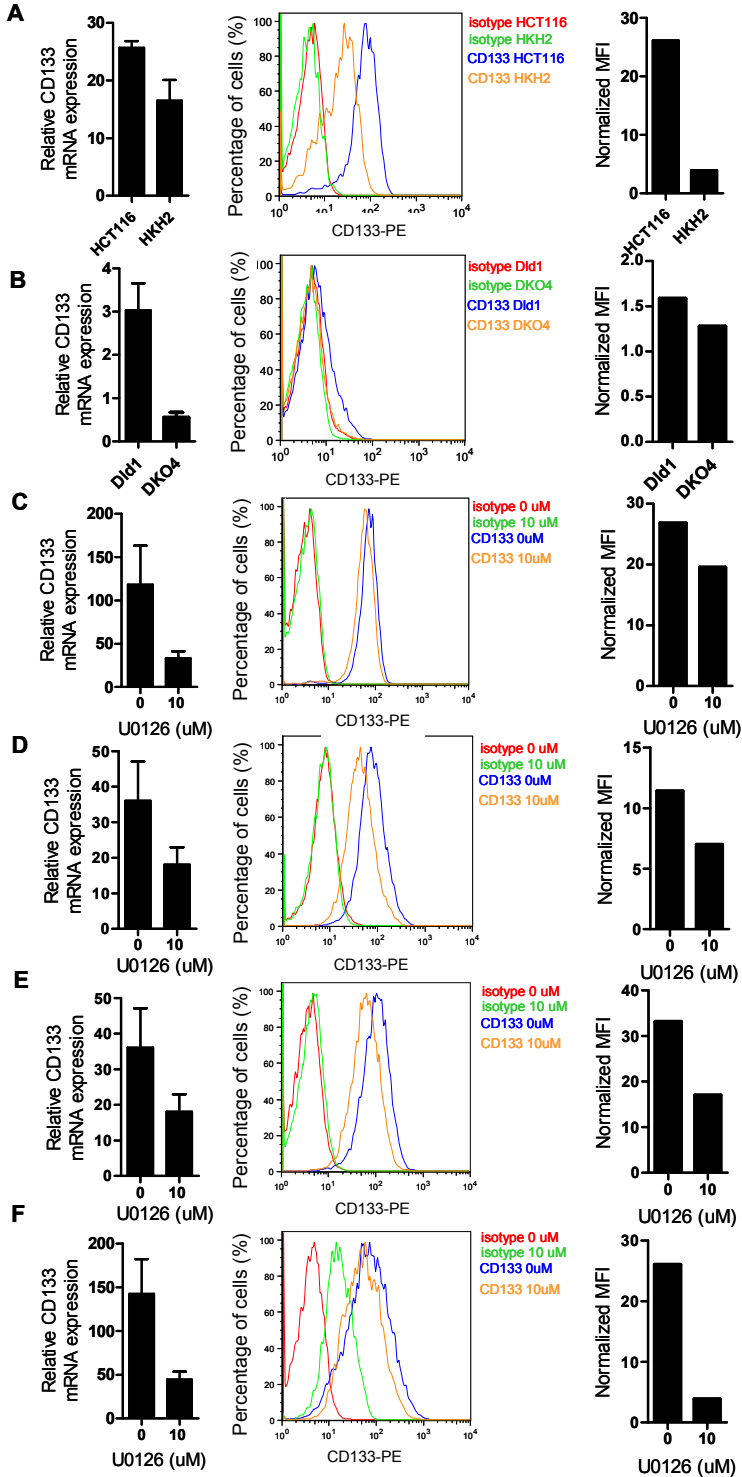| Variable	*Univariate analysis*			*Multivariate analysis #1*			*Multivariate analysis #2*		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
CD133	3,546	1,155-10,885	0,027	2,720	0,805-9,189	0,107	3,933	1,250-12,374	0,019
K-Ras mutation	1,422	0,501-4,036	0,509	1,489	0,402-5,278	0,537	ND		
B-Raf mutation	2,233	0,826-6,041	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

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

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

As it has been shown that activation of the Ras-Raf-MEK-ERK pathway results in activation of CD133 promoter 5, thereby inducing CD133 expression[38], mutations in K-Ras/B-Raf could induce high CD133 expression. To investigate this, two CRC cell lines HCT116 and Dld1were used, 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[37]. These wild-type K-Ras HCT116 and Dld1 lines display reduced growth *in vitro* and *in vivo*[37]. 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 wild-type K-Ras HKH2 (Figure 3A). In Dld1, CD133 is repressed by promoter methylation[39]. Therefore, we 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 population of CD133^{+} cells. However, the K-Ras wild-type DKO4 cells displayed no CD133-positivity, also reflected in the low mRNA expression (Figure 3B). So, disruption of mutant K-Ras is decreasing the expression of CD133 both on mRNA and protein.

Since these isogenic lines might differ in more aspects than only their Ras status, we decided to treat CRC lines with MEK inhibitor U0126, a potent inhibitor of the Ras-Raf-MEK-ERK pathway. Inhibition of ERK phosphorylation reduced the CD133 mRNA and protein expression in HCT116 and LS411N (Figure 3C-D). Also, treatment of two established colon spheroid cultures derived from a primary CRC[16], which have high CD133 expression, with U0126 resulted in reduced CD133 mRNA and protein expression (Figure 3F-G). These data show that CD133 expression is dependent on the activity of the Ras-Raf-MEK-ERK pathway.

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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). **B.** 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. **C-F.** 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. **C.** K-Ras mutant HCT116. **D.** B-Raf mutant LS411N **E.** K-Ras mutant CSC line C002 and **F.** K-Ras mutant CSC line LM5.

Activated K-Ras gene expression signature correlates to CD133 expression

As mutant K-Ras/B-Raf can induce hyperactivation of the Ras-Raf-MEK-ERK pathway causing CD133 upregulation, we hypothesized that an activated K-Ras gene expression signature would correlate with CD133 expression. A previously generated activated K-Ras gene expression signature was used[35] to perform k-means clustering in our patient set. After obtaining these clusters, the activated K-Ras cluster was identified by gene set enrichment analysis (GSEA) (Supplementary Figure 3A). In the activated K-Ras group, 26 patients with mutations in K-Ras or B-Raf clustered, 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. Tumors in the activated K-Ras cluster had a 1.5-fold higher CD133 expression compared to the control cluster (Figure 4A). Also, the activated K-Ras signature was able to predict the relapse-free survival in our patient series (p=0.0016, Figure 4B). So, there seems to be 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 (Figure 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.

To extend our findings to different malignancies, we analyzed whether an active K-Ras gene expression signature correlates with high CD133 expression in other tumor types as well. Therefore, k-means clustering with the K-Ras signature was performed on four additional tumor collections, namely a neuroblastoma, glioma, breast carcinoma and ovarian cancer dataset. 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 (Figure 4D, Supplementary Figure 3B-E), indicating that CD133 is also regulated by the Ras-Raf-MEK-ERK pathway in other tumor types. When the datasets were divided in 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 trend was observed (Supplementary Figure 4). So, CD133 expression seems to be regulated by hyperactivation of K-Ras and its downstream signaling pathway, providing an alternative reason for the prognostic value of CD133.
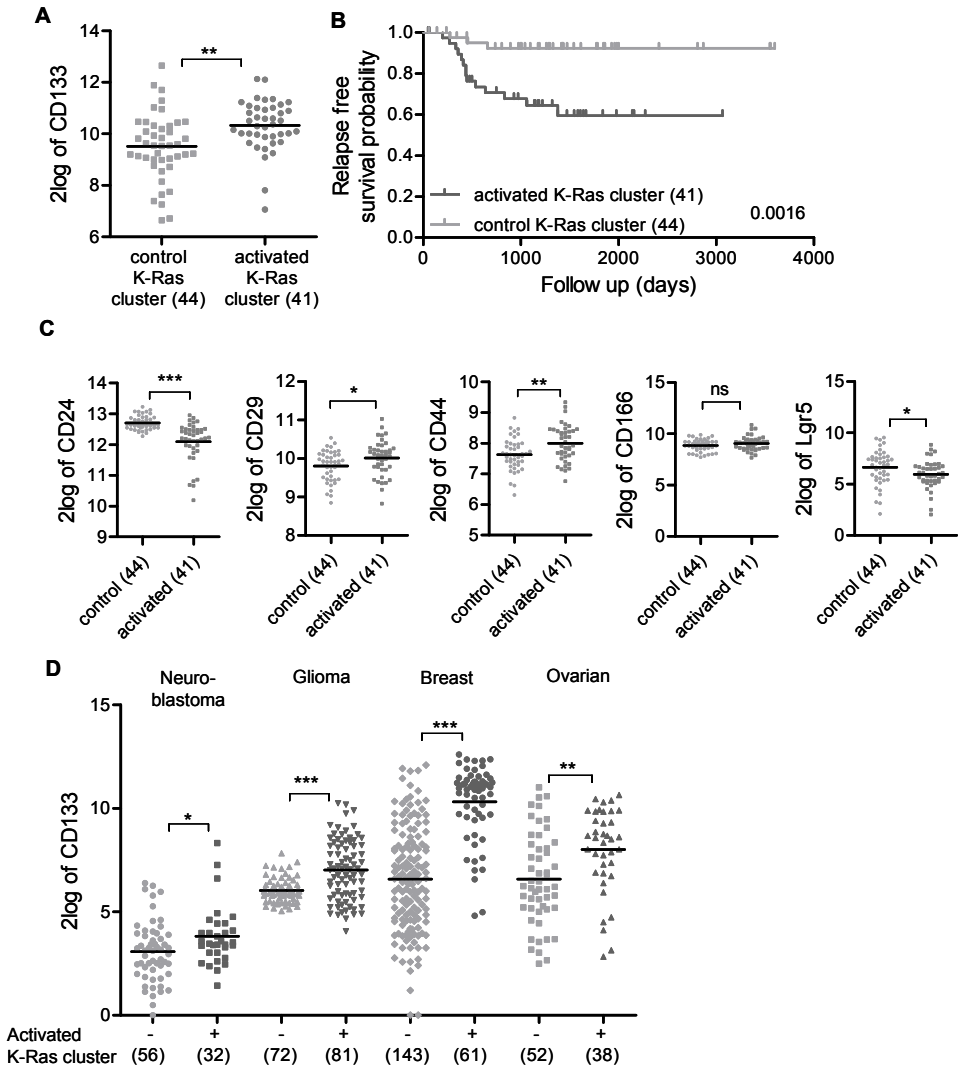
Figure 4. Mutant K-Ras gene expression signature correlates to CD133. A. An activated K-Ras gene expression signature [176] was used for K-means clustering of our CRC patient database. The gene signature was correlated to the correct cluster by GSEA (Supplementary Figure 2). The CD133 expression was analysed 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.

Discussion

Our data confirm that CD133 mRNA expression can predict the relapse-free survival in stage II CRC patients. These observations are in accordance with previous observations[2-7,13,15]. However, we show here that the prognostic value of CD133 is probably independent of its use as a CSC marker. First of all, CD133 expression did not correlate with the expression of any of the other frequently used colon CSC markers. Secondly, neither of these 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[40]. 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, showing that CD133 is regulated by the Ras-Raf-MEK-ERK pathway. In addition, an activated K-Ras gene expression signature[35] 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. Since mutations in B-Raf and K-Ras are known to correlate with a worse prognosis in CRC[29-31], but also in other tumor types[41-44], we concluded that CD133 can be used as a prognostic marker because it relates to hyperactivation of the Ras-Raf-MEK-ERK pathway.

So, the prognostic value of CD133 is not related to its function as CSC marker. Interestingly, CSC isolations using CD133 as marker are performed by FACS analysis, whereas the prognostic values are determined by either mRNA expression or IHC staining. We have shown before that differentiation of colon CSCs does not reduce total CD133 protein or mRNA expression, while the recognition by the AC133 antibody analyzed by FACS is decreased. We hypothesized that this is due to differential folding of CD133 on the surface of CSC, which may be determined by glycosylation. In addition, we found that different IHC protocols resulted in completely different CD133 stainings caused by differential accessibility of the CD133 epitope[26]. We therefore concluded that the level of CD133 mRNA or protein is not a reliable marker for the presence of CSC and should not be used to enumerate CSC. Also, mRNA expression levels of other CSC markers like CD24, CD29, CD44, CD166 and Lgr5 do not correlate with CD133 or each other, indicating that mRNA expression of CSC markers does not necessarily reflect the number of CSCs in a tumor.

On the other hand, CD133 and these other CSC markers can still be used to identify and select CSCs. As mentioned above, CD133 is used for isolating CSCs by either FACS or MACS sorting. In this procedure, cells acquired after dissociation of the primary tumor are sorted for high expression of CD133, whereas the low-expressers are regarded as non-CSCs. First of all, the expression level of CD133 in these tumors is not seen as an on- or off-state, but a high versus low expression difference. Secondly, the difference in expression levels of CD133 between different tumors is not taken into account here. A tumor may have in total a lower expression level of CD133, but within this tumor, the CD133high cells can still identify the CSCs. This same hypothesis can be posed for other CSC markers: mRNA and protein expression may vary between tumors, but the cells within a tumor that express high levels of these markers can be still identified as CSCs. Thirdly, the enumeration of CSCs does not reflect the mutation status of these CSCs, meaning that for instance tumors with CSCs carrying a B-Raf mutation are more likely to escape therapy and relapse than CSCs without this mutation. Clonal variation within a tumor might therefore be more important than the amount of CSCs for the prognosis of the patient. Lastly, CSCs are thought to be a small population in the tumor, whereas the bulk of the tumor consists of differentiated cells. So, studying CD133 expression in the total tumor by qPCR or microarray to enumerate CSCs might therefore be quite hard, especially since the expression differences are small. Therefore we suggest that the association between CD133 expression levels and patient prognosis is not reflecting the presence of CSCs, but rather points to a differential activity of the Ras pathway within these tumors.

Acknowledgements

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Supplementary Figures

A

B

C

D

E
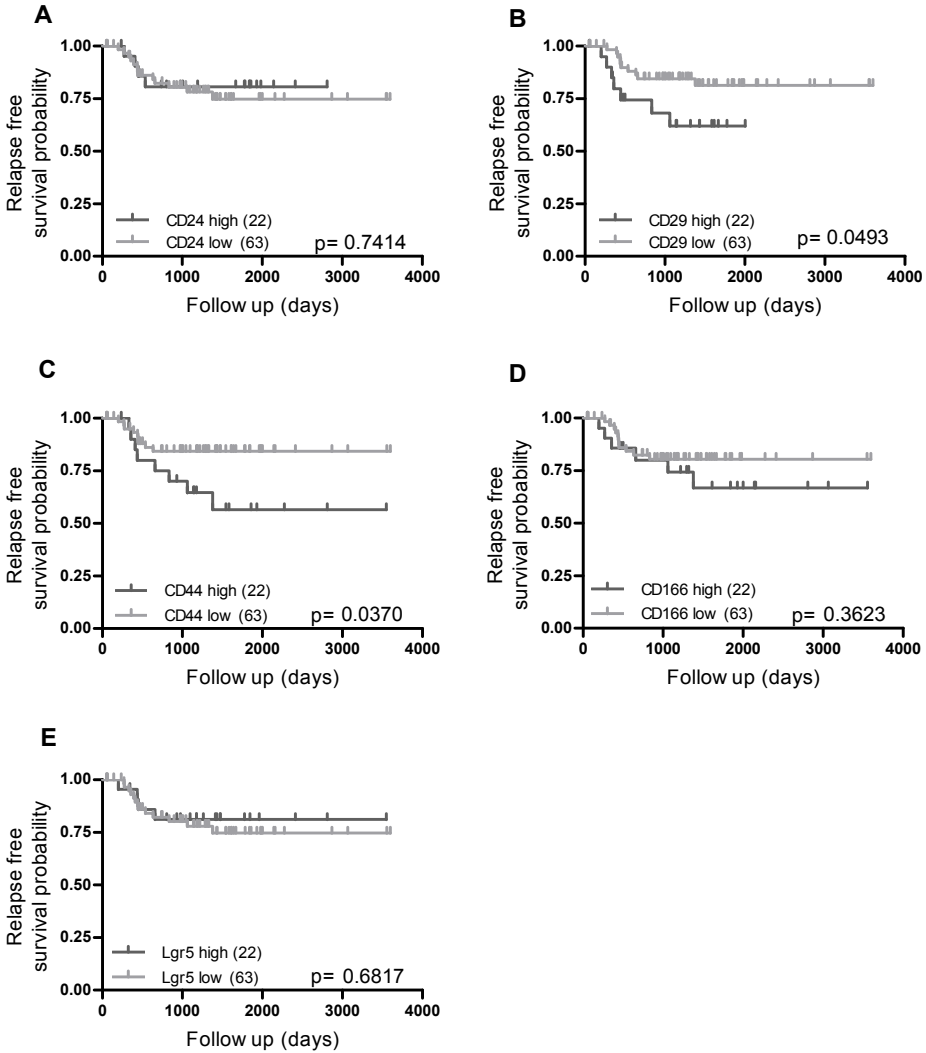
Supplementary Figure 1. Only CD44 is prognostic when highest quartile is used to separate the groups. The highest quartile of expression was used to divide the patient set in two groups after which the relapse-free survival probability was analyzed for each group. Only CD44 displayed the capacity to predict prognosis, whereas the other CSC markers did not. **A.** CD24, **B.** CD29, **C.** CD44, **D.** CD166, **E.** Lgr5

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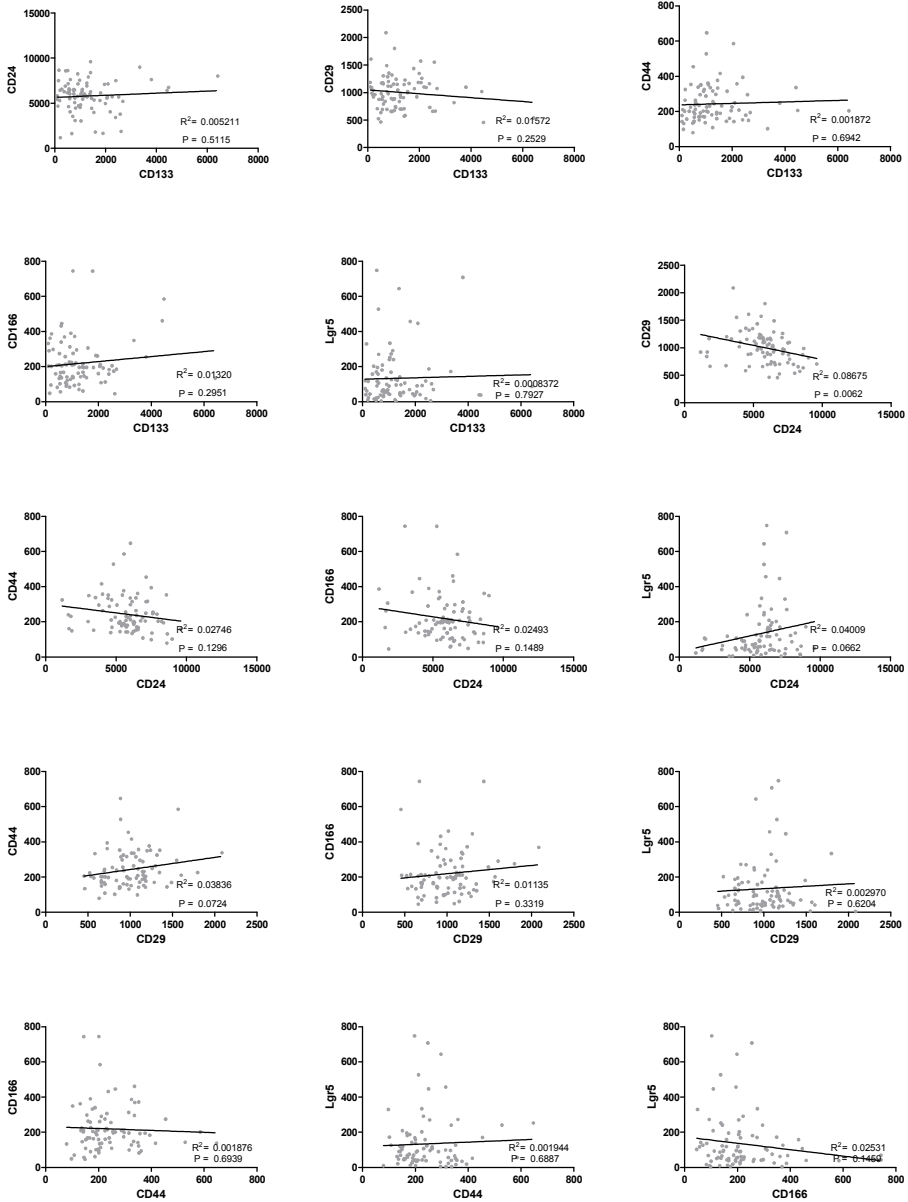
Supplementary Figure 2. Correlation in expression levels between different CSC markers. Expression levels of different CSC markers were plotted against each other. Linear regression was used to study if there is a significant correlation. **A.** CD133 versus CD24, **B.** CD133 versus CD29, **C.** CD133 versus CD44, **D.** CD133 versus CD166, **E.** CD133 versus Lgr5, **F.** CD24 versus CD29, **G.** CD24 versus CD44, **H.** CD24 versus CD166, **I.** CD24 versus Lgr5, **J.** CD29 versus CD44, **K.** CD29 versus CD166, **L.** CD29 versus Lgr5, **M.** CD44 versus CD166, **N.** CD44 versus Lgr5, **O.** CD166 versus Lgr5.
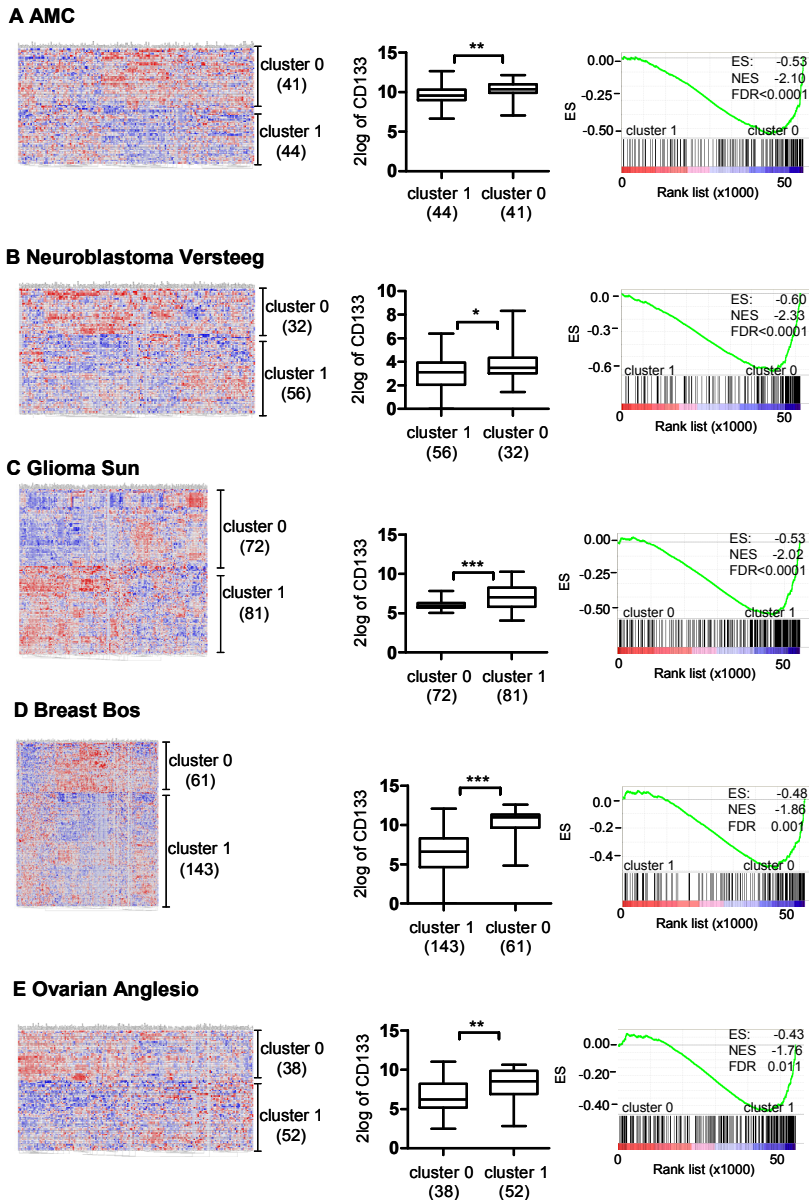
Supplementary Figure 3. Gene set enrichment analysis after K-means clustering with activated K-Ras gene expression signature. An activated K-Ras gene expression signature was used to segregate ours as well as other patient collections in two groups by k-means clustering (left panel). CD133 expression consequently studied in these two clusters (middle panel). To identify which cluster resembled the activated K-Ras profile, gene set enrichment analysis was used (right panel). **A.** our CRC patient cohort (n=85), **B.** a neuroblastoma collection (n=88), **C.** a glioma cohort (n=153), **D.** a breast cancer cohort (n=204) and **E.** an ovarian cancer collection (n=90). ES: enrichment score; NES: normalized enrichment score, FDR: false discovery rate.
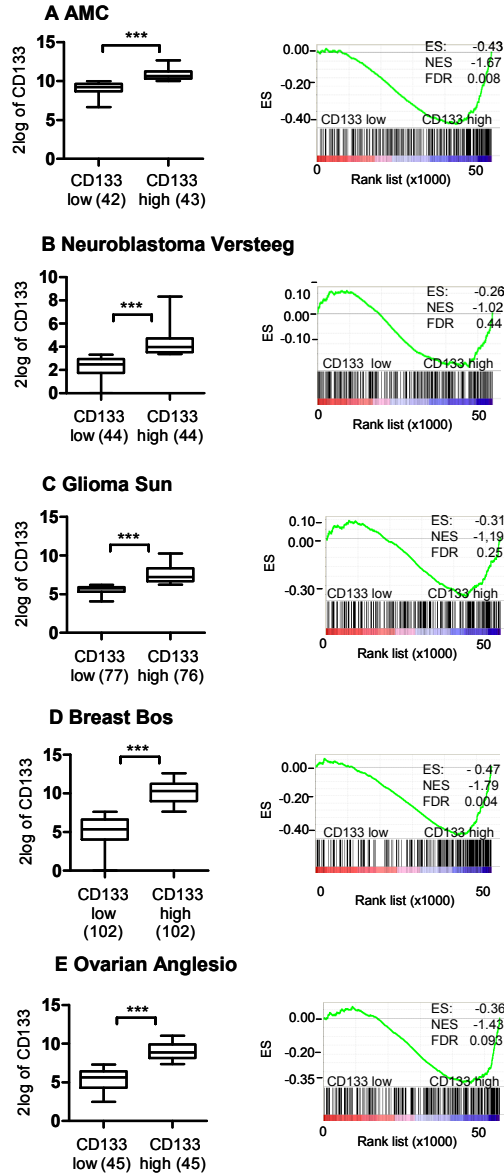
Supplementary Figure 4. Gene set enrichment analysis after dividing the patient cohort in CD133 low versus CD133 high for activated K-Ras gene expression signature. The patient cohorts were divided in two equal groups based upon the median of the CD133 expression (left panel). Then, by gene set enrichment analysis, it was studied which cluster had the most overlap with the mutant K-Ras gene expression profile (right panel). **A.** our CRC patient cohort (n=85), **B.** a neuroblastoma collection (n=88), **C.** a glioma cohort (n=153), **D.** a breast cancer cohort (n=204) and **E.** an ovarian cancer collection (n=90). ES: enrichment score; NES: normalized enrichment score, FDR: false discovery rate.

Supplementary Table 1: CSC marker CD133 as prognostic marker

CRC	Stage	Analysis	High CD133 associates with	Reference
Rectal	II-III	qPCR	Low recurrence-free survival	7
Colon	T2-3 (N0, M0)	IHC with AC133	Low survival	13
Colon	IIIB	IHC with Ab19898	Poor prognosis	4
Colon	I-III	qPCR	Shorter relapse-free and overall survival	2
Colon	I-IV	IHC with AC133	Low overall survival	15
Colorectal	I-IV	IHC with AC133	Worse survival	5
Rectal	II-III	qPCR	Low recurrence-free survival after CRT	6
Colon	I-IV	PCR	Poorer survival	3

Supplementary Table 2: Primers

Name	Forward	Reverse
K-Ras	5'-ACTGAATATAAACTTGTGGTAGTTGG-3'	5'-TGACCTGCTGTGTCGAGAAT-3'
B-Raf	5'-TGATTTTTGTGAATACTGGGAAC-3'	5'-TGCTTGCTCTGATAGGAAAATG-3'
P53-exon1	5'- GCTTTCCACGACGGTGACA-3'	5'-TTGTTGAGGGCAGGGGAGTA-3',
P53-P33	5'-TGTCATCTTCTGTCCCTTCCC-3'	5'-GATGGTGGTACAGTCAGAGC-3'
P53-P31	5'-TTGCGTGTGGAGTATTTGGA-3'	5'-GCAAGCAAGGGTTCAAAGACC-3'
P53-P120	5'-CAGTGGTTTCTTCTTTGGCTG-3'	5'-AAGAAAGGGGAGCCTCACCA-3'
CD133	5′ -GCATTGGCATCTTCTATGGTT-3′	5′ -CGCCTTGTCCTTGGTAGTGT-′ 3
GAPHD	5′ -AAGGTGAAGGTCGGAGTCAAC-3′	5′ -TGGAAGATGGTGATGGGATT-3′ .