Prognostic significance of CD44 variant 2 upregulation in colorectal cancer

M Ozawa*,1, Y Ichikawa2,7, Y-W Zheng3,7, T Oshima4, H Miyata5, K Nakazawa3, H-B Guan3, M Shiozawa5, M Akaike5, K Watanabe4, M Ota4, S Fujii4, C Kunisaki4, T Ishikawa6, K Tanaka1, H Akiyama1, I Endo1 and H Taniguchi*,3

1Department of Gastroenterological Surgery, Yokohama City University, 3-9 Fukaura Kanazawa-ku Yokohama-shi, Kanagawa-ken 236-0004, Japan; 2Department of Clinical Oncology, Yokohama City University, 3-9 Fukaura Kanazawa-ku Yokohama-shi, Kanagawa-ken 236-0004, Japan; 3Department of Regenerative Medicine, Yokohama City University, 3-9 Fukaura Kanazawa-ku Yokohama-shi, Kanagawa-ken 236-0004, Japan; 4Gastroenterological Center, Yokohama City University Medical Center, 4-57 Urafune Minami-ku Yokohama-shi, Kanagawa-ken 232-0024, Japan; 5Department of Surgery, Kanagawa Cancer Center, 2-3-2 Nakao Asahi-ku Yokohama-shi, Kanagawa-ken 241-8515, Japan and 6Breast and Thyroid Surgery, Yokohama City University Medical Center, 4-57 Urafune Minami-ku Yokohama-shi, Kanagawa-ken 232-0024, Japan

Background: CD133 and CD44 are putative cancer stem cell (CSC) markers in colorectal cancer (CRC). However, their clinical significance is currently unclear. Here, we evaluated primary CRC cell isolates to determine the significance of several CSC markers, including CD133 and CD44, as predictors of tumorigenesis and prognosis.

Methods: CD133- and CD44-positive cells from fresh clinical samples of 77 CRCs were selected by flow cytometric sorting and evaluated for tumourigenicity following subcutaneous transplantation into NOD/SCID mice. Cancer stem cell marker expression was examined in both xenografts and a complementary DNA library compiled from 167 CRC patient samples.

Results: CD44*, CD133* and CD133*CD44* sub-populations were significantly more tumourigenic than the total cell population. The clinical samples expressed several transcript variants of CD44. Variant 2 was specifically overexpressed in both primary tumours and xenografts in comparison with the normal mucosa. A prognostic assay using qRT–PCR showed that the CD44v2high group (n = 84, 5-year survival rate (5-OS): 0.74) had a significantly worse prognosis (P = 0.041) than the CD44v2low group (n = 83, 5-OS: 0.88).

Conclusions: CD44 is an important CSC marker in CRC patients. Furthermore, CRC patients with high expression of CD44v2 have a poorer prognosis than patients with other CD44 variants.

Within many solid cancers, there is considerable heterogeneity in phenotypic, genetic and epigenetic traits. The plasticity of a minor sub-population of cancer stem cells (CSCs) is thought to contribute to intercellular heterogeneity and the emergence of therapeutic resistance. Therefore, CSCs are potentially critical targets for novel cancer treatments (Reya et al, 2001). Although the presence of CSCs may, in some cases, be a strong predictor of tumourigenesis and prognosis (Jessup et al, 1989), there is no ‘standard’ genetic or epigenetic marker for CSCs across tumour types.

Several markers are associated with CSC in colorectal cancer (CRC), including CD24, CD29, CD44, CD133, CD166, the epithelial cell adhesion molecule, Musashi-1, aldehyde dehydrogenase-1 (ALDH1) and the leucine-rich-repeat containing G protein-coupled receptor 5 (LGR5; Todaro et al, 2010). CD133+ and CD44+ cell populations represent a particular subset of aggressive CRC cells (Dalerba et al, 2007; O’Brien et al, 2007; Ricci-Vitiani et al, 2007; Du et al, 2008). However, there has been insufficient evaluation of these markers using clinical samples in...
standard tumourigenicity assays. To address this deficiency, we used cancer cells derived directly from clinical samples of primary CRC lesions to evaluate the impact of several CSC markers, including CD133 and CD44 variants, on tumourigenesis and prognosis.

### MATERIALS AND METHODS

**Experiment 1.** The tumourigenicity of enriched CD133- and CD44-positive cells from CRC patients was estimated using a xenograft model in NOD/SCID mice.

**Samples.** Suitable clinical samples (volume > 1 cm³), including CRC and adjacent normal mucosa, were obtained following surgical resection in 77 CRC patients at Yokohama City University Hospital, Yokohama City University Medical Center and Kanagawa Cancer Center from April 2007 to August 2011. We followed the approved guidelines set by the ethical committee at each institution (permits no. 18-28-14, no. 21142 and no. 9). The cancer specimens were immediately minced and filtered through 100-μm nylon mesh and washed with calcium/magnesium-free PBS. To dissociate the cells, the specimens were incubated in Hank’s balanced salt solution with 1% EGTA and 1% antibiotic-antimycotic (Life Technologies, Inc., Rockville, MD, USA) at 37 °C for 30 min and then placed in DMEM with 10% FBS supplemented with 2 mg ml⁻¹ type IV collagenase (Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 1 h. The cells were then filtered through a 40-μm nylon mesh and washed twice.

**Flow cytometric sorting.** The dissociated cells of each sample were analysed and separated by a MoFlo cell sorter (Beckman Coulter, Brea, CA, USA). Single-cell suspensions were incubated with allophycocyanin (APC)-conjugated anti-human CD133/1 (clone AC133; Miltenyi-Biotec, Bergisch Gladbach, Germany), phycoerythrin (PE)-conjugated anti-human CD44 (clone G44-26; BD Pharmingen, BD Biosciences, San Jose, CA, USA), biotinylated anti-human CD133/1 (clone AC133; Miltenyi-Biotec), PE-conjugated anti-human CD44 MAb (clone G44-26; BD Pharmingen) and anti-human Ki67 MAb (clone MIB-1; Dako, Glostrup, Denmark).

**Gene expression assay.** The expression of stem cell marker-related mRNAs was examined using five sets of normal mucosa, primary tumour tissues and xenografts derived from the same patients. Total RNA was isolated from each sample using an RNeasy Mini kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesised from 1 μg of total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed with a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland). The PCR primer sequences are shown in Supplementary Table 1.

**RT–PCR and exon-specific PCR.** qRT–PCR was performed for standard and variant isoforms of CD44 (CD44s and CD44v). Variant isoform 1 (exon 6) contains a stop codon in humans; thus, CD44v1 is not expressed (Solis et al., 2012). Therefore, the remaining variant exons 2–10 (v2–10) were examined.

**Primer design (Supplementary Table 2) and nested PCR were performed as described previously (Konig et al., 1996 and Rajarajan et al., 2012).**

First-strand cDNA was synthesised from 0.5 μg of total RNA using 10 ng μl⁻¹ oligo (dT) 12–18 primers (Life Technologies, Inc., Paisley, UK) with an initial denaturation step at 65 °C for 5 min. The samples were then placed on ice at least for 1 min, and 5× first-strand buffer, 5 mM dithiothreitol (Life Technologies, Inc., Rockville, MD, USA), and each dNTP at 0.5 mM were added to each sample. SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was added at a final concentration of 10 U μl⁻¹ and the sample was incubated for 60 min at 37 °C. A 15-min incubation at 70 °C was performed to terminate the reaction.

Hot-start PCR was performed using cDNA primers for total CD44, individual variant exons (CD44v R and CD44v F, exon-specific forward primers for v2 to v10) (Konig et al., 1996; Rajarajan et al., 2012) and GAPDH (see Supplementary Table 2 for primer sequences).

A sample containing 1 μl of cDNA, 5 μl Tris–HCl (pH 7.5), 3.2 mM MgCl₂, each dNTP at 40 μM, and each forward and reverse primer at 5 μM was denatured for 2 min at 94 °C, and 1.0 U μl⁻¹ KOD FX (Toyobo, Osaka, Japan) was added. Amplification of cDNA was performed in a GeneAMP PCR System 9700 using 30 cycles for total CD44 and 25 cycles for GAPDH, respectively. Each cycle included denaturation for 10 s at 98 °C, annealing for 30 s at 57 °C and elongation for 2 min at 72 °C. A second PCR reaction was performed for individual exon-specific PCRs from v2 to v10 and total variants using 20 cycles of denaturation for 10 s at 98 °C, annealing for 30 s at 58 °C and elongation for 1.5 min at 72 °C. The reaction products were resolved by electrophoresis on a 1.5% agarose gel.

**Experiment 2.** A previously developed cDNA library (Oshima et al., 2008) that included 167 pairs of CRCs and their adjacent

CD44v2 is prognostic marker for colorectal cancer
normal mucosa was utilised. Quantitative PCR was performed as described above (see ‘Gene expression assay’ section). The PCR primer sequences were as follows: CD133 (Prominin1) (forward: 5'-ACAACACTACAAGGACAGG-3', reverse: 5'-GGACCTAA TCTCATCAAGGACAGG-3'); CD44 (forward: 5'-CAGGAAGA GGATGGATATGG-3', reverse: 5'-ATTACTCTGCTGGGTGTGC-3'); and LGR5 (forward: 5'-CCCGACGGCAGGATGTTG-3', reverse: 5'-GGAGGTGAAGACGCTGAGG-3').

A CD44 primer was designed to determine whether variant isoform 2 (exon 7) was associated with the patients’ prognosis. The data associated with this primer are referred to as ‘CD44v2’.

The clinicopathological characteristics of patients were classified according to the TNM classification for malignant tumours (Sobin et al., 2009).

Statistical analysis. Categorical variables are presented as the mean ± s.d. A univariate analysis was performed using the Mann–Whitney nonparametric test, where appropriate, for continuous variables, and the χ² test was used for categorical variables. A multivariate analysis was performed using the Cox proportional hazard regression model. Overall survival (OS) rates were calculated using the Kaplan–Meier method, and differences were evaluated using the log-rank test. Significance tests were two-tailed, and the level for significance was P < 0.05. The SPSS 18 statistical package was used for all calculations (SPSS Inc., Chicago, IL, USA).

A multivariate analysis should ideally include a maximum of 3 variables and analyse 10 events per variable. Therefore, the TNM factor was defined as the TNM stage and analysed in association with adjuvant chemotherapy and CD44 expression.

RESULTS

CD133⁺, CD44⁺ and CD133⁺ CD44⁺ cells isolated from CRC samples are tumorigenic. We first compared the frequency of cells with different immunophenotypes in cancerous (n = 77) vs normal mucosa (n = 20). The following frequencies were observed: CD133⁻ CD44⁻, 57.4 ± 22.3% vs 70.9 ± 3.0% (P = 0.001); CD133⁻ CD44⁺, 3.2 ± 5.0% vs 1.2 ± 0.8% (P = 0.001); CD133⁺ CD44⁻, 21.1 ± 14.9% vs 25.2 ± 10.8% (P = 0.249); CD133⁺ CD44⁺ was a very minor sub-population in both the cancerous and normal mucosa (1.7 ± 1.6% vs 1.4 ± 1.2%; P = 0.468; Figure 1A). We used flow cytometry to enrich populations of cells expressing the various CSC markers in 77 CRC samples and obtained an adequate cell number in 63 out of 77 samples for use

Figure 1. Flow cytometric analysis of CRC cells and immunofluorescent staining of normal mucosa, primary tumour and xenograft tissue. Total CRC cells were stained with monoclonal antibodies and analysed by FACS (A). Haematopoietic cells were excluded with a leukocyte marker (CD45) and the erythroid cell marker CD235a. Non-haematopoietic (CD45⁻ CD235a⁻) cells were fractionated with antibodies directed against CD133 and CD44. The ratios of the gated cells are included in the panel (mean ± s.d., n = 77). Staining patterns of CD133 and CD44 in normal human colonic mucosa (B). CD44 immunostaining was restricted to cells at the base of the crypt. CD133 staining was present from the crypt base to the transmembrane lesion (C). CD133 staining was present from the crypt base to the transmembrane lesion (C). CD133 and CD44 were broadly observed in human colonic cancer and xenografts (D and E). Scale bar, 300 µm in (B) and 50 µm in (C–E).
in tumourigenicity assays. The profiles of the 63 patients whose cells were used in the tumourigenesis assay are shown in Table 1. We found that 21 samples were tumourigenic, whereas the remaining 42 samples failed to produce palpable tumours during the 20-week experiment.

The results of the tumourigenesis assay are shown in Table 2. Calculation of the CSC frequency revealed that the CD44+ and CD133+ fractions were significantly more tumourigenic than the non-enriched population. In contrast, CD133− and CD44− cells were less tumourigenic in this context. The CD133+CD44− fraction displayed the highest CSC frequency (1 out of 3160), and only the CD44+ and CD133+CD44− fractions were able to initiate tumours upon injection of the lowest number (102) of cells. There was no statistical difference between the CD44+ and CD44+CD133− fractions with regard to tumourigenicity.

Distribution of CD133+ and CD44+ cells in clinical CRC samples. Immunofluorescence revealed broad CD133 expression from the crypt base to the epithelial cells in normal colonic mucosa. In contrast, CD44 expression was limited to cells between the crypt base and the transiently amplifying cell compartment (Figure 1B and C). Colorectal cancer samples presented high expression of CD44 throughout the mucosa. Some cells were also positive for CD133 (Figure 1D). This immunophenotype was also recapitulated in the xenografts (Figure 1E).

CD44+ and CD133+CD44− fractions are highly enriched in CSC xenografts. Flow cytometric analysis of the xenografts revealed that the proportion of CD44+ and CD133+CD44− cells was significantly higher in xenografts than in primary lesion (P = 0.014, P = 0.020; Figure 2A). No significant differences in the frequencies of other markers were found in comparisons between primary lesions and xenografts.

Expression of other stem cell–related markers, including LGR5, Musashi-1, CD166, ALDH1, EphrinB2 and EphrinB3, tended to be higher in xenografts than in the primary lesion; however, this difference was not statistically significant (Figure 2B).

Proliferation of the CD44+ fraction was significantly higher in xenografts than in primary lesions. The frequency of Ki67 positivity was significantly higher in the CD133+CD44− and CD133−CD44+ cells of xenografts than in cells from the primary lesions (Figure 2C). In contrast, the lack of Ki67 immunoreactivity in subsets of CD133+CD44− cells from both primary lesions and xenografts suggests that this population is non-cycling.

CD44 variant 2 is overexpressed in primary cancer and xenograft samples. The expression of CD44s and CD44v

| Pathologic factors | Tumourigenicity (n = 21) | No tumourigenicity (n = 42) | P-value |
|--------------------|--------------------------|-----------------------------|---------|
| Age, years (range) | 61.3 (40–85) | 68.0 (44–86) | 0.023 |
| Gender | | |
| Male/female | 12/9 | 28/14 | 0.58 |
| Differentiation | Well/moderately poorly/undifferentiated | 8/11/2/0 | 15/22/0/5 | 0.317 |
| Tumour location | Colon/rectum | 16/5 | 26/16 | 0.028 |
| Depth of invasion | T1/T2/T3/T4 | 0/3/12/6 | 4/4/24/10 | 0.492 |
| Lymph node metastasis | Negative/positive | 6/15 | 20/22 | 0.182 |
| Liver metastasis | Negative/positive | 17/4 | 37/5 | 0.466 |
| TNM stage | 1/2/3/4 | 1/5/11/4 | 5/14/18/5 | 0.718 |
| FACS sub-population (%) | CD133+CD44− | 1.26 | 1.64 | 0.356 |
| | CD133+CD44+ | 0.78 | 2.26 | 0.035 |
| | CD133−CD44+ | 16.65 | 21.7 | 0.275 |
| | CD133−CD44− | 51.9 | 55.0 | 0.656 |

Abbreviations: CRC = colorectal cancer; FACS = fluorescence-activated cell sorting; TNM stage = The TNM Classification of Malignant Tumours 7th edition.

Table 2. Tumourigenicity of CD133, CD44 and each of the combined sub-populations in NOD/SCID mouse xenografts

| Cell populations | 10^2 | 10^3 | 10^4 | 10^5 | 10^6 | Cancer stem cell frequency (95% CI) | P-value (vs total cells) |
|------------------|-----|-----|-----|-----|-----|-----------------------------------|-----------------------|
| Total cells | 2/5 (40%) | 13/19 (68%) | 16/21 (76%) | 6/8 (75%) | 1/39 318 (1/63 691–1/24 272) | — |
| CD133+ | 0/2 (0%) | 3/13 (23%) | 1/3 (33%) | — | 1/894/0 (1/26 657–1/2999) | 0.022 |
| CD133− | 0/1 (0%) | 0/1 (0%) | 3/16 (19%) | 2/3 (66%) | 1/64 145 (1/165 715–1/24 829) | 0.311 |
| CD44+ | 1/3 (33%) | 5/13 (38%) | 0/2 (0%) | — | 1/511/1 (1/143 317–1/1825) | <0.001 |
| CD44− | 0/4 (0%) | 1/5 (20%) | 2/13 (15%) | 0/2 (0%) | 1/108 249 (1/392 006–1/29 892) | 0.059 |
| CD133+CD44+ | 1/4 (25%) | 4/8 (50%) | 1/2 (50%) | — | 1/3160 (1/9518–1/1050) | <0.001 |
| CD133+CD44− | 0/6 (0%) | 0/4 (0%) | 0/2 (0%) | — | — | — |
| CD133−CD44− | 0/2 (0%) | 0/9 (0%) | 2/6 (33%) | — | 1/29 317 (1/116 179–1/73 938) | 0.703 |
| CD133−CD44+ | 0/2 (0%) | 1/5 (20%) | 1/5 (20%) | 0/1 (0%) | 1/74794 (1/409 064–1/13 676) | 0.338 |

Abbreviations: CI = confidence interval; CRC = colorectal cancer; NOD-SCID = non-obese diabetic–severe combined immunodeficient. Limiting dilution analysis of the repopulating frequency of each fraction of sorted cells or unsorted total cells from CRC in clinical specimens. The cells were injected subcutaneously into NOD/SCID mice. Tumourigenesis was evaluated by measuring the size of the xenograft with an external calliper every week up to 20 weeks after injection.
Figure 2. Comparison of cell sub-populations and gene expression in primary tumours and xenografts. The fraction of CD44⁺ and CD133⁻ CD44⁺ cells is expanded in xenograft tumours (*P = 0.014, **P = 0.020) (A). qRT–PCR analysis of normal colon mucosa, primary tumours and xenografts (n = 5). The expression was normalised to that of B2M. Stemness genes were overexpressed in the xenografts (B). Cell proliferation indices were estimated in the primary tumours and xenografts by Ki67 immunohistochemistry. Subsets of CD133⁺CD44⁺ and CD133⁻CD44⁻ cells in xenografts showed a significantly higher proliferative capacity than cells of primary tumours (n = 5, *P = 0.002, **P < 0.001). Scale bar, 50 µm (C). RT–PCR analyses of CD44v2 expression. All five sets showed a similar increasing trend of CD44v2 overexpression in primary tumours and xenografts in comparison with the normal mucosa. Note the variant 2-specific bands at 1.2 kb. L = ladder; N = normal mucosa; P = primary tumour; X = xenograft (n = 5) (D). The expression of standard total CD44, variant total CD44 and the individual exon-specific variants is shown in Supplementary Figure 1.
(v2–10) was compared across five patient-derived sets of normal mucosa, primary cancer tissue and xenografts. As expected, the expression of CD44s was observed in all normal tissues and primary cancers; however, expression was difficult to detect in xenografts. CD44v3–10 was expressed in all samples, including xenografts (Supplementary Figure 1B). CD44v2 was specifically overexpressed in the cancerous tissue, particularly in the primary tumours and xenografts, when compared with the normal mucosa (Figure 2D).

CD44v2 is a clinical prognostic factor in CRC. The profiles of 167 patients are shown in Table 3, and the Kaplan–Meier curves in relation to the TNM stage are shown in Figure 3. These samples did have expression patterns similar to those shown in the set of 63 samples in Table 1. A comparison of stem cell-related markers revealed that the expression of CD133, CD44v2, LGR5 (Figure 4A), EphB2 and Musashi-1 (data not shown) was significantly higher in cancerous lesions than in normal mucosa, whereas there was no difference in the expression of Bmi1 and NANOG (data not shown). We did not find any significant correlations among the expression levels of CD133, CD44v2, LGR5, EphB2 and Musashi-1.

The median value for the expression of each marker was determined as a cut-off value, and this threshold was used to divide the 167 patients into two groups of ‘high’ and ‘low’ expression. Overall survival was then compared between these two groups for each of the markers, and the results of the Kaplan–Meier analysis are shown in Figure 4B. The prognosis of the CD44v2high group (n = 84, 5-year survival rate (5-OS): 0.74) was significantly worse (P = 0.041) than that of the CD44v2low group (n = 83, 5-OS: 0.88). There were 32 deaths among the 167 patients in the cohort. Multivariate analysis revealed that CD44v2 was an independent prognostic factor for survival (Table 4). Stratification based on expression of CD133, LGR5, EphB2 and Musashi-1 was not a successful prognostic indicator (data not shown). Each SC marker was combined with each of the different markers and the OS of each group was calculated. The OS of patients in the CD133highCD44v2high group (n = 56, 5-OS: 0.77) was not significantly worse than that of patients in the other groups (n = 111, 5-OS: 0.83; Figure 4B). However, the prognosis of the CD44v2highLGR5high group (n = 48, 5-OS: 0.68) was significantly worse than that of the other groups (n = 132, 5-OS: 0.86). The CD133highCD44v2highLGR5high group (n = 35 5-OS: 0.68) also showed a worse prognosis than the other groups (n = 132, 5-OS: 0.84). No other combination of markers with either CD133 or CD44 was correlated with prognosis.

Table 3. Patients’ profiles (n = 167)

| Pathologic factors                  | n (frequency or range) |
|-------------------------------------|------------------------|
| Age, years                          | 65.4 (40–90)           |
| Gender                              |                        |
| Male                                | 92 (55%)               |
| Female                              | 75 (45%)               |
| Median follow-up period (months)    | 38.8 (0.8–70)          |
| Viability (5 years)                 |                        |
| Died                                | 32 (19%)               |
| Alive                               | 135 (81%)              |
| Tumour diameter, mm                 | 46.1 (13–130)          |
| Histological differentiation        |                        |
| Well                                | 51 (31%)               |
| Moderately                          | 98 (59%)               |
| Poorly                              | 8 (5%)                 |
| Undifferentiated                    | 10 (6%)                |
| Tumour location                     |                        |
| Colon                               | 93 (56%)               |
| Rectum                              | 74 (44%)               |
| Depth of invasion                   |                        |
| T1                                  | 16 (10%)               |
| T2                                  | 26 (16%)               |
| T3                                  | 69 (41%)               |
| T4                                  | 55 (33%)               |
| Lymph node metastasis               |                        |
| Negative                            | 89 (53%)               |
| Positive                            | 78 (47%)               |
| Lymphatic invasion                  |                        |
| Negative                            | 109 (65%)              |
| Positive                            | 58 (35%)               |
| Vascular invasion                   |                        |
| Negative                            | 62 (37%)               |
| Positive                            | 105 (63%)              |
| Liver metastasis                    |                        |
| Negative                            | 109 (65%)              |
| Positive                            | 58 (35%)               |
| TNM stage                           |                        |
| 1                                   | 32 (19%)               |
| 2                                   | 42 (25%)               |
| 3                                   | 35 (21%)               |
| 4                                   | 58 (35%)               |
| Adjuvant chemotherapy               |                        |
| Negative                            | 123 (74%)              |
| Positive                            | 44 (26%)               |
| 5-Year survival rate (%)            |                        |
| Stage 1                             | 100%                   |
| Stage 2                             | 95.20%                 |
| Stage 3                             | 88.60%                 |
| Stage 4                             | 55.20%                 |

Figure 3. Kaplan–Meier curves by TMN stage.
The influence of CD44v2 was analysed in relation to disease stage. In stage IV patients, the CD44 high immunophenotype showed a significantly poorer prognosis than the CD44 low group \( (n = 28 \text{ OS: } 0.36 \text{ vs } n = 30 \text{ OS: } 0.73; P = 0.03, \text{ respectively}) \).

There was no clear distinction based on marker expression in patients with stages I–III disease.

**DISCUSSION**

Here, we confirm that CD44 and CD133 are important CSC markers, and that CD44 is a significant predictor of tumourigenic potential and prognosis in primary CRC lesions. CD133 \(^+\) cells were previously identified as tumour-initiating (TICs) in CRCs using 17 clinical samples (O’Brien et al, 2007), which was supported by the finding that CD133 has a role in the tumourigenicity of CRC cell lines (Yang et al, 2011). However, whether CD133 is strictly required for tumour initiation is unclear, as a CRC-derived CD133 \(^+\)/CD0 cancer cell line was found to retain tumour-initiating potential (Navarro-Alvarez et al, 2010). CD44 is also an important determinant of CRC malignant behaviour. Expression of CD44 splice variants in CRC was suggested to be a marker of cancer malignant potential (Jackson et al, 1992; Screaton et al, 1992; Wielenga et al, 1993). However, studies of CD44 are also contradictory, as firm conclusions are hampered by nonspecific CD44 variant isoforms found in different tissue sources and in different laboratories (Al-Maghrabi et al, 2012; Ohata et al, 2012; Saito et al, 2013). Until this study, the importance of CD44v2 in cancer initiation and tumour cell maintenance was unclear. Here, we found that CD44v2 expression increased in xenograft tumours derived from clinical CRC specimens. However, our data do not diminish the importance of CD44v3 or v6, which are well-established markers in CRC. Future studies may identify new factors to improve the prediction of poor outcome. In tumourigenicity assays, CD44 was previously identified as a marker of stem-like TIC in colon cancer using 15 clinical samples (Chu et al, 2009). The CD133 \(^+\) CD44 \(^+\) phenotype has also been reported to be associated with a highly enriched population of cancer-initiating cells (Haraguchi et al, 2008) and may also represent a metastatic subset of CRCs (Chen et al, 2011). Here, we found that both CD133 \(^+\) cells and CD44 \(^+\) cells are significantly more tumourigenic than total (non-enriched) cells and CD44 \(^+\) cells. Indeed, 1 TIC was found for every 5111 cells (Table 2), a frequency higher than that observed for CD133 \(^+\) cells (1 out of 8940);
however, the difference was not statistically significant. CD133⁺ CD44⁺ cells were also highly tumourigenic (1 out of 3160) in comparison with CD133⁺ CD44⁻ cells, whereas CD133⁻ CD44⁺ cells were weakly tumourigenic (1 out of 29317). The significant increase in the fraction of CD44⁺ and CD133⁺ CD44⁺ cells in xenografts compared with primary tumours (Figure 2A) also underscores the important role of these sub-populations during tumourigenesis. Notably, the CD133⁺ fraction did not undergo any

### Table 4. Prognostic factors for overall survival of CRCs

| Variables                        | n   | Hazard ratio (95% CI) | 5-year OS (%) | P-value | Hazard ratio (95% CI) | P-value |
|----------------------------------|-----|-----------------------|---------------|---------|-----------------------|---------|
| **Univariate analysis**          |     |                       |               |         |                       |         |
| **Age**                          |     |                       |               |         |                       |         |
| <70                              | 107 | 1                     |               |         |                       |         |
| >70                              | 60  | 1.390 (0.685–2.820)   | 82.2          | 0.362   |                       |         |
| **Gender**                       |     |                       |               |         |                       |         |
| Female                           | 75  | 1                     |               |         |                       |         |
| Male                             | 92  | 1.092 (0.542–2.198)   | 81.3          | 0.8058  |                       |         |
| **Tumour diameter (mm)**         |     |                       |               |         |                       |         |
| <50                              | 96  | 1                     |               |         |                       |         |
| >50                              | 71  | 3.887 (1.798–8.403)   | 90.6          | <0.001  |                       |         |
| **Differentiation**              |     |                       |               |         |                       |         |
| Poorly/undifferentiated          | 18  | 1                     |               |         |                       |         |
| Well/moderately                  | 149 | 0.538 (0.207–1.400)   | 72.2          | 0.2043  |                       |         |
| **Depth of invasion**            |     |                       |               |         |                       |         |
| T1, T2                           | 42  | 1                     |               |         |                       |         |
| T3, T4                           | 124 | 1.190 (1.624–87.155)  | 92.6          | 0.0148  |                       |         |
| **Tumour location**              |     |                       |               |         |                       |         |
| Colon                            | 41  | 1                     |               |         |                       |         |
| Rectum                           | 125 | 1.262 (0.631–2.524)   | 80.5          | 0.8915  |                       |         |
| **Lymph node metastasis**        |     |                       |               |         |                       |         |
| Negative                         | 89  | 1                     |               |         |                       |         |
| Positive                         | 78  | 5.041 (2.176–11.678)  | 92.1          | <0.001  |                       |         |
| **Liver metastasis**             |     |                       |               |         |                       |         |
| Negative                         | 109 | 1                     |               |         |                       |         |
| Positive                         | 58  | 11.400 (4.660–27.886) | 94.5          | <0.001  |                       |         |
| **TNM stage**                    |     |                       |               |         |                       |         |
| 1/2                              | 74  | 1                     |               |         |                       |         |
| 3/4                              | 93  | 15.423 (3.675–64.721) | 97.3          | <0.001  |                       | <0.001  |
| **Adjuvant chemotherapy**        |     |                       |               |         |                       |         |
| Negative                         | 123 | 1                     |               |         |                       |         |
| Positive                         | 44  | 0.166 (0.196–1.323)   | 78.1          | 0.1576  |                       | <0.001  |
| **CD133 expression**             |     |                       |               |         |                       |         |
| Low                              | 75  | 1                     |               |         |                       |         |
| High                             | 92  | 1.556 (0.759–3.193)   | 84            | 0.2238  |                       |         |
| **CD44 expression**              |     |                       |               |         |                       |         |
| Low                              | 83  | 1                     |               |         |                       |         |
| High                             | 84  | 2.140 (1.013–4.522)   | 88            | 0.0462  |                       | 0.0155  |
| **LGR5 expression**              |     |                       |               |         |                       |         |
| Low                              | 82  | 1                     |               |         |                       |         |
| High                             | 95  | 1.541 (0.761–3.122)   | 84.2          | 0.2263  |                       |         |

Abbreviations: CI = confidence interval; CRC = colorectal cancer; LGR5 = leucine-rich-repeat containing G protein-coupled receptor 5; OS = overall survival.
obvious expansion. Although the CD133⁺ CD44⁺ immunophenotype has previously been associated with the most potent TICs in CRC (Haraguchi et al. 2008), we did not find any difference in their tumour-initiating potential when compared with CD44⁺ cells. Haraguchi et al. (2008) also reported that the CD133⁺ CD44⁺ population was not tumourigenic. Du et al. (2008) compared the tumourigenicity of cells in which either CD44 or CD133 was silenced by lentiviral delivery of shRNA. Specifically, knockdown of CD44, but not CD133, inhibited clonal outgrowth and blocked tumourigenicity. Together, these results indicate that CD44 + is a robust marker of CSCs.

Our current study provides a clinically relevant perspective, as the tumourigeness assay was performed exclusively with CRC cells derived from primary tumours. It has been suggested that cell lines do not recapitulate all aspects of primary tumours (O’Brien et al., 2007). Furthermore, most xenogeneic colon cancer models utilise cell lines, which have been selected for growth in culture and may therefore be more ‘homogenous’ than cells that constitute the primary tumour. Indeed, there is likely to be considerable heterogeneity both within and between tumours from different patients with CRC. In an attempt to more faithfully reproduce the process of tumour initiation from cancer-initiating cells, tumourigeness assays using clinical samples have been performed (Chu et al., 2009). However, this study had limitations, as the cells were first cultured as xenografts in mice. Therefore, CRCs in metastatic lesions or in xenografts that express the same marker show significantly different biological behaviour relative to the cells in the primary tumour. Thus, we suggest that modelling the disease using the strategy described in this report is a more effective experimental approach.

The fraction of CD133- or CD44-positive cells is higher in metastatic liver disease than in CRC (O’Brien et al., 2007; Bellizzii et al., 2012). Here, we found that the proportion of K67-positive cells in the CD133⁺ CD44⁺ and CD133⁻ CD44⁺ fractions was higher in xenografts than in primary lesions (Figure 2C). Thus, our study confirms that both CD133 and CD44 are robust CSC markers in primary CRC lesions.

In addition to CD44 and CD133, many other molecules have been proposed to be CSC markers, including LGR5, MSI-1, CD166, ALDH1, NANOg, BM11, ABCB1 and ABCG2 (Sanders and Majumdar, 2011). In the this study, the expression of LGR5, MSI-1, CD166, ALDH1, NANOg and Ephrin receptors tended to be higher in xenografts than in primary tumours and normal mucosa; however, this trend was not statistically significant (Figure 2B). We were thus unable to confirm the relevance of TIC markers other than CD133 and CD44.

There are contradictory reports regarding the prognostic significance of several CSC markers. For example, a high proportion of CD133⁺ cells is significantly associated with poor prognosis in some cases (Wang et al., 2009; Takahashi et al., 2011). However, one report suggested that CD133 overexpression is not associated with poor prognosis (Choi et al., 2009). Similarly, controversy exists regarding the utility of LGR5 and CD44 as prognostic markers (Horst et al., 2009; Saigusa et al., 2012). Combined expression of CD133 and CD44 is reportedly a prognostic indicator for disease-free survival in patients with CRC (Galizia et al., 2012). We found that expression of CD133, CD44 and LGR5 was significantly higher in tumours than in normal tissue (Figure 4A), whereas expression of NANOg and BM11 was equivocal. CD44 expression was an independent prognostic marker of poor OS, whereas expression of CD133, LGR5, EPHB2 and MSI-1 did not correlate with survival. Although the combination of CD133/CD44 was not a prognostic factor, the co-expression of CD133, CD44 and LGR5 was an independent prognostic factor. Despite the correlation in the latter case, the OS of the ‘poor prognosis’ group based on a CD133$h^+$CD44$h^+$LGR5$h^+$ phenotype was almost the same as that in patients expressing CD44$h^+$ alone.

In conclusion, the expression of CD44, particularly CD44 variant 2, is a well-established CSC and prognostic marker. Further research into the signalling pathways associated with CD44 upregulation will therefore provide a potential avenue for treatment and prognosis in patients with CRC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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