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Genome and Transcriptome Profiles of CD133-Positive Colorectal Cancer Cells

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Colorectal carcinomas (CRC) might be organized hierarchically and contain a subpopulation of tumorigenic, putative cancer stem cells that are CD133 positive. We studied the biological and genetic characteristics of such cells in CRC cell lines and primary tumors. Three CRC cell lines were sorted in CD133 positive and negative fractions. The respective genetic aberration profiles were studied using array comparative genomic hybridization (aCGH) and expression profiling. Tumorigenicity for each cellular population was tested by injection into nude mice. Additionally, we compared CD133+ and CD133− cells of 12 primary colorectal tumors using laser capture microdissection and aCGH. Three of five CRC cell lines displayed both CD133+ and CD133− cells, but tumorigenicity of these subfractions did not differ significantly and aCGH revealed essentially identical genomic imbalances. However, 96 genes were differentially expressed between the two populations. Array comparative genomic hybridization analysis after laser capture microdissection of CD133+ and CD133− areas in primary colorectal tumors revealed genetic differences in 7 of 12 cases. The use of cell lines for studying genomic alterations that define cancer stem cell characteristics, therefore, seems questionable. In contrast, CD133+ cells in primary cancer samples showed a unique genomic aberration profile. In conclusion, our data suggest that CD133 positivity defines a genetically distinct cellular compartment in primary CRC, which potentially includes tumor initiating cells. ***(Am J Pathol 2011, 178:1478–1488; DOI: 10.1016/j.ajpath.2010.12.036)***

Traditional models of carcinogenesis assert that cancer can originate in virtually any cell of a given tissue through a series of genetic events that promote cellular proliferation. Malignant transformation is the eventual result of increased cellular proliferation and inhibited apoptosis.[1] Regarding colorectal cancer (CRC), this process begins in epithelial cells lining the gastrointestinal tract undergoing sequential mutations in specific key genes including *APC*, *MYC*, *KRAS*, *P53,* and *SMAD2*.[2] These mutations, in concert with specific chromosomal aneuploidies drive the transition of functional colonic epithelia to dysplastic cells and finally to colorectal cancer. This process is called the "adenoma-carcinoma sequence."[3–5]

Until a few years ago, all neoplastic cells in a malignant neoplasm were considered to have tumorigenic potential, but recent findings suggested a hierarchy, hypothesizing that only a more or less rare population of cancer stem cells (CSCs) can in fact replenish a tumor.[6] CSCs were first described in human leukemia. Lapidot et al[7] demonstrated that human acute myeloid leukemia harbored a subfraction of cells exclusively capable of tumor initiation in severe combined immunodeficiency mice. More recently, compelling evidence suggested that a wide variety of solid tumors,[8–14] including colorectal cancers, also

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contain a tumor initiating fraction of CSCs.[15,16] It has yet to be investigated whether cancer stem cells exhibit the same chromosomal changes as the other cells in the tumor or present with a specific genetic makeup.

Supposedly, CSCs can be identified and enriched by staining for specific cell surface markers. One of the most frequently used markers is CD133, which has been implied as a marker for CSC in different tumor entities.[17,18] Originally described in the context of normal hematopoietic stem cells,[19] CD133 gained recognition as a marker for CSCs in medulloblastoma and glioblastoma,[13] and subsequently for tumors of epithelial origin, such as breast, lung, and pancreas.[20–22] *CD133*, also known as *PROM1* (*prominin 1*) or *AC133*, maps to chromosome 4p15 and codes for a 120 kDa transmembrane pentaspan protein. The precise function is still unclear. Studies of consanguineous pedigrees from India with retinal degeneration revealed a frame shift mutation in the *PROM1* gene in these individuals.[23] PROM1 is concentrated in the membrane evaginations at the base of the outer segment of rod photoreceptor cells. Therefore, it has been proposed that this protein has a role in establishing and/or maintaining certain plasma membrane protrusions, which is consistent with the apical membrane expression pattern in CRC cells.[24]

Recently, two groups identified two subsets of cells selected from colon cancer samples based on CD133 expression. In a series of studies, CD133-positive (CD133+) cells were shown to be capable of initiating tumor growth in murine xenograft models, while CD133-negative (CD133−) cells were not. Therefore, the authors concluded that the propagation of colorectal cancer depends on this small subset of CD133+ CSCs.[25,26]

However, this hypothesis was challenged by Shmelkov et al[27] who observed that CD133− cells isolated from colon cancer metastases were also able to initiate tumors in nonobese diabetic/severe combined immunodeficiency mice. Furthermore, the same group found that CD133 is not only expressed in CSCs but also in differentiated tumor cells.[27]

In summary, the exact role of CD133 as a CSC marker for colorectal cancers still remains elusive.[28,29] The goal of the present study was to investigate the biological role and in particular the genetic characteristics of CD133+ and CD133− cells in CRC cell lines and primary tumor samples. We analyzed isolated cell populations, both from CRC cell lines and primary tumors using array comparative genomic hybridization (aCGH) to determine whether CD133+ from CD133− cells exhibit distinct differences in their genomic aberration profiles.

Materials and Methods

Tissue Collection and Cell Lines

The five CRC cell lines (Caco-2, HCT 116, NCI-H508, LS174T, and HT-29) were purchased from the American Type Culture Collection (ATCC, Manassas, VA).

All cell lines were cultured in complete media (with fetal bovine serum) as recommended by ATCC. Paraffin–embedded tissues from diagnostic colon cancer samples were obtained from the archive of the Institute for Pathology, Paracelsus Medical University, Salzburg, Austria. The study was conducted in accordance with the regulation of the local ethics committee. All specimens were diagnosed according to the latest TNM classification by two board certified pathologists (see Supplemental Table S1 at *http://ajp.amjpathol.org*).

Immunohistochemistry and Immunofluorescence

Immunohistochemistry (IHC) and immunofluorescence were performed on 4-μm thick sections of formalin-fixed, paraffin-embedded tumor samples. Anti-CD133 rabbit monoclonal antibody (1:100 for IHC; 1:20 for IF; clone C24B9; Cell Signaling, Danvers, MA) and anti-CD133, mouse monoclonal antibody (1:40, clone AC133, Miltenyi Biotech, Bergisch Gladbach, Germany) were used as primary antibodies for CD133 detection. Slides were deparaffinized and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. Slides were subjected to citrate-based antigen retrieval (0.01 mol/L of citric acid, pH 6.0, for 5 minutes) in a pressure cooker (Keystone Manufacturing, Buffalo, NY) followed by slow cooling for 20 minutes, and incubation at 4°C for 12 hours with a primary antibody diluted in PBS containing 0.2% bovine serum albumin and 5% goat serum. Subsequently, the slides were washed in 1 × PBS. For immunofluorescence, the slides were incubated for 1 hour at room temperature with a goat anti-rabbit IgG-fluorescein isothiocyanate as secondary antibody (dilution 1:200; clone 4030-02; SouthernBiotech, Birmingham, AL). For IHC, the EnVision-Plus Kit (DAKO, Carpinteria, CA) with diaminobenzidine as chromogen was used for detection.

Flow Cytometry and Cell Sorting

Cells from CRC cell lines were detached using 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA) in PBS, counted with a hemocytometer and washed in 0.1% bovine serum albumin in PBS. Cells from the mouse xenografts were prepared using a protocol for preparation of single-cell suspensions.[30]

At least 500,000 cells (in 100 μL PBS/0.5% bovine serum albumin) were incubated with phosphatidylethanolamine-labeled mouse anti-human CD133 monoclonal antibody (1:10; clone AC133; Miltenyi Biotech) at 4°C for 30 minutes in the dark. Unstained cells and cells stained with an isotype control were used as reference. After the washing steps, labeled cells were analyzed by flow cytometry using a FACS-Calibur (BD Biosciences, San Jose, CA). A minimum of 20,000 membrane intact cells was recorded and analyzed with CellQuest Pro (BD Biosciences) or FloJo software (Tree Star Inc, Ashland, OR). CD133+ and CD133− cells were sorted by fluorescence-activated cell sorting for further experiments using a FACSAria II system and FACSDiva software. The staining protocol was chosen according to earlier studies with primary tumor material to allow direct comparison with the original CD133-related CRC literature.[25–27] An improved staining procedure has been published by us recently.[31]

aCGH from Flow Cytometry-Sorted Cells

After fluorescence-activated cell sorting (FACS), DNA was isolated from CD133+ and CD133− HT-29, Caco-2, and HCT 116 cells, and hybridized to aCGH arrays with genomically normal DNA as a reference following published protocols.[32] Briefly, 3 μg test DNA and 3 μg normal genetic reference DNA (Genomic DNA, Promega, Madison, WI) were differentially labeled with dCTP-Cy5 and dCTP-Cy3, respectively (Perkin Elmer, Waltham, MA). Genome wide analysis of DNA copy number changes was performed using Human Genome CGH Microarray Kit 105A (Agilent, Santa Clara, CA) with 21.7 Kb overall median probe spacing resolution according to the manufacturer's protocol version 6.0 (Agilent). Slides were scanned with microarray scanner G2505B (Agilent) and analyzed using CGH Analytics software 4.0.76.

Laser Microdissection or FFPE Block Punch Biopsy and aCGH

Five consecutive sections each (first and last section on glass slides, section two to four on membrane slides) were prepared from each of the 12 primary CRC cases exhibiting a CD133+ subfraction. The first and the fifth slide were stained for CD133 by IHC and used to guide the laser capture microdissection (LCM) performed on the sections two to four mounted on membrane slides. This approach ensured that IHC-positive cells were selectively dissected without compromising their DNA quality due to staining artifacts. CD133+ and CD133− tumor cells were selected and the glands were dissected using Arcturus XT (Arcturus Engineering Inc., Mountain View, CA) with a UV and IR laser. To rule out random genomic heterogeneity in the tumor samples we also performed punch biopsies (0.6 mm in diameter) from eight tissue blocks. We choose two areas (0.6 mm in diameter and approximately 5 mm between the two areas) in either the CD133+ or CD133− section and dissected the tissue with a needle (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). The tissue punches were placed in a tube containing 1 mL xylene. Proteinase K digestions were performed at 56°C. After DNA preparation using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) the samples were labeled with a Bioprime Array CGH Genomic Labeling Kit according to the manufacturer's instructions (Invitrogen). Briefly 200 ng test DNA and 200 ng normal genomic reference DNA were differentially labeled with dCTP-Cy5 and dCTP-Cy3, respectively (GE Health care, Piscataway, NJ). Genome wide analysis of DNA copy number changes was performed using SurePrint G3 Human CGH Microarray Kit 8 × 60K (Agilent) with 41.5 Kb overall median probe spacing resolution according to the manufacturer's protocol version 6.0 (Agilent).

Expression Array

One μg of total RNA of each FACS sorted CD133+ and CD133− cells (HT-29, Caco-2, and HCT 116) were labeled with Cy3, using a T7 RNA polymerase according to the manufacturer's protocol version 6.0 (Agilent), and hybridized to the 44 K oligonucleotide-based Whole Human Genome Microarray (Agilent). Microarrays were washed and processed using a G2565BA scanner. Data were quality controlled and extracted using Technologies' Feature Extraction (Agilent, version 9.1).

Transplantation of Cancer Cells and Tumorigenicity Assay

Each 2000 and 20,000 CD133± sorted cells (HT-29, Caco-2, and HCT 116) were resuspended in 50 μL of PBS after sorting, and cell aliquots were diluted in 1:1 with Growth Factor Reduced Matrigel Matrix (BD Biosciences) before subcutaneous injection into the flanks of athymic NCr-*nu/nu* (nude) mice (five mice per cell line and per CD133 fraction).

All mice were bred and housed in a pathogen-free environment and used in experiments in accordance with institutional guidelines at the Center for Cancer Research, National Cancer Institute, National Institutes of Health. All experimental procedures conducted in this study were approved by the Animal Care and Use Committee (National Institutes of Health). Tumor sizes were measured in two dimensions two times per week, and volumes were calculated using the formula for a rotational ellipsoid $v = \pi/6 \times a \times b^2$.[33] Mice were sacrificed once the tumor diameter had reached 2 cm in either length or width.

Tumors were removed and prepared for flow cytometric analyses.[30] A fraction of each specimen was also fixed in 10% neutral buffered formalin for histopathological examination.

Statistical Analyses

Differences between groups were estimated by Student's *t*-test and repeated measures analysis of variance analysis. Normality tests were done on the gene expressions for samples from each CD133+ or CD133− cell line using the Shapiro-Wilk test. After adjusting the *P* values for multiple comparisons using the false discovery rate method of Benjamini and Hochberg[34], no genes passed significance for non-normality. For multivariate analysis, possible factors correlating with CD133 IHC were identified by multivariate linear regression analysis. All differences were deemed significant when reaching the level of $P < 0.05$. For each cell line, we selected genes differentially expressed between CD133+ versus CD133− groups using Student's *t*-test with a threshold *P* value < 0.05 (using R version 2.10).

Results

CD133 Expression Pattern in Primary Colorectal Cancers and CRC Cell Lines

We first examined the expression and the topology of CD133 in tissue sections of primary human tumors using

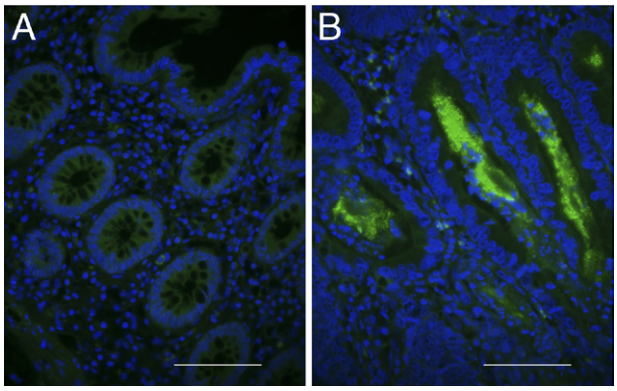

Figure 1. Immunohistochemistry of primary tissue sections from normal mucosa (**A**) and colorectal cancer (**B**) with an antibody against CD133 (fluorescein isothiocanate) and nuclear counterstaining (DAPI). While normal mucosa did not stain for CD133, a strongly CD133+ tumor (herein 85% CD133+ glands) shows apical luminal staining of the tumor cells and staining of intraglandular debris. Scale bars = 100 μm.

immunohistochemistry. Consistent with previous reports, CD133 expressing cells were located in the apical luminal surface, and/or in the intraglandular lumen (Figure 1).[35] Both antibodies (C24B9 and AC133) used for IHC showed comparable staining patterns supporting previous reports.[35] Twelve of 15 stained tumor samples (80%) showed CD133+ tumor cells with percentages ranging from 5 to 95% positive cells. The remaining three tumors showed no CD133 expression (see Supplemental Table S1 at *http://ajp.amjpathol.org*). CD133 positivity was not correlated to clinical parameters, including nodal status or TNM classification (data not shown). As a next step, we investigated whether the frequency of CD133+ cells in the primary tumors was maintained in CRC cell lines. Using flow cytometry under standardized serum supplemented conditions we found that three of five cell lines showed clear expression of the marker with both positive and negative CD133 populations. The proportion of cells above isotype with a clear CD133+ staining ranged from 9% ± 3 (HT-29), 62 ± 8 (HCT 116) to 80% ± 15 (Caco-2). LS174T and NCI-H508 did not contain a CD133+ fraction (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). With the exception of HT-29, these percentages are consistent with previous reports.[31,36,37] The differences observed for HT-29 are likely due to different culture conditions, frozen subclones, or the staining procedure.

After renewed, short-term culture of the different fractions isolated from HCT 116, the percentages of CD133+ versus CD133− cells changed only slightly and confirmed earlier observations.[31] However, in long-term cultures, we observed changes in the CD133 distribution throughout culturing (see Supplemental Figure S3 at *http://ajp.amjpathol.org*). Taken together, these results confirm the presence of CD133+ cell fractions in primary human colon cancers and CRC cell lines. The heterogeneity among the investigated samples indicated that CD133+ may potentially mark genetically or epigenetically distinct tumor populations and served as a basis for our subsequent analyses. The three CRC cell lines that contained positive fractions were used for subsequent experiments.

Tumorigenic Capacity of CD133+ and CD133− Cells after Xenotransplantation

The most important functional property ascribed to CSC is the capacity to initiate tumor growth *in vivo*. To examine whether the status of CD133 results in differences in tumor initiation *in vivo*, we transplanted flow sorted CD133+ and CD133− cells from three cell lines (HCT 116, Caco-2, and HT-29) subcutaneously into the flank of nude mice. For Caco-2, which is considered to be weakly tumorigenic,[38] only 2 of 20 injections resulted in a tumor after 8 weeks. All other injections failed to initiate tumor growth (observation time >5 months) (see Supplemental Table S2 at *http://ajp.amjpathol.org*). For HCT 116, all 20 injections resulted in a tumor after 3 to 4 weeks. There was no difference in tumor initiating capacity (P = 0.997) or growth rate

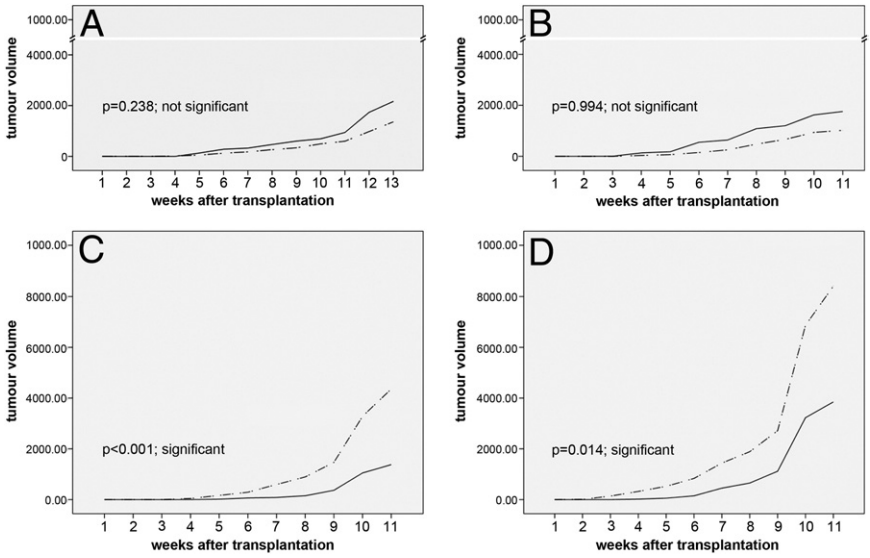

Figure 2. Evaluation of the tumor initiating potential of CD133+ (dotted line) and CD133− (solid line) cells of colon cancer cell lines on subcutaneous injection of cells into nude mice. Y-axis, tumor volume in mm^3. X-axis, time after injection in weeks. **A:** HCT 116, 2000 cells. **B:** HCT 116, 20,000 cells. **C:** HT-29, 2000 cells. **D:** HT-29, 20,000 cells.

($P = 0.238$; $P = 0.994$) between the two fractions in the athymic NCr-*nu/nu* indicating that for these two cell lines CD133 is indeed unable to discriminate between tumorigenic and nontumorigenic cells (Figure 2A and B, and Supplemental Table S2 at *http://ajp.amjpathol.org*).

For HT-29, 6 of 10 injections with CD133− and 9 of 10 injections with CD133+ cells resulted in tumor growth, again with no significant difference between the fractions ($P = 0.135$) (see Supplemental Table S2 at *http://ajp.amjpathol.org*). However, the CD133+ injected cells showed a faster tumor growth rate than the CD133− cells (Figure 2, C and D) ($P \leq 0.001$; $P = 0.014$), independent of the injected tumor cell numbers (2000 or 20,000).

Next, we examined whether the CD133 status of the injected cells was maintained in the xenografts by dissociating the tumors into single cell suspensions followed by staining for CD133 using flow cytometry. After injection and tumor formation the percentages changed: primary CD133+ injected cells were able to generate the corresponding negative cell fraction. The CD133+ cell fraction of HCT 116 showed only 29% ± 5 CD133+ cells after xenotransplantation, originally the cell line contained 62% ± 8 CD133+. The CD133+ cell fraction of HT-29 showed 49% ± 7 CD133+ cells after xenotransplantation, originally this cell line contained only 9% ± 3% CD133+ cells. These data indicate a high variability for CD133 expression in nude mice assays, probably caused by the changes in environmental conditions. Conversely, the CD133− cell population also showed an enrichment of CD133+ tumor cells (see Supplemental Figure S4 at *http://ajp.amjpathol.org*) after injection and tumor formation.

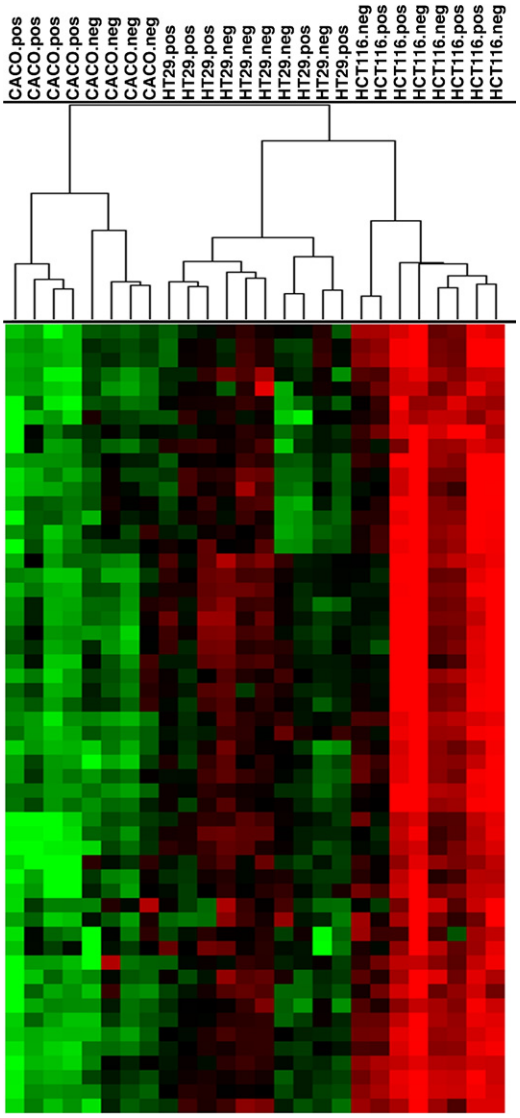

Figure 3. Unsupervised hierarchical clustering based on gene expression profiles of CD133+ and CD133− fractions from Caco-2, HCT 116, and HT-29 cell lines, data expressed using Euclidean metrics and the complete linkage algorithm. Samples cluster by cell line first. Only in the cell line Caco-2 were the different CD133 fractions clearly separated.

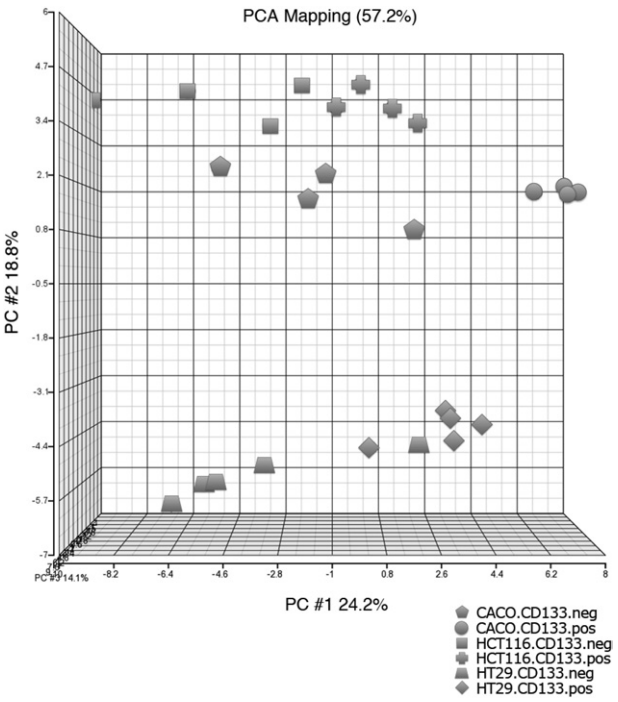

Figure 4. Supervised principal component analysis (PCA) of gene expression arrays using 96 genes up or down-regulated in CD133+ versus CD133− fractions for HCT 116 ($n = 4$), Caco-2 ($n = 4$), and HT-29 ($n = 5$). The supervised PCA shows a clear separation of CD133+ and CD133− along the first principal component for all samples, except one outlier (one HT-29 CD133− sample).

Gene Expression Profiles of CD133+ and CD133− Cells in HCT 116, Caco-2, and HT-29

Then, we went on to examine whether the CD133 status in our CRC cell lines would be reflected in specific gene expression profiles. To select those genes that are consistently differentially expressed between CD133+ and CD133− fractions, we built two intersection gene lists, with each intersection gene list retaining only those genes that are i) up-regulated in CD133+ fraction for the

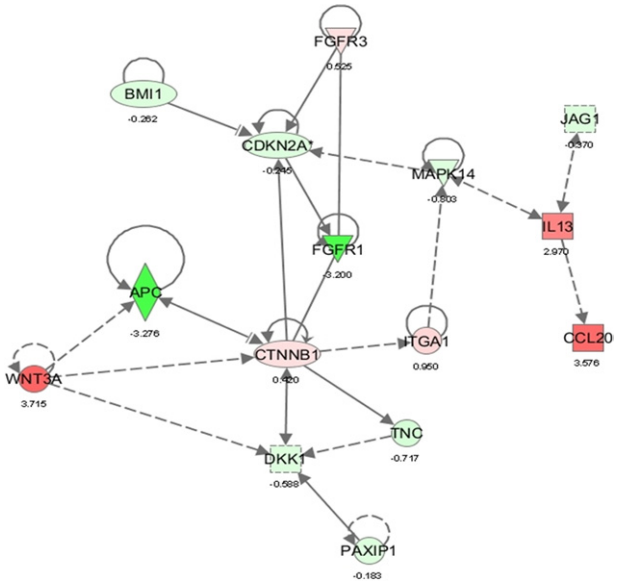

Figure 5. Network annotation of genes differentially expressed according to the CD133 status in HT-29 using ingenuity pathway analyses. Red, genes up-regulated in CD133+; green, genes down-regulated in CD133+. Dark red or green shade, genes with > threefold differential expression; light red or green shade, genes with lower difference in expression. All genes spotted were deregulated significantly ($P < 0.0001$).

three cell lines or ii) down-regulated in CD133+ fraction for the three cell lines.

We used a χ^2 goodness of fit test to evaluate if the intersection gene lists are larger than expected by random chance and found that the *P* value is highly significant (*P* value $< 2.2 \times 10^{-16}$). We identified 96 genes (86 up-regulated and 10 down-regulated) that were consistently differentially expressed between CD133+ and CD133− fractions for all three cell lines.

Reassuringly, *CD133* ranked among the most significant up-regulated genes (see Supplemental Table S3 at *http://ajp.amjpathol.org*). While the gene expression differences between the fractions were not as pronounced as the one from one cell line to another (Figure 3), a principal component analyses based on the 96 genes could differentiate the CD133+ and CD133− fractions (Figure 4).

The corresponding functional annotation of the 96 differentially expressed genes and their affiliation with specific genetic pathways was interrogated using the Ingenuity Pathway Analysis Software (Ingenuity Systems, Redwood City, CA) and revealed the genes up-regulated in CD133+ cells mapped to the pathways lipid metabolism, small molecule biochemistry, and cancer ($P < 0.05$).

Because of the growth rate differences observed for CD133+ versus CD133− in HT-29, we also performed an Ingenuity Pathway Analysis Software (Ingenuity Systems) analyses for these subsets, demonstrating that the stem cell proliferation pathway is enhanced in the CD133+ cells. Key players in this pathway are *APC*, *CTNNB1*, and *WNT3A* (Figure 5). This is in line with findings that *CTNNB1* was upregulated in CD133+ SW620 cells, thereby also explaining growth rate differences.[39]

aCGH from Flow Cytometry-Sorted Cells from Colon Cancer Cell Lines

To determine whether the observed changes in gene expression could be explained by distinct genomic aberration profiles, we performed aCGH from unsorted and sorted CD133+ and CD133− cells from HCT 116, HT-29, and Caco-2. We observed no differences among the fractions (Figure 6). These results imply that the specific transcriptional differences we observed were not attributable to differences in the underlying genomic aberrations.

Genetic Profiling of CD133+ and CD133− Populations in Primary Human Colorectal Cancers by Array CGH after LCM

Since our aCGH analysis of CRC cell lines did not reveal genomic differences between CD133+ and CD133− cells, we extended our experiments to tissue sections of primary tumor specimens. CD133+ crypts, identified using IHC, from 12 samples were microdissected with LCM and analyzed by aCGH.

All 12 cancer samples had chromosomal imbalances that were characteristic for colon cancer.[4] Copy number increases were most prominent on chromosomes 13 (67%), 7 (58%), 8q (50%), 1q (33%), and 20 (33%), and chromosomal losses on chromosomes 8p (50%), 18 (42%), 1p (42%), 15 (42%), and 4 (33%) (Table 1). We detected differences between the CD133+ and CD133− cell fraction in 7 of 12 (58%) cases. The remaining cases showed identical aberration profiles (Table 1). The significance of these findings was further substantiated by our control experiments, in which we extracted DNA from two randomly selected punch biopsies of tissue sections from eight patients (in four cases from CD133+ areas, and in another set of four from CD133− areas on the slides). In all cases, aCGH showed identical aberration profiles. The differences between the different CD133 fractions can therefore not be attributed to general sample heterogeneity.

The gain of 13q occurred in three of the seven cases with genetic differences (Table 1). We evaluated whether the presence of this unique difference between CD133+ and CD133− was higher than what would be expected by chance. For this analysis, we used a re-sampling method to compute a *P* value. For each patient we pooled the chromosome arms that are changed in either CD133+ or CD133− samples. Then we selected zero or one arm that is randomly assigned as being changed in CD133+ but not in CD133− cells. We also selected zero or one arm that is randomly assigned as changed in CD133− but not in CD133+. This was done in a way that ensures there are exactly 11 arms changed in one region but not in the other when summed across all seven patients (R script is available on request). This re-sampling approach resulted in a *P* value of 0.024 for the probability that the same gain or loss emerges as different between the two regions in three or more of the seven patients. These data indicate that CD133 in the majority of colo-

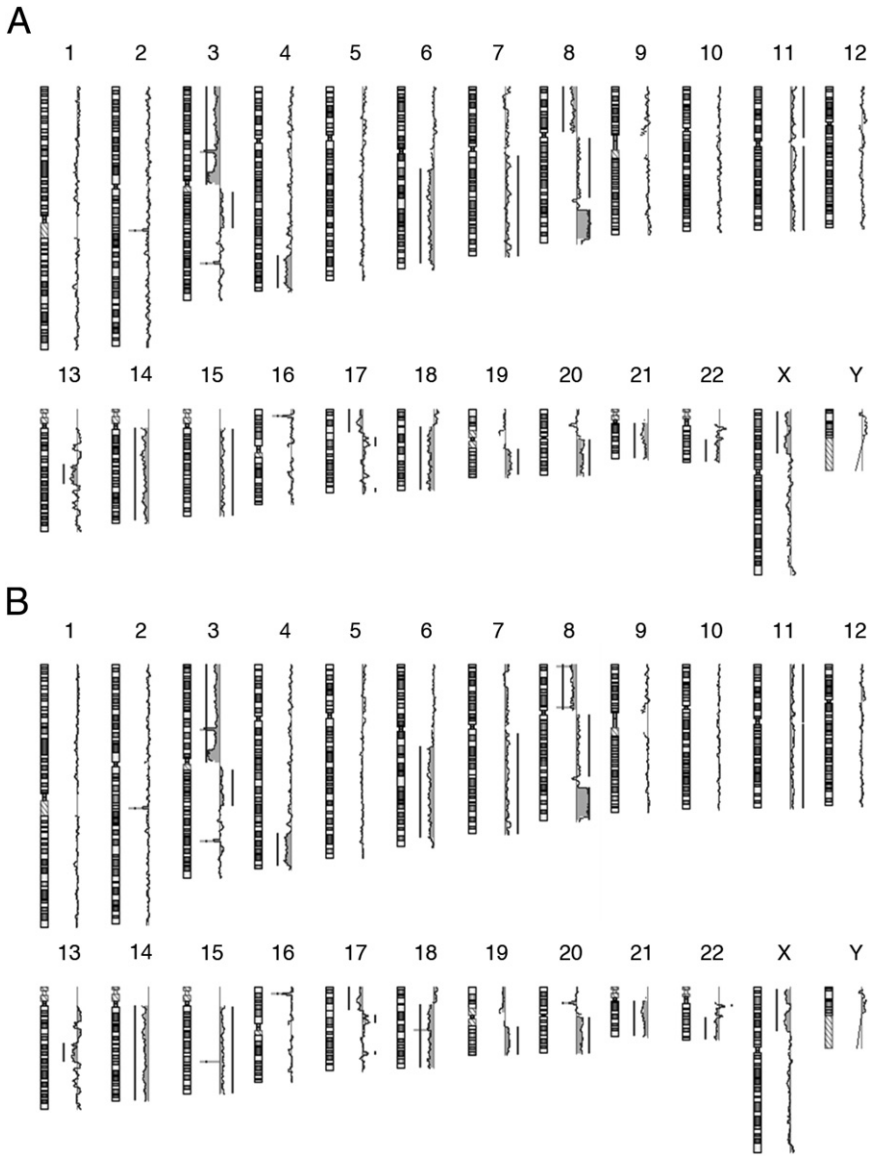

Figure 6. Ideogram of array comparative genomic hybridization chromosomal gains and losses for HT-29. (**A**) CD133+ and (**B**) CD133− cells. The aberration profile looked identical.

rectal cancer cases studied herein (58%) marked genetically distinct cell populations.

Discussion

Colorectal cancer is the third most common cancer in the United States resulting in approximately 50,000 deaths every year.[40] While curative surgical treatment is possible at early stages, the presence of synchronous metastases at the time of diagnosis dramatically worsens prognosis.[41,42] Adjuvant chemotherapy often leads to temporary remission but is frequently followed by disease recurrence. This could possibly be due to the fact that the majority of anti-cancer therapies are targeting rapidly dividing cells. In other words, conventional chemotherapies only target the transit amplifying and differentiated cells that form more than 99% of the tumor, yet spare the resting tumor initiating cells. CSCs, which are supposed to initiate new tumors, are slow cycling and are therefore less affected by anti-proliferative therapies.[43–45] In addition to chemotherapy resistance,[46] CSCs are often refractory to standard radiotherapy regimes.[47]

In colon cancer, CSCs were described as being contained in a fraction of cells positive for the surface marker CD133.[25,26] Furthermore, it has been reported that CD133+ CSCs increase in proportion after therapy.[43,45,48–50] It is tempting to speculate that residual colon cancer stem cells are responsible for loco regional recurrence, a hypothesis supported by the finding that indeed high levels of CD44 and CD133 expression were associated with poor prognosis in colorectal cancer.[51,52]

There is a pressing need to develop new therapies that can target this unique subpopulation of cancer cells. One of the first steps to achieve this would be a molecular characterization of these stem cells.

The maintenance of a recurrent pattern of chromosomal aneuploidy in the bulk of the tumor suggests that

Table 1. List of Genetic Alterations in Primary Colorectal Tumor Samples Depending on the CD133 Immunophenotype

Pt. number	Genetic alterations in all tumor cells	Δ CD133+	Δ CD133−
H12017/09	2+, 7+, 8+, 17p−, 17q+, X+	13q+	−
H14515/09	1p−, 3p−, 4−, 5q−, 6q−, 7q+, 8−, 9q−, 10−, 15q−, 17−, 18−, 21q−, 22q−	14q−	1q−
H21254/09	1p−, 4q+, 7+, 9p+, 13q+, 15q−, 16p−, 17−, 18−, 19−, 20+, 21q−, 22q−	8+	8p−
H35810/07	1p−, 4−, 5q−, 6−, 7+, 8p−, 8q+, 9−, 13q+, 14q−	10q−	5p+
H1676/08	7+, 8+, Y+	−	−
H12291/09	1q+, 18q+, Y+	−	−
H13103/09	5q−, 7q+, 8p−, 10p−, 11q−, 13q+, 14q−, 15q−, 18p−, 20q+, 21q−, 22q−	−	−
H25110/08	1p−, 4−, 8p−, 8q+, 15q−, 17p−, 18−	13q+	−
H25542/08	1p−, 1q+, 2q+, 4p−, 6q−, 7+, 9p−, 13q+, 15q−, 18−, 20q+	−	−
H32373/08	1q+, 2+, 3q+, 5q+, 7+, 9q+, 10+, 11+, 12+, 15q+, 16+, 20+	13q+	6q+
H33961/07	1+, 4q−, 7+, 8p−, 8q+, 13q+, 17−, 18q−, X−	−	−
H35810/07	8q+	−	9+

Δ CD133+, genetic alterations only observed in CD133+ tumor cells; Δ CD133−, alterations only observed in CD133− tumor cells; Pt. number, individual patient tumor histology number.

cells with this specific pattern of aberrations have a growth advantage. Therefore, one could also argue that this very pattern of aneuploidies originates in the stem cell compartment, and when present, converts a stem cell into a tumor stem cell. This hypothesis could be tested by analyzing the genomic aberration profile of suspected stem cell populations.

We found, in concordance with the literature, that not all CRC lines contained CD133+ cells. Of the five examined cell lines, three showed both populations.[31] The high percentage of CD133+ cells in HCT 116 and Caco-2, however, is somewhat surprising because stem cells were thought to be rare.[53] An explanation is that cell culture conditions provide a suitable milieu for amplifying cancer stem cells, and therefore artificially increase the stem cell compartment.

Our aCGH analyses that followed FACS for CD133± revealed an identical genomic aberration profile in HT-29, Caco-2, and HCT 116, arguing against a hierarchical organization based on different subclones. This is consistent with our observation that there were no differences in the tumorigenic potential of CD133+ versus CD133− subpopulations in the three cell lines.[31,54] We only noticed a subtly faster tumor growth rate for CD133+ cells versus CD133− cells in HT-29, which was also observed by Ieta et al.[54]

However, an increased tumor growth rate for the CD133+ HT-29 fraction is not an argument for stemness, because tumor initiation, the most important defining feature of stem cells, is not necessarily linked to proliferative activity. In addition, the morphology of tumors after xenotransplantation was identical regardless of the CD133 status.

Analyses of the xenografted tumors with flow cytometry revealed that CD133+ tumors had now gained a significant percentage of CD133− cells, while CD133− tumors acquired a certain number of CD133+ tumor cells, therefore reconstituting the biphasic distribution in the cell lines before sorting, possibly reflecting differences in the growth environment.

To investigate the molecular consequences of CD133 positivity, we performed global gene expression analyses of FACS fractions. This revealed an overexpression of genes involved in lipid metabolism in CD133+ cells. This finding is intuitive because PROM1 is associated with a cholesterol-based membrane microdomain in which PROM1 interacts directly and specifically with plasma membrane cholesterol.[55–57]

In conclusion, the *in vitro* results demonstrate a high variability of CD133 expression and do not support an association with stemness.

The results also indicate that established cell lines are most likely not appropriate to study stemness defining molecular features, and that such analyses require the use of primary human colorectal cancers, including recurring and metastatic tumors. This considerable amount of variability of CD133 expression could be possibly due to an adaptation of cells to tissue culture conditions. In three-dimensional cultures (spheroids) of primary tumors, such differences might not be as pronounced.

Then we approached the question as to whether CD133+ cells are different from negative fractions in primary colorectal carcinomas by combining IHC, LCM, and aCGH on tissue sections. The percentage of CD133 IHC-positive tumor cells in our FFPE-embedded CRC cases ranged from 0 to nearly 95% and revealed earlier findings.[35] Usually, several CD133+ tumor glands were grouped together with intervening CD133− glands.

In tumor glands with CD133+ tumor cells, the intraglandular cellular debris was always also CD133+. The amount of positive cells was considerably higher than the approximately 2 to 5% positive glands published by Ricci-Vitiani et al.[25] The cause of that discrepancy could be a different IHC staining procedure and embedding strategy. Ricci-Vitiani et al[25] used fresh frozen cryostat-sectioned material and not FFPE, which might affect IHC results.

After LCM of the CD133+ and CD133− tumor cells and aCGH of each fraction, we observed a genomic aberration profile in all 24 samples that was typical for

colorectal carcinoma.[58] Specifically, each of the 12 analyzed cancer samples showed recurrent losses (≥ 35%) of chromosomes 5q, 8p, 17p, 18p, 18q, and 20p, and recurrent gains (≥ 35%) of chromosomes 7p, 7q, 8q, 11q, and 20q.

While 42% (5 of 12) of the cases showed an identical aberration profile in the subfractions, we found different patterns in 58% (7 of 12) of the cases. To exclude that genetic differences in the subfractions were caused by polygenomic tumors without any association to a surface marker, we also performed independent aCGHs in the same tumor area. None of the performed control cases showed genetic differences within the chosen areas, thereby ruling out that the observed differences were attributed to random heterogeneity.

Our observations could be explained by the existence of different cell clones in primary tumors. The distribution of these clones does not appear to be coincidental, but defined by CD133 status. By showing a distinct aberration profile in CD133+ cells, we provide an explanation for the different properties of CD133+ cancer cells observed by Ricci-Vitiani et al.[25] and O'Brien et al.[26]

However, in 42% of the tumor samples, the genomic aberrations profile was identical in the two fractions, possibly suggesting that CD133 status does not identify a putative CSC fraction in all colorectal tumors. This has also been shown in breast cancer.[59] Therefore, it might be necessary to group colorectal tumors in two general classes of tumors, namely the monogenomic or polygenomic ones. It would be interesting to correlate this observation to therapy response and disease-free and overall survival.

Among the genetic alterations found, gain of 13q was detected in 3 of 7 cases (43%) that showed genetic differences between the CD133 positive and negative subfractions, thereby reaching a significant level. The gain of 13q is one of the major factors associated with the progression from colorectal adenoma to adenocarcinoma in chromosomal instable tumors.[60] It was also demonstrated that 13q gain correlates particularly with metastasis, hence underlining that this is an important genetic region.[61] Furthermore, it has been demonstrated that gain of 13q is associated with increased microRNA-17 to 92 cluster expression.[62]

Our results could now, for the first time, link this chromosomal aberration to cancer stem cells, which might have important implications for future therapeutic studies. Further studies in our laboratory will identify the role and function of candidate genes located on the chromosome of interest and potentially demonstrate an association with cancer stem cell pathways.

In summary, to the best of our knowledge, we were able to demonstrate for the first time that cancer stem cells, defined by the surface marker CD133, show a different genetic profile than the rest of the tumor cells in primary tumor samples.

While cell lines do not seem to be an appropriate model system for the characterization of cancer stem cells, CD133 expression in primary cancer samples was able to define a cell type that carries a specific aberration pattern in the majority of tumors investigated in this study. Given that not all cancer samples showed genetic differences in CD133± cells, it will be crucial to characterize the samples with gene expression and epigenetic assays. These data support the interpretation that CD133 positivity defines a tumor hierarchy, based on a distinct aberration profile, which could denote the tumor initiating cellular compartment.

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