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Tumour CD133 mRNA expression and clinical outcome in surgically resected colorectal cancer patients

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ABSTRACT

Background: Human prominin-1 (CD133) is a novel pentaspan membrane protein which was originally classified as a marker of primitive haematopoietic and neural stem cells. Cancer stem cells have been isolated and expanded from leukaemia and several solid tumours, and have been associated with metastasis, chemoresistance and relapse. CD133 is recognised as a stem cell marker and is capable of identifying a tumour-initiating subpopulation in brain, colon, melanoma and other solid tumours.

Methods: We assessed CD133 mRNA expression levels by RT-QPCR in tumour and matched normal tissue from 64 stages I–III colorectal cancer (CRC) patients and correlated tumour CD133 levels with clinicopathological characteristics and clinical outcome.

Results: In four patients, CD133 mRNA was not expressed in tumour or in normal tissue. In the remaining 60 patients, expression levels were higher in tumour than in normal tissue ($p = 0.001$). Higher levels of CD133 expression were associated with shorter relapse-free interval (RFI) ($p = 0.004$) and overall survival (OS) ($p < 0.0001$). In the multivariate analyses, CD133 levels emerged as a prognostic marker for RFI and OS.

Conclusions: We have observed longer RFI and OS in patients with lower levels of CD133, regardless of adjuvant treatment and other clinical characteristics. If these findings are confirmed in larger prospective studies, CD133 assessment may prove useful for new diagnostic and therapeutic procedures for CRC patients.

1. Introduction

Colorectal cancer (CRC) remains the second most common cause of cancer-related death in the western world. Fluoropyrimidines, including 5-fluorouracil (5-FU), have been shown to improve outcome in metastatic CRC[1] and are also a fundamental component of adjuvant treatment.[2] A pooled analysis of three studies found that adjuvant chemotherapy with 5-FU + leucovorin led to a 22% reduction in mortality compared with no treatment.[3] An analysis of stages II–III CRC patient data from 18 trials found an OS and disease-free survival benefit for 5-FU-based chemotherapy.[4] However, although adjuvant treatment may improve outcomes for patients with respected CRC,[5] the benefit, especially in stage II disease, is

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not clear, and the American Society of Clinical Oncology guidelines do not recommend its routine use for all patients.[6] Furthermore, 5-FU provokes 20–25% grades 3 and 4 toxicities and 0.2–0.4% toxic deaths, often in patients in adjuvant settings, who may have already been cured by surgery before chemotherapy or who can receive no benefit from adjuvant treatment.[7] Moreover, notwithstanding the potential benefits of adjuvant chemotherapy, approximately 50% of patients relapse within 3 years of surgical resection,[8] indicating that some cancer cells are not eradicated by current therapies.

There is an emerging body of evidence that tumour cells resistant to chemotherapy represent a subpopulation of cells from the original tumour that are molecularly and phenotypically distinct.[9] Three recent studies provide evidence for the existence of a small proportion of tumour-initiating cells or 'cancer stem cells' (CSCs)[10–12] that are capable of proliferation, self-renewal and differentiation into various cell types seen in the bulk tumour. According to the CSC model, cancer cannot be viewed as simple monoclonal expansions of functionally equal tumour cells. Instead, despite their monoclonal origin, only a small minority of tumour cells, the CSCs, have the ability to maintain the malignant population.[9,13] These CSCs may be associated with metastasis, treatment resistance and recurrence.[14] Recent findings in CRC suggest that a CD133-positive subpopulation of cancer cells is highly enriched in CSCs.[12]

CD133 (also known as human prominin-1), located on chromosome 4, is a member of the prominin family of pentaspan membrane proteins.[15] It has been used as a marker in mice to detect embryonic stem cells[16] and in humans to detect adult stem cells.[17,18] Results of experimental models indicate that cells with CD133 expression are responsible for CRC tumour growth in mice.[11] In human tumours, CD133 expression has been found in retinoblastoma,[19] teratocarcinoma,[19] leukaemia,[19] brain,[20] hepatocellular carcinoma[21] and in CRC,[12] where CD133 expression by immunohistochemistry (IHC) has been shown to be an independent prognostic marker for overall survival.[22,23]

To date, no study has compared the potential effect of tumour CD133 mRNA expression on outcome in resected CRC patients receiving either adjuvant chemotherapy or no adjuvant treatment. In the present study, we have analysed CD133 mRNA in tumour and matched normal tissue from resected CRC patients in order to examine the potential relationship between clinicopathological characteristics, CD133 mRNA expression levels and clinical outcome to surgery alone or to surgery followed by adjuvant chemotherapy.

2. Patients and methods

2.1. Tumour and normal tissue samples

Between August 2003 and August 2006, 64 stages I–III CRC patients underwent surgical resection at the Municipal Hospital of Badalona, Spain. Tumour and normal fresh tissue were obtained from each patient and preserved in liquid nitrogen. RNA was obtained from the samples after histopathological confirmation of neoplastic and normal tissues. All patients gave their signed informed consent.

2.2. Treatment plan

Stage I patients received no adjuvant treatment. Stages II–III patients received either no adjuvant treatment or adjuvant chemotherapy, at the discretion of the attending physician (Table 1). Eleven patients received Mayo (5-FU/leucovorin), 15 received FOLFOX (oxaliplatin/capecitabine) and 22 received capecitabine alone. In addition to chemotherapy, rectal cancer patients received sequential and/or concomitant radiotherapy, at the discretion of the attending physician. Follow-up was carried out every 4 months during the first 3 years and every 6 months thereafter.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from tumour tissue and normal fresh tissue using the commercial RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration purity and amount of total RNA were determined by UV spectrophotometry, and the integrity and quality of RNA were assessed by electrophoresis on 1% agarose gel. RNA from samples was quantified in duplicate using a NanoDrop 1000 Spectrophotometer.

cDNA was synthesised using TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City CA). Reverse transcription was performed using 330 ng of total RNA in 10 μl of TaqMan RT Buffer, 22 ml of 25 mM magnesium chloride, 20 μl dNTPs, 5 μl Random Hexamers, 2 μl RNAse Inhibitor, 2.5 μl MultiScribe Reverse Transcription and RNA sample plus RNAse-free water, for a final volume of 100 μl, in the following thermal cycler conditions: 10 min 25 °C, 48 min 30 °C and 5 min 95 °C.

2.4. Gene expression and quantification with real-time quantitative PCR (RT-QPCR)

Primers and probes to identify the CD133.s1 isoform (Hs00195682_m1) and to determine CD133 mRNA levels (Hs01009247_m1) were supplied by Applied Biosystems. Primers were labelled at the 5′ end with the reporter dye molecule FAM. 18s gene probe labelled at the 5′ end with the reporter dye molecule FAM (Hs99999901_s1; Applied Biosystems) was used as housekeeping gene.

RT-QPCR was performed in a total volume of 20 μl in the ABI Prism 7700 Sequence Detection System (Applied Biosystems). All samples for each gene were run in duplicate for 40 cycles using the following master mix and thermal cycler conditions: 10 μl of the TaqMan universal PCR master mix, 1 μl of the primers and probes, 2 μl of the cDNA and 7 μl of the RNAse-free water; about 2 min 50 °C, 10 min 95 °C, 15 s 95 °C and 1 min 60 °C. Genomic DNA was used as negative control in each run. Fluorescent emission data were captured, and mRNA concentrations were quantified by using the critical threshold value and $2^{-\Delta\Delta Ct}$.

2.5. Statistical analyses

This study was designed to examine the levels of CD133 mRNA expression in tumour and normal tissue from CRC patients and to correlate the levels of tumour CD133

Table 1 – Clinicopathological characteristics of CRC patients

	All patients (N = 64) N (%)	All patients with CD133 mRNA expression in tumour (N = 60) N (%)	Patients with low CD133 (N = 45) N (%)	Patients with high CD133 (N = 15) N (%)
Gender				
Male	32 (50%)	29 (48.33)	21 (46.67%)	8 (53.33%)
Female	32 (50%)	31 (51.67%)	24 (53.33%)	7 (46.67%)
Age, median (range)	70 (39–88)	67.27 (39–88)	63 (39–88)	79 (53–83)
Karnofsky index				
⩾80	58 (90.63%)	55 (91.67%)	43 (95.56%)	12 (80%)
<80	6 (9.37%)	5 (8.33%)	2 (4.44%)	3 (20%)
Tumour site				
Right colon	23 (35.94%)	22 (36.67%)	14 (31.11%)	8 (53.33%)
Left colon	41 (64.06%)	38 (63.33%)	31 (68.89%)	7 (46.67%)
Histologic grade				
A	9 (14.06%)	8 (13.33%)	7 (15.55%)	1 (6.67%)
B	55 (85.94%)	52 (86.67%)	38 (84.45%)	14 (93.33%)
Perineural permeation				
Yes	5 (7.81%)	5 (8.33%)	4 (8.89%)	1 (6.67%)
No	59 (92.19%)	55 (91.67%)	41 (91.11%)	14 (93.33%)
Vascular permeation				
Yes	7 (10.94%)	6 (10%)	5 (11.11%)	1 (6.67%)
No	57 (89.06%)	54 (90%)	40 (88.89%)	14 (93.33%)
Lymphatic permeation				
Yes	8 (12.5%)	7 (11.67%)	4 (8.89%)	3 (20%)
No	56 (87.5%)	53 (88.33%)	41 (91.11%)	12 (80%)
Preexisting polyp				
Yes	17 (26.56%)	16 (26.67%)	12 (26.67%)	4 (26.67%)
No	47 (73.44%)	44 (73.33%)	33 (73.33%)	11 (73.33%)
Lymph node status				
N0	35 (54.68%)	33 (55%)	25 (55.55%)	8 (53.33%)
N1	19 (29.69%)	18 (30%)	13 (28.90%)	5 (33.33%)
N2	10 (15.63%)	9 (15%)	7 (15.55%)	2 (13.34%)
Stage				
I	3 (4.69%)	3 (5.00%)	3 (6.67%)	0 (0%)
II	31 (48.44%)	30 (50%)	22 (48.89%)	8 (53.33%)
III	30 (46.87%)	27 (45%)	20 (44.44%)	7 (46.67%)
Chemotherapy				
Yes	48 (75%)	44 (73.33%)	36 (80%)	8 (53.33%)
FOLFOX	15 (31.25%)	14 (23.33%)	12 (33.33%)	2 (25%)
Mayo	11 (22.92%)	11 (18.33%)	10 (27.78%)	1 (12.5%)
Capecitabine	22 (45.83%)	19 (31.67%)	14 (38.89%)	5 (62.5%)
No	16 (25%)	16 (26.67%)	9 (20%)	7 (46.67%)
CD133 mRNA in tumour				
Yes	0	60 (100%)	45 (100%)	15 (100%)
No	4 (6.66%)	0	0	0

expression with clinicopathological characteristics and clinical outcome. High and low mRNA expression levels in tumour tissue were set using the maxstat package of R.[24] In order to define the cutpoint that provides the best separation into two groups, maxstat computes the maximally selected log-rank statistic for cutpoints between the 10% and 90% quantile of the mean expression following the method of Hothorn and Lausen.[24] Relapse-free interval (RFI) was defined as the time from surgery until relapse, and overall survival as the time from surgery until death. RFIs and overall survival times were compared using the Logrank test. Survival curves were drawn using the Kaplan Meier method. The Cox proportional hazards model was used to assess the independent relative risks (RR) and their 95% confidence intervals (CIs) for RFI and OS. Statistical procedures were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, United States of America). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Patient characteristics

Median age for all 64 patients was 70 years; 32 were males and 32 females; in 23 patients, the tumour was located in the right colon and in 41 patients in the left colon. Three patients had stage I disease, 31 stage II and 30 stage III. Thirty-five patients had no lymph node involvement, 19 were N1 and 10 were N2. Nine had poorly differentiated disease and 55 had moderately or well-differentiated disease. Forty-eight patients received adjuvant chemotherapy, and 16 received no treatment after surgery (Table 1).

3.2. CD133 mRNA expression levels and clinical outcome

CD133 mRNA was present in tumour in 60 of 64 patients (93.75%). In 53 of these 60 patients (88.33%), CD133 was present in both tumour (Ct mean, 30.14 [range, 24–32]) and normal (Ct mean, 33.1 [range, 28–37]) tissue. Levels were overall higher in the tumour than in the normal tissue samples ($p = 0.001$). Of the 53 patients with CD133 expression in both tumour and normal tissues, 37 (69.81%) had higher CD133 levels in tumour, compared to 16 (30.19%) with higher levels in normal tissue (Fig. 1). The 60 patients with CD133 mRNA expression in tumour were included in the analyses of clinical outcome. Using the cutoff selected by the maxstat package of R, the 60 patients were divided into two groups: 45 had low CD133 levels (<1.04) and 15 had high levels (>1.04) (Table 1). No significant difference in clinicopathological characteristics was observed between patients with high and low CD133 levels.

After a median follow-up of 44 months (6.5–77), RFI was 68.6 months (95% CI: 63.17–74.04) for all 60 patients. In the univariate analysis, three factors were significantly associated with shorter RFI: perineural permeation, lymphatic permeation and high CD133 mRNA expression levels. RFI for

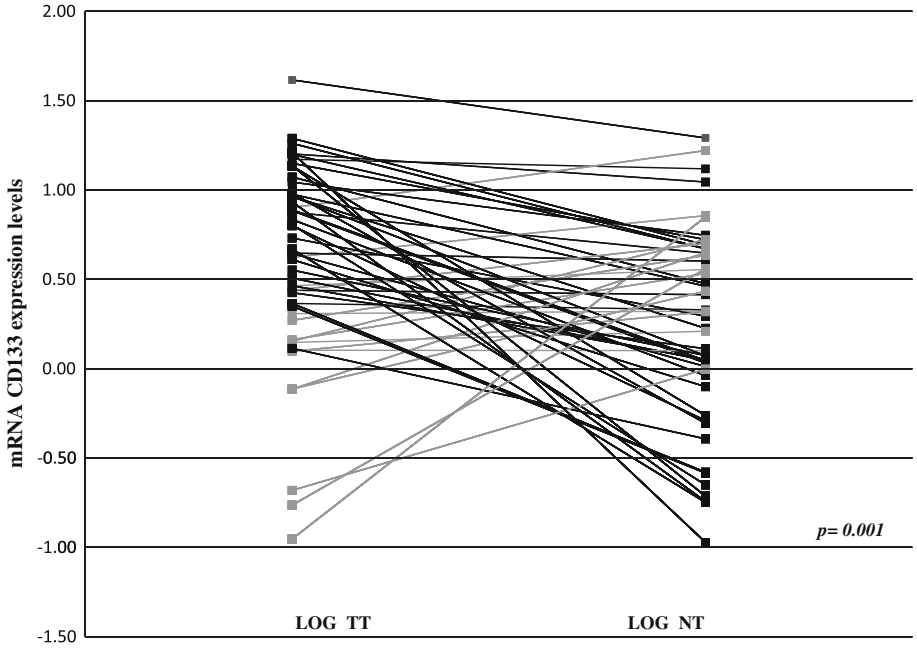

Fig. 1 – Comparison of relative expression of CD133 mRNA between tumour and paired normal tissues ($p = 0.001$) in 53 patients with CD133 expression in both tumour and normal tissues. Black lines indicate higher expression in tumour than in normal tissue; grey lines indicate higher expression in normal than in tumour tissue. Thirty-seven patients had higher CD133 levels in tumour, compared to 16 patients with higher levels in normal tissue.

Table 2 – Relapse-free interval (RFI) and overall survival (OS) in 60 patients with CD133 mRNA expression in tumour, according to CD133 mRNA levels

	RFI months (95% CI)			OS months (95% CI)		
	Low CD133 mRNA (<1.04)	High CD133 mRNA (>1.04)	*p*-Value	Low CD133 mRNA (<1.04)	High CD133 mRNA (>1.04)	*p*-Value
All patients (*N* = 60)	72.7 (68.1–77.4)	47.9 (34.8–61.0)	0.004	71.9 (67.2–76.6)	44.4 (31.6–57.3)	<0.0001
Patients receiving adjuvant chemotherapy (*N* = 44)	71.7 (65.9–77.4)	46.7 (28.7–64.6)	0.029	71.4 (65.7–76.5)	34.9 (20.7–49.2)	<0.0001
Patients receiving no adjuvant treatment (*N* = 16)	NR	46 (32.5–59.5)	0.062	NR	NR	–

NR = not reached.

the five patients with perineural permeation was 48.7 months (95% CI: 19.7–77.7), while for those without perineural permeation, it was 70.4 months (95% CI: 65.3–75.4) ($p = 0.038$). RFI was 40.9 months (95% CI: 18.9–62.9) for the seven patients with lymphatic permeation and 72.3 months (95% CI: 67.9–76.7) for those without lymphatic permeation ($p < 0.0001$). RFI was 47.9 months (95% CI: 34.8–61.0 months) for the 15 patients with high CD133 levels and 72.7 months (95% CI: 68.1–77.4 months) for those with low CD133 levels ($p = 0.004$) (Table 2, Fig. 2A). No correlation was observed between CD133 expression levels and perineural or lymphatic permeation. No other significant correlation was found between RFI and clinical characteristics.

OS was 69.14 months (95% CI: 64.0–74.2) for all 60 patients. In the univariate analysis, four factors were significantly associated with shorter OS: stage III disease, Karnofsky index (<80), younger age (<66.5 years) and high CD133 mRNA expression levels. For the 27 patients with stage III disease, OS was 57.0 months (95% CI: 47.5–66.5), while it was 74.0 months (95% CI: 70.2–77.9) for those with stages I–II disease ($p = 0.008$). For the five patients with Karnofsky index <80, OS was 58.9 months (95% CI: 49.2–68.6), while it was 70.6 months (95% CI: 64.9–76.3) for those with =>80 ($p = 0.027$). For the 31 patients older than 66.5 years, OS was 59.4 months (95% CI: 50.6–68.2), while it was 71.7 months (95% CI: 66.1–77.2) for those younger than 66.5 years ($p = 0.043$). OS was 44.4 months (95% CI: 31.6–57.3 months) for patients with high CD133 levels and 71.9 months (95% CI: 67.2–76.6 months) for those with low CD133 levels ($p < 0.0001$) (Table 2, Fig. 3A).

All variables that were significant in the univarate analyses, as well as gender and tumour site, were included in the multivariate analyses for RFI and OS. Only lymphatic permeation (RR: 7.54; 95% CI: 1.8–31.4; $p = 0.005$) and high CD133 levels (RR, 4.74; 95% CI: 1.09–20.56; $p = 0.038$) emerged as significant prognostic markers for shorter RFI, while stage III disease (RR: 8.33; 95% CI: 1.75–39.6; $p = 0.008$) and high CD133 levels (RR: 11.4; 95% CI: 3.3–39.0; $p = 0.0001$) were significant prognostic markers for poor OS (Table 3).

3.3. *Outcome according to CD133 levels in patients receiving adjuvant chemotherapy or no adjuvant treatment*

Of the 60 patients with CD133 mRNA expression in tumour, 44 patients received adjuvant chemotherapy while 16 did not. RFI was 68.3 months (95% CI: 61.8–74.8) and OS was 64 months (95% CI: 57.5–70.6) for all 44 patients receiving chemotherapy. RFI was 46.7 months (95% CI: 28.7–64.6) for the eight patients with high CD133 levels and 71.7 months (95% CI: 65.9–77.4) for those with low CD133 levels ($p = 0.029$) (Table 2, Fig. 2B). OS was 34.9 months (95% CI: 20.7–49.2) for patients with high CD133 levels and 71.4 months (95% CI: 65.7–76.5) for those with low CD133 levels ($p < 0.0001$) (Table 2, Fig. 3B).

Amongst the 16 patients who did not receive adjuvant treatment, RFI was 53 months (95% CI: 47–59) and OS was 54.3 months (95% CI: 48.25–60.36) for all 16 patients. None of the nine patients with low CD133 mRNA levels relapsed, while RFI was 46.0 months (95% CI: 32.5–59.5) for the seven patients with high CD133 levels ($p = 0.062$) (Table 2, Fig. 2C). One patient died of causes unrelated to the disease, and all the remaining 15 patients were alive at the time of writing this manuscript.

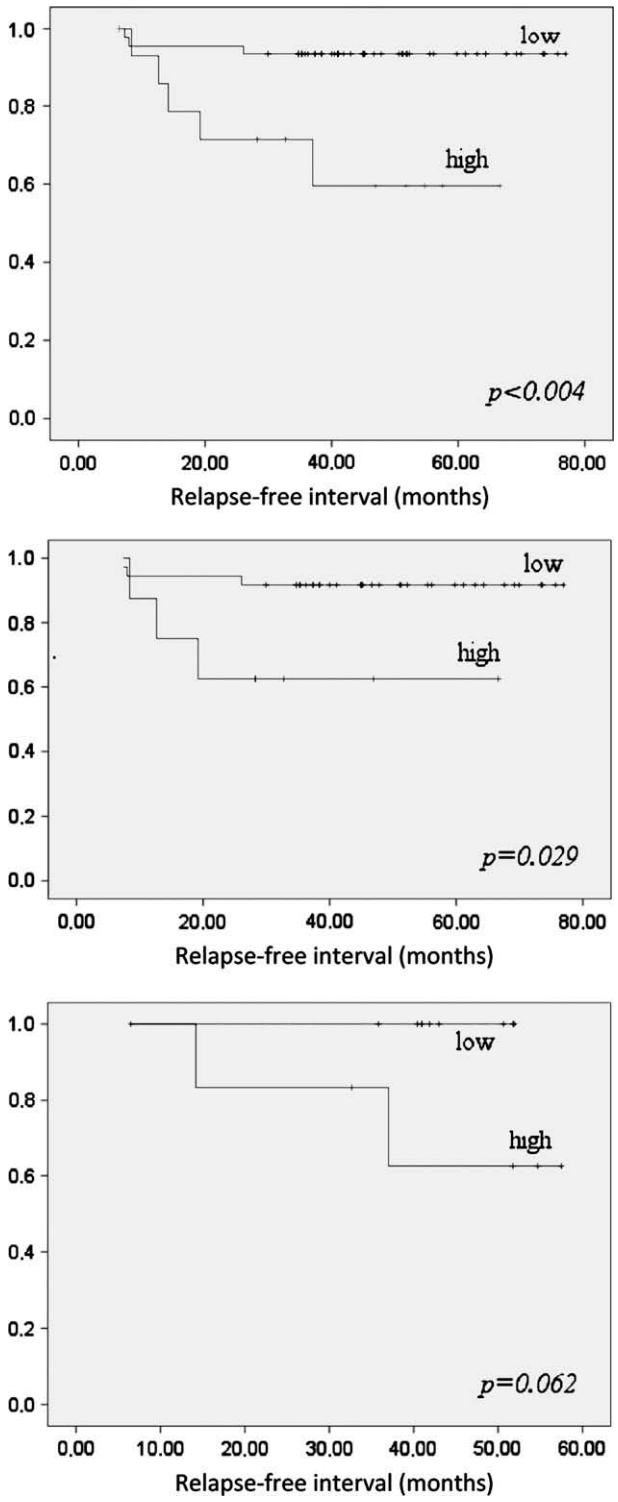

Fig. 2 – Relapse-free interval in 60 CRC patients with CD133 mRNA expression in tumour. Patients with high levels of CD133 mRNA had shorter relapse-free interval than those with low levels. (A) All 60 patients ($p = 0.004$). (B) Forty-four patients receiving adjuvant chemotherapy ($p = 0.029$). (C) Sixteen patients receiving no adjuvant treatment ($p = 0.062$).

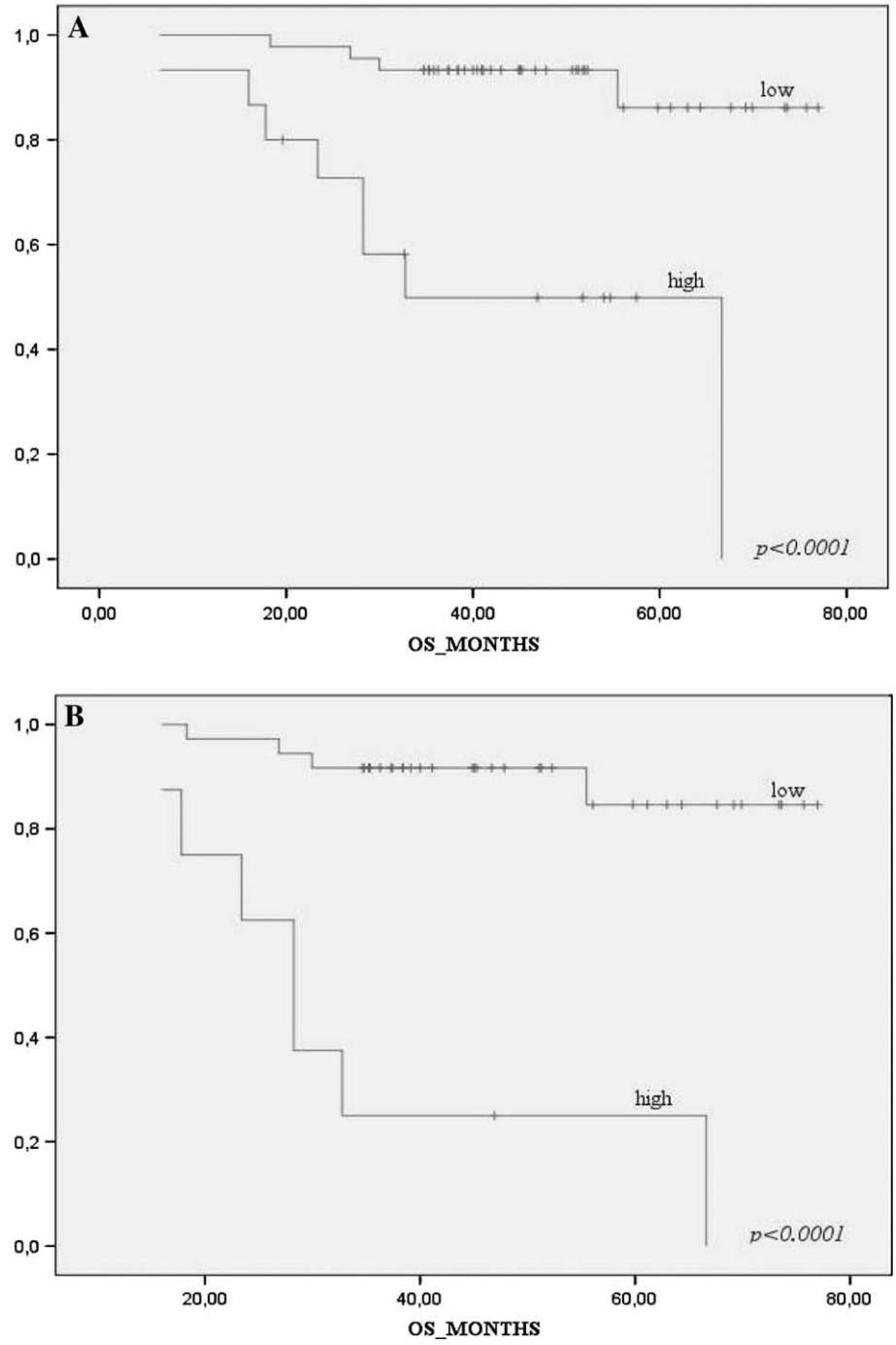

Fig. 3 – Overall survival in 60 CRC patients with CD133 mRNA expression in tumour. Patients with high levels of CD133 mRNA had shorter survival than those with low levels. (A) All 60 patients ($p < 0.0001$). (B) Forty-four patients receiving adjuvant chemotherapy ($p < 0.0001$). All 16 patients receiving no adjuvant treatment were alive at the time of writing this manuscript, with the exception of one patient who died of causes unrelated to the disease.

Table 3 – Multivariate analysis for relapse-free interval (RFI) and overall survival (OS) (N = 60)

	RR (95% CI)	p Value
RFI		
Lymphatic permeation	7.54 (1.8–31.4)	0.005
CD133 mRNA > 1.04	4.74 (1.09–20.56)	0.038
OS		
Stage III	8.33 (1.75–39.6)	0.008
CD133 mRNA > 1.04	11.4 (3.3–39.0)	0.0001

4. Discussion

CD133 is present in different types of stem cells and several cancers, and is down-regulated in differentiated cells.[12] For the first time, we have examined the presence of CD133 mRNA in matched tumour and normal colon tissue from 64 resected CRC patients. Higher levels of CD133 mRNA were observed in tumour than in normal tissue. This is in line with results from a preclinical study where CD133-postive cells were both necessary and sufficient to initiate tumour growth in immunodeficient mice, indicating that CD133 may be a useful marker for the detection of CSCs.[11]

A previous study of CD133 expression by IHC in 77 CRC patients found that 26% of the tumours had high levels of CD133 and 74% had low levels of CD133.[22] These percentages are similar to our findings by RT-QPCR in the present study, where 25% of patients had high and 75% had low levels of CD133. Also in line with our findings, high CD133 levels by IHC were a prognostic marker for shorter survival.[22] In addition, CD133 mRNA expression in peripheral blood was shown to be higher in patients with recurrent disease than in those without recurrence.[25] In another study, using real-time nuclear acid sequence-based amplification, CD133 was increased in patients with bone metastases, and patients with high CD133 had shorter survival.[26] Our findings provide support for the hypothesis that CSC expression levels in a tumour may correlate with more aggressive clinicopathological features and worse outcome.[14] Interestingly, although disease stage is generally recognised as a prognostic marker,[27] in our study, CD133 expression levels trumped stage in the multivariate analyses of RFI and OS.

The benefits of FU-based adjuvant chemotherapy in reducing recurrence and in prolonging survival are generally accepted for stage III but not for stage II disease,[6] and the decision to offer adjuvant therapy for stage II disease thus needs to be individualised to the circumstances of each patient. Dallas et al.[28] reported that both 5-FU- and oxaliplatin-resistant cells were significantly enriched for the CSC markers CD133 and CD44. In the present study, high levels of CD133 mRNA correlated with poor RFI and OS, both in all patients and in the subgroup of those receiving adjuvant chemotherapy. In light of these findings, CD133 levels should also be taken into consideration at the time of deciding on the need for adjuvant chemotherapy.

Although CD133-positive cells are associated with chemoresistance,[28] it is not clear whether CD133 is simply a marker of resistant cells or whether high expression of CD133 in CSCs can itself contribute to treatment resistance.[29] One possible explanation for the poor outcome observed in patients with high CD133 levels may be due to the fact that cytotoxic drugs do not target the CSCs, which then are able to recapitulate the bulk tumour population.[12]

In conclusion, we have observed that high levels of CD133 are associated with shorter RFI and OS in resected CRC patients. If our findings are confirmed in larger prospective studies, they may lead to the development of new diagnostic and therapeutic procedures for CRC patients.

Conflict of interest statement

None declared.

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