Progenitor Marker CD133 mRNA Is Elevated in Peripheral Blood of Cancer Patients with Bone Metastases

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Abstract

Purpose: We examined whether RNA expression of CD133, a surface molecule expressed on progenitors from hematopoietic and endothelial lineages, and CD146, a pan-endothelial marker, are increased in the blood of cancer patients and whether these factors correlate with patient characteristics and are predictive factors of survival.

Experimental Design: We developed a real-time quantification method (nuclear acid sequence-based amplification) to determine expression of CD146 and CD133 mRNA in the peripheral blood mononuclear cells of 131 progressive cancer patients, 37 healthy volunteers, and 5 patients who received granulocyte colony-stimulating factor. Overall survival and other clinicopathologic variables were obtained. Cox proportional hazards studies were done.

Results: We show that patients with metastatic disease have a significant increase in CD133 mRNA ($P = 0.03$), specifically patients with bone metastasis ($P < 0.001$). Cancer patients with high CD133 mRNA expression, using a defined cutoff value, show a decreased survival compared with patients with low or undetectable CD133 expression (21% versus 45% cumulative survival, respectively, after 20 months; $P = 0.01$). Among patients with metastasis to the bone, cumulative survival was 22%, compared with 61% for patients with high or low CD133 levels ($P = 0.004$). Multivariate analysis showed that CD133 expression is an independent predictor for overall survival in patients with bone metastases. CD146 mRNA was not increased in patients with cancer, nor did it correlate with clinical variables or survival.

Conclusion: CD133, but not CD146, mRNA expression is increased in cancer patients with metastatic disease, specifically with bone metastasis. In addition, CD133 mRNA expression seems to be an independent prognostic factor for overall survival.

Blood vessel growth is critical for the progression and spread of tumors. Tumor vessel growth can arise by sprouting of preexisting vessels or by incorporation and differentiation of bone marrow–derived stem cells into angiogenic vessels. Several studies suggest that these processes may be monitored in the peripheral blood by the quantification of endothelial cells and endothelial progenitor cells.

Circulating endothelial cells are increased in patients with progressive cancer (1–4). These circulating endothelial cells may originate from the bone marrow or may be shed from the tumor vasculature. Preclinical data show a significant increase of mature circulating endothelial cells in the blood of tumor-bearing mice and a correlation of circulating endothelial cell numbers with tumor size (5). Circulating endothelial cell levels can be altered by chemotherapeutic and antiangiogenic treatment (6, 7). The bone marrow–derived endothelial progenitor cells express stem cell and endothelial markers, and can be recruited by hypoxia and cytokines (8–12) and subsequently incorporate into sites of active neovascularization (11, 13, 14). Experiments with endothelial progenitor cells in mice indicate that these cells contribute to tumor angiogenesis and that inhibition of endothelial progenitor cell mobilization results in retardation of tumor growth (6, 12, 15–18). Recent data also indicate that hematopoietic bone marrow–derived progenitor cells facilitate metastasis by initiating the premetastatic niche (19).

Therefore, circulating endothelial cells, and bone marrow–derived hematopoietic and endothelial progenitor cells, are being evaluated as surrogate markers of blood vessel growth, tumor progression, and response markers for antiangiogenic therapy in clinical trials (20–22). The clinical relevance of increased endothelial progenitor cells in human tumor angiogenesis is, however, still controversial. First, there are no reports of increased endothelial progenitor cells in the peripheral blood of patients with solid tumors, although an increase has been

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reported in patients with myelodysplastic disorders (23), multiple myeloma (24), and infantile hemangioma (25). Second, increased endothelial progenitor cells have not been implicated as a predictor of poor prognosis in patients with cancer. Nevertheless, a recent study with six cancer patients indicated that endothelial progenitor cell do contribute to tumor neoangiogenesis. Bone marrow–derived progenitor cell differentiation into tumor endothelium was found to vary from 1% to 12% (14). This study indicates that there may be large differences in the contribution of endothelial progenitor cells in human cancers and that xenograft models may overestimate the role of endothelial progenitor cells in tumor neovascularization.

We hypothesized that increased CD133 mRNA or CD146 mRNA expression in the peripheral blood compartment correlates with patient survival. To test this hypothesis, we developed a real-time mRNA quantification assay, based on nucleic acid sequence-based amplification (NASBA) technology for high-throughput analysis of CD133, a marker of bone marrow–derived precursor cells and of CD146, a pan-endothelial cell marker. This method is highly reproducible and can be done on samples that have been frozen and therefore may be a good alternative for the flow cytometry (1, 5–7, 23, 25–27) and magnetic bead assays (3, 28–31) for monitoring circulating endothelial cell, and hematopoietic and endothelial progenitor cell levels in clinical trials.

We quantified these markers in the peripheral blood mononuclear fraction of 131 patients with solid tumors and 37 healthy controls. Our results show a significant increase of CD133 mRNA in patients with metastasis to the bone, regardless of their primary tumor. We further observed that CD133 mRNA expression correlates with clinical characteristics and high expression is associated with a greater risk of death.

Patients and Methods

Characterization of study patients and healthy volunteers. Between February 2003 and August 2005, blood samples were collected from new or relapsed patients visiting the outpatient clinic of the Department of Medical Oncology, or patients hospitalized at the University Medical Center in Utrecht, the Netherlands.

Tumor types are summarized in Table 1. In addition, five healthy subjects, who volunteered as bone marrow donor and were receiving granulocyte colony-stimulating factor, were included as *in vivo* positive controls for CD133 mRNA (32). Blood samples were also acquired from six cancer patients before, during, and after infusion of gemcitabine and cisplatin (0, 2, 4, 8, 24, and 48 hours) for *in vivo* positive controls of CD146 mRNA. A previous study revealed that infusion of gemcitabine and cisplatin increased the numbers of CD146-positive cells found in peripheral blood.[4] All prostate cancer patients had hormone refractory disease and were on maintenance hormonal therapy. Other cancer patients were either naïve for cancer treatment or did not receive chemotherapeutic or radiotherapeutic treatment for at least 1 month before blood collection. Healthy subjects of similar age (n = 37) served as controls.

The following variables were obtained from the medical records of the 131 patients: sex, age, previous antitumor treatment, location of metastasis (by radiological tests and physical examination within 1 month of blood draw), hemoglobin and leukocyte levels, platelet count, alkaline phosphatase, lactate dehydrogenase, and prostate-specific antigen levels. Patient survival was noted after a median observation period of 16 months (range 3-33 months).

Table 1. Characteristics of 131 untreated cancer patients

Age (y)	
Mean ± SD	63.5 ± 9.8
Range	31-88
Sex	
Male	107 (82%)
Cancer patients	
Renal cell carcinoma	43 (34%)
Prostate cancer	34 (26%)
Head and neck cancer	27 (21%)
Colorectal cancer	27 (21%)
Metastasis	
No	14
Yes	111
Bone	50 (38%)
Liver	27 (21%)
Lung	37 (28%)
Lymph node	36 (28%)
Other	28 (20%)
Unknown	6
Survival (mo)	
Median	12.5
Interquartile range (25-75%)	5.3-27.6

Blood collection and isolation of nucleic acids. Peripheral blood mononuclear cells (PBMC) were isolated from blood of patients by using a Vacutainer CPT Cell Preparation Tube with sodium citrate (Becton Dickinson, Mountain View, CA), according to the recommendations of the manufacturer. The mononuclear cells were washed once in serum-free RPMI medium (Life Technologies Invitrogen, Breda, the Netherlands) and resuspended in 520 μL serum-free medium. Fifteen microliters of PBMC in medium were diluted 5-fold and analyzed using the Cell Dyn 1700 (Abbott Laboratories, Abbott Park, IL) to establish WBC counts, and RBC and platelet contamination. PBMCs (500 μL) in medium were added to 4,500 μL L6 lysis buffer (containing guanidine thiocyanate, Triton X-100, EDTA, and Tris-HCl), aliquoted per milliliter, and stored directly at −80°C until further use.

For nucleic acid isolation, we added ~300,000 mononuclear cells from the lysed PBMC solution and filled the volume to 1 mL with lysis buffer. The nucleic acid now present in the lysis buffer was further purified with the method described by Boom et al. (33). The isolated nucleic acid was eluted in 50 μL elution buffer. Usually, a dilution was made such that the equivalent of 10,000 cells/5 μL was used as input in NASBA amplification reactions.

Nucleic acid sequence-based amplification. For the quantification of CD133 and CD146 mRNA, we used a one-tube, real-time detection and quantification method based on NASBA (34–36). For patient samples, U1A DNA was also amplified as internal control for the cell input, amplification, and isolation for each sample.

Standard NASBA nucleic acid amplification reactions were done in a 20 μL reaction volume and contained the following: 40 mmol/L Tris (pH 8.5), 90 mmol/L KCl, 12 mmol/L $MgCl_2$, 5 mmol/L dithiotreitol, 1 mmol/L deoxynucleotide triphosphate (each), 2 mmol/L rNTPs (each), 0.2 μmol/L primer P1 (Invitrogen, Breda, the Netherlands), 0.2 μmol/L primer P2 (Invitrogen), 0.05 μmol/L molecular beacon (Eurogentec, Maastricht, the Netherlands), 375 mmol/L sorbitol, 0.105 μg/μL bovine serum albumin, 6.4 units AMV RT (Seikagaku, Chiyoda-ku Tokyo, Japan), 32 units T7 RNA polymerase (Invitrogen), 0.08 units RNase H (Life Technologies Invitrogen), and input nucleic acid. For the amplification of RNA, the mixture (without the enzymes) was heated to 65°C for 3 minutes to denature any secondary structure in the RNA and to allow the primers to anneal. In the case of DNA, 2 units of *Msp*I

[4] Unpublished result.

(40 units/μL, Roche Diagnostics Nederland BV, Almere, the Netherlands) were added and the mix was incubated at 37°C for 15 minutes, followed by denaturation at 95°C for 3 minutes. After cooling the mixtures to 41°C, the enzymes were added. The amplification took place at 41°C for 90 minutes in a thermostated fluorimeter (RetinAlyzer, Primagen, Amsterdam, the Netherlands). The molecular beacons used in these experiments are labeled with 6-fluorescine (fluorescent label) at its 5′ end and with {[4-(dimethylamino)phenyl] azo}benzoic acid (quencher) at its 3′ end. Primer and beacon sequence of CD146, CD133, and U1A are shown in Table 2.

The reaction mixtures were excited at 485 nm, fluorescence was measured at 530 nm, and the fluorescent signal of the molecular beacon probe was measured every 45 seconds. Readings were normalized to the background of a reaction mixture containing TE (10 mmol/L Tris and 1 mmol/L EDTA) instead of template.

The amount of target sequence present in samples was calculated using a standard curve generated from *in vitro* RNA (CD133 and CD146) or plasmid DNA (U1A) standards that indicated the relation between time to positivity and input amount. The standards were used in serial dilutions ranging from 5×10^5 to 67 copies (CD133), 1×10^6 to 100 copies (CD146), or 3×10^5 to 500 (U1A) copies per reaction mixture. Human umbilical endothelial cells and the immortalized human microvascular endothelial cell-line (HMEC-1; ref. 37) were used as positive control for CD146 mRNA. The NT-2 teratocarcinoma cell line (38) was used as a positive control for CD133 mRNA. The technical lowest detection for the CD133 assay (67 copies *in vitro* RNA) corresponds to 25 spiked NT-2 cells. The minimal technical detection for the CD146 assay (100 copies *in vitro* RNA) corresponds to 33 NT-2 cells.

All amplifications were done in duplicate. The average of these duplicate amplifications was considered as the value for the sample. The concentration of target sequence in the samples was expressed as log copies per reaction mixture. If the difference between duplicate amplifications was >0.5 log value, the amplification for that sample was repeated.

Cell culture and spike experiment. The NT-2 teratocarcinoma cell line was cultured in RPMI medium (Life Technologies Invitrogen), supplemented with penicillin, streptomycin, glutamate, and 5% FCS. HMEC-1 was cultured with M199 (Life Technologies Invitrogen), supplemented with penicillin, streptomycin, glutamate, 10% FCS, and 10% human serum. Cells were grown in 75 cm^2 flasks to confluence, trypsinized, and split 1:3. Three days before the flow cytometry experiments, the medium was replaced with phenol-free medium to reduce autofluorescence.

For the spike experiments, NT-2 and HMEC-1 cells were resuspended as single cells in PBMCs and quantified using a Bűrker-Turk hemacytometer. For the NASBA experiments, 0, 10, 30,100, 300, or 1,000 cells were spiked in 100 μL serum-free RPMI medium containing 200,000 PBMCs. Subsequently, cells were lysed in 900 μL L6 buffer and frozen at −80°C.

For flow cytometry, an equivalent of the spiked cells in the NASBA assay were added to 5×10^5 or 1×10^6 cells. Two hundred thousand events were acquired for analysis (FACSCalibur, Becton Dickinson, San Jose, CA). For NT-2 and HMEC-1 spike experiments, the CD133-2 APC conjugated antibody (Miltenyi Biotec, Bergisch-Gladbach, Germany) and the P1H12 phycoerythrin-conjugated antibody (Chemicon, Temecula, CA) were used. The results were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Circulating endothelial cell quantification. For circulating endothelial cell isolation, 0, 10, 30, 100, or 300 HMEC-1 cells were spiked in 1 mL peripheral blood and diluted with 3 mL physiologic salt. The isolation of circulating endothelial cells from whole blood was done as previously described (3, 28, 30, 31, 39). Briefly, magnetic beads (Dynal M450 IgG1, Dynal AS, Oslo, Norway) conjugated to an anti-CD146 monoclonal antibody (Kordia Life Sciences, Leiden, the Netherlands) bind CD146-expressing cells, which allows magnetic separation from unbound, CD146-negative cells. Viable, CD146-positive cells can subsequently be quantified by counting CalceinAM (Molecular Probes, Eugene, OR) fluorescently labeled cells in a Nageotte hemacytometer.

Statistical analysis. Median copy numbers of CD133 and CD146 mRNA and interquartile ranges were compared between patients with different cancer types and controls. The same was done comparing median copy numbers between patients with and without metastases at different sites, and patients of different sex. Differences were tested using the Mann-Whitney test.

To estimate correlations between CD133 and CD146 mRNA copy number and age, leukocyte and hemoglobin levels, platelet count, alkaline phosphatase, lactate dehydrogenase, and prostate-specific antigen, the Spearman's correlation coefficient was used.

A receiver operating characteristic was constructed by calculating the proportion of positive tests among those who died ("sensitivity") and the proportion of positive tests among those who survived ("1 − specificity") for each cutoff point of CD133 levels. The cutoff point with the combination of highest sensitivity and specificity was 200 copies (sensitivity and specificity of 64% and 71%, respectively, in patients with bone metastasis). This cutoff point was used in all subsequent analyses.

Differences in survival between cancer patients with CD133 levels below and above 200 copies were evaluated according to the method of Kaplan and Meier. Kaplan-Meier curves were truncated when fewer than five patients are left at risk in one or both arms of the curve. Differences in Kaplan-Meier curves were tested using the log-rank statistic.

To identify independent factors influencing survival, univariate and multivariate risk factor assessments were done using the Cox proportional hazards model. This was first done for the total group of patients and then for patients with bone metastases only. Hazard ratios with their corresponding 95% confidence intervals were estimated. Variables that showed P values <0.10 in the univariate analysis were selected and entered into a multivariate model.

The laboratory variables hemoglobin, leukocyte, and platelet count were not determined in four patients, whereas lactate dehydrogenase and alkaline phosphatase were not determined in 39 and 27 cases, respectively. Correlative testing and Cox proportional hazards analysis for these variables were done with fewer than 131 cases.

All analyses were done using SPSS software (version 12.0.1) and $P < 0.05$ (two-sided) was considered statistically significant. All error bars depicted are SEs of the mean.

Table 2. Primer and beacon sequences

Name	Primer	Sequence 5′-3′
CD133	P1	*AATTCTAATACGACTCACTATAGGG* AA-GAACAGGGATGATGTTGGGTCTCA
	P2	TTTCAAGGACTTGCGAACTCTCTTGA
	MB	**CGATCC**AAGGACAAGGCGTTCACA**GGATCG**
CD146	P1	*AATTCTAATACGACTCACTATAGGG* AGAG-GGGCTAATGCCTCAGATCGATGTA
	P2	CCCCCGTCTCGTAAGAGCGAA
	MB	**CGATCC**TTAAGTCAGATAAGCTCCC**GGATCG**
U1A	P1	*AATTCTAATACGACTCACTATAGGG* AGAGGC-CCGGCATGTGGTGCATAA
	P2	TGCGCCTCTTTCTGGGTGTT
	MB	**CGCATGC**TGTAACCACGCACTCTCCTC**GCATGCG**

NOTE: The T7 promoter moiety of primer P1 sequences is shown in italics, the stem sequences of the molecular beacon probes are shown in bold.

Results

Validation of CD133 and CD146 real-time mRNA amplification assay. *In vitro* translated RNA of CD146 and CD133 cDNA was added at different concentrations to a background of

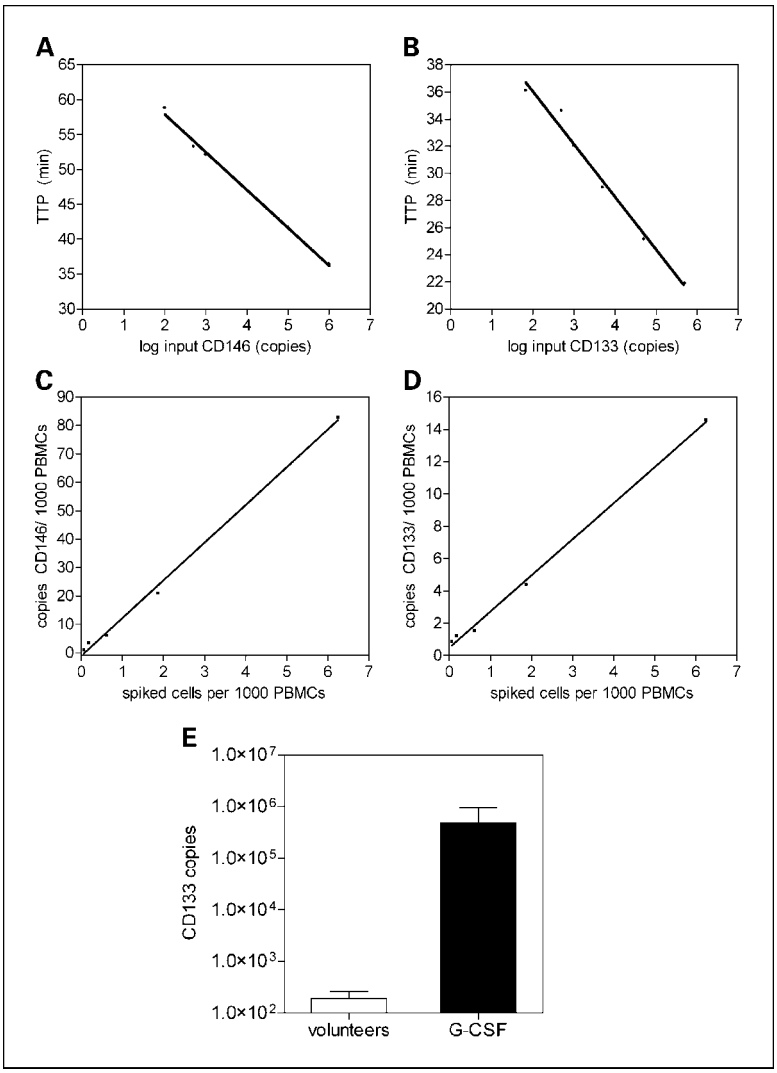

Fig. 1. Validation of CD133 and CD146 real-time mRNA amplification. CD133 (*A*) and CD146 (*B*) standard curve with *in vitro* translated RNA. NT-2 cells (*C*) or HMEC-1 (*D*) cells spiked in a background of PBMCs. CD133 mRNA measured in PBMCs of healthy volunteers (n = 37) and healthy subjects treated with granulocyte-colony stimulating factor (*G-CSF*; n = 5), Mann-Whitney test, P = 0.0001 (*E*).

nucleic acids from mononuclear cells to achieve a standard curve for input in relation to time to positivity (Fig. 1A and B). The sensitivity and linearity of the CD133 assay was tested using NT-2 teratocarcinoma cells spiked in a background of human PBMCs (Fig. 1C). There was a strong linear correlation between input and copies CD133 measured (r = 0.99, P < 0.001) in PBMCs. The sensitivity of the test was ~0.05 cells per 1,000 mononuclear cells or 50 cells/mL peripheral blood (assuming ~1 × 10^6 lymphocytes and monocytes per milliliter of peripheral blood). The sensitivity and linearity of the CD146 assay was determined by measuring HMEC-1 cells spiked in a background of human PBMCs. The CD146 assay had a strong linear correlation between input and CD146 copies measured (r = 1.00, P < 0.001; Fig. 1D). The sensitivity for the CD146 assay was ~0.05 cells per 1,000 PBMCs or 50 cells/mL peripheral blood.

Next, we compared the sensitivity of CD146 and CD133 mRNA quantification to other commonly used detection assays, such as flow cytometry and the magnetic beads assay. NT-2 cells were quantified by flow cytometry and NASBA after they were spiked in a background of human PBMCs. The sensitivity of both assays was comparable (0.18 cells per 1,000 PBMCs for the NASBA assay and 0.15 cells per 1,000 PBMCs for flow cytometric quantification). We found very strong correlation between quantification of spiked cells using NASBA and flow cytometry (r = 0.96, P < 0.001).

HMEC-1 cells were spiked in peripheral blood or PBMCs and were quantified subsequently by magnetic bead extraction, fluorescence-activated cell sorting, and NASBA. There was a strong correlation between endothelial cell quantification using magnetic bead isolation and NASBA (r = 0.96, P < 0.001), and between fluorescence-activated cell sorting analysis and NASBA (r = 0.99, P = 0.005). Using magnetic bead isolation, the lower limit of detection is ~10 spiked endothelial cells per milliliter of blood, which is ~15-fold more sensitive than NASBA and flow cytometry.

In vivo *validation of real-time mRNA amplification assays.* To assess our assay *in vivo*, we analyzed CD133 expression in the PBMCs of five healthy subjects who volunteered as bone marrow donor and had received granulocyte-colony stimulating factor for stem cell mobilization (Fig. 1E). There was a 2,500-fold increase in CD133 mRNA expression in the PBMCs of the bone marrow donors compared with healthy controls (P < 0.001).

Table 3. CD133 RNA copies according to tumor type and site of metastasis

	Reference	Median CD133 copy number (IQ range of tumor type)	Percentage of subjects with CD133 copy > detection limit	Median CD133 copy number (IQ range of reference)	Percentage of subjects with CD133 copy > detection limit	Comparison of CD133 copies between tumor type and reference, *P* (MW)
Tumor type						
All cancers (*n* = 131)	Controls (*n* = 37)	0 (0-265)	45%	0 (0-281)	38%	0.40
RCC (*n* = 43)	Controls (*n* = 37)	0 (0-270)	49%			0.45
Prostate (*n* = 34)	Controls (*n* = 37)	153 (0-1,088)	61%			0.03
H&N (*n* = 27)	Controls (*n* = 37)	0 (0-208)	37%			0.94
CRC (*n* = 27)	Controls (*n* = 37)	0 (0-76)	37%			0.62
Site of metastases						
All metastases (*n* = 110)	No metastases (*n* = 14)	53 (0-323)	51%	0 (0-15)	20%	0.03
Bone (*n* = 50)	No bone (*n* = 81)	163 (0-721)	63%	0 (0-41)	37%	0.0002
Liver (*n* = 27)	No liver (*n* = 103)	0 (0-76)	37%	28 (0-310)	50%	0.09
Lung (*n* = 37)	No lung (*n* = 93)	0 (0-197)	41%	18 (0-435)	50%	0.96
Lymph (*n* = 36)	No lymph (*n* = 94)	0 (0-584)	42%	0 (0-243)	49%	0.15
Other (*n* = 20)	No other (*n* = 110)	0 (0-297)	35%	0 (0-245)	49%	0.98

Abbreviations: RCC, renal cell carcinoma; H&N, head and neck cancer; CRC, colorectal cancer; IQ, interquartile range (25-75%); MW, Mann-Whitney test.

To evaluate the CD146 assay *in vivo*, we isolated CD146-positive endothelial cells from six patients receiving gemcitabine and cisplatin using an antibody against CD146 bound to magnetic beads, and quantified all viable endothelial cells by counting calcein-AM–positive rosetted bead-bound cells under a fluorescence microscope. We subsequently measured CD146 mRNA expression in the PBMCs and found a significant correlation (r = 0.89, P = 0.019) between these two methods.

Bone metastasis is a determinant for high CD133 expression. The mononuclear fraction of peripheral blood from 131 cancer patients and 37 volunteers was tested on CD133 mRNA, CD146 mRNA, and U1A DNA as internal reference for cell input, amplification, and isolation. Levels of CD133 and CD146 were detectable in 45% and 16% of the cancer patients and in 38% and 22% of the controls, respectively. There was no significant difference in expression of CD133 or CD146 mRNA between the total cancer patient group and the controls (P = 0.395 and P = 0.311, respectively). However, when analyzed according to tumor type, patients with prostate cancer had statistically significantly increased CD133 mRNA levels compared with healthy controls (median 154 copies with interquartile range 0-1,088 and median 0 copies with interquartile range 0-281, respectively; P = 0.033). Patients with renal cancer, head and neck cancer, and colorectal cancer did not have an increase in CD133 expression when compared with healthy controls (CD133 results are shown in Table 3). Further analysis revealed that bone involvement was related to high CD133 levels in the peripheral blood. Patients with bone metastasis (n = 50) had significantly elevated CD133 levels compared with patients without bone metastasis (median 163 copies with interquartile range 0-721 and median 0 with interquartile range 0-41, respectively; P = 0.0002). As most patients with bone metastasis were prostate cancer patients (n = 32), we also analyzed patients with renal cell carcinoma, head and neck carcinoma, and colorectal cancer with metastasis to the bone

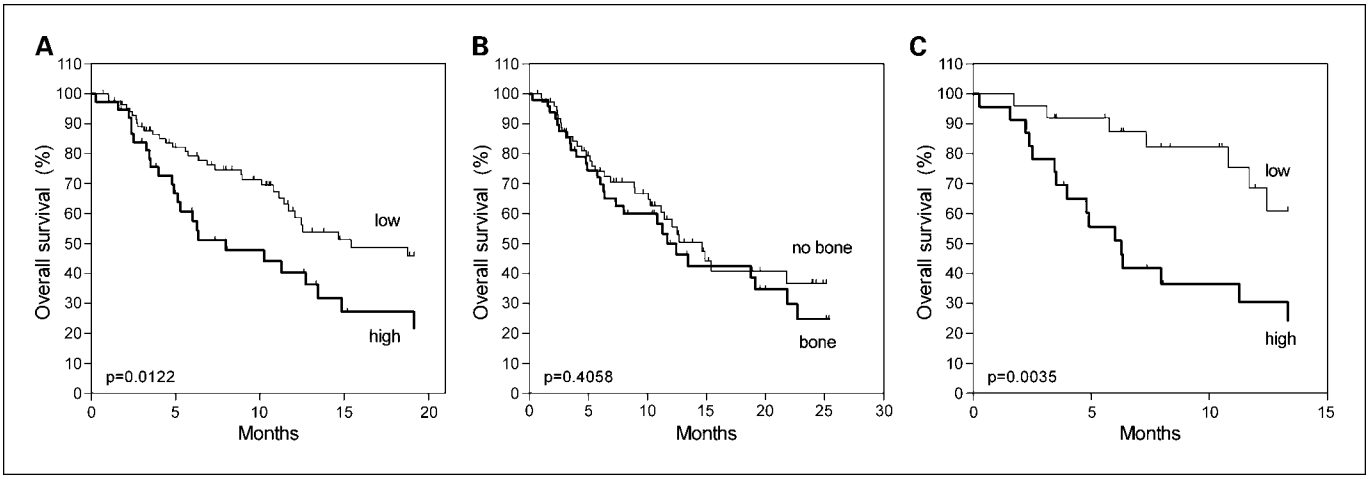

Fig. 2. Patients with elevated CD133 mRNA expression have decreased survival. The Kaplan-Meier survival curves for the 131 cancer patients evaluated according to dichotomized CD133 mRNA expression (>200 copies or "high" versus <200 copies or "low"; *A*). Survival curves for all 131 cancer patients evaluated according to the presence or absence of bone metastasis (*B*). Survival curves of the 50 patients with bone metastasis, evaluated according to high or low CD133 mRNA expression (*C*).

separately (n = 18), and similarly found a significant increase in comparison with patients without bone lesions (P = 0.034).

CD133 mRNA expression correlates with markers of disease progression. There was no relationship of CD146 or CD133 mRNA expression with patient age or sex. There was a negative correlation between CD133 and hemoglobin level (r = −0.21 with P = 0.019, n = 126), but none with leukocyte levels, platelet count, lactate dehydrogenase, or alkaline phosphatase. In the 34 patients with prostate cancer, we found a positive correlation between number of CD133 copies and prostate-specific antigen (r = 0.37, P = 0.040). CD146 mRNA expression had no relation with any laboratory variable tested.

High CD133 mRNA expression is associated with decreased survival. Patients had a median follow-up of 16 months after blood draw. Of the 131 patients, 64 patients were alive, 62 patients died due to disease progression, 3 patients had a noncancer related death, and 2 patients were lost to follow-up.

Kaplan-Meier estimates of overall survival using a cutoff of 200 copies CD133 mRNA per 10,000 copies U1A DNA showed a significant decrease (P = 0.012) in survival in patients with high CD133 (more copies than the cutoff) compared with patients with low CD133 expression (lower or undetectable levels). Median survival for patients with low CD133 expression was 15.4 months, whereas patients with high CD133 levels had a median survival of 8.0 months (Fig. 2A). After ∼20 months of follow-up, the cumulative survival of patients with low and high CD133 copies was 45% and 21%, respectively. The survival curves of patients with or without metastases to the bone were roughly overlapping (Fig. 2B).

Many patients with bone metastasis had increased CD133 mRNA expression compared with healthy volunteers. The range of CD133 mRNA expression varied from 0 to ∼1,000 copies. We evaluated whether survival in this group was also affected by high or low CD133 mRNA expression. After ∼13.5 months, the cumulative survival was 61% in the patients below the cutoff, compared with 22% of patients above the cutoff (P = 0.004; Fig. 2C).

CD133 expression is an independent predictor for survival. In the upper part of Table 4, hazard ratios are presented for patient characteristics as determined in the univariate analysis. When the total group of cancer patients was evaluated, low hemoglobin levels, high levels of leukocytes, lactate dehydrogenase and alkaline phosphatase, naivety for cancer treatment, and a high number of CD133 copies were associated with increased mortality (P < 0.10) and included in the multivariate model. When only patients with bone metastases were evaluated, the same patient characteristics, except for leukocytes, were selected for inclusion in the multivariate model.

In the multivariate models (lower part of Table 4), only lactate dehydrogenase and naivety for cancer treatment seemed to be independent, statistically significant predictors of survival (hazard ratio 1.07 per 100 units and 0.38, respectively; 95% confidence interval, 1.04-1.11 and 0.18-0.79, respectively), whereas hemoglobin levels showed a borderline effect

Table 4. Results of univariate and multivariate cox proportional hazards analysis

Variable	Univariate Analysis			
	All patients		Bone metastasis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	1.19 (0.63-2.24)	0.58	3.30 (0.75-14.55)	0.12
Age	1.00 (0.97-1.03)	0.90	0.98 (0.93-1.03)	0.45
Hemoglobin	0.58 (0.47-0.72)	<0.0001	0.63 (0.45-0.88)	0.006
Leukocytes	1.09 (1.02-1.18)	0.02	1.03 (0.93-1.14)	0.56
Platelets*	1.06 (0.96-1.18)	0.26	1.10 (0.80-1.51)	0.57
Lactate dehydrogenase*	1.07 (1.04-1.10)	<0.0001	1.33 (0.10-1.79)	0.05
Alkaline phosphatase*	1.46 (1.20-1.77)	0.0002	1.59 (1.14-2.23)	0.007
Treatment naïve	0.40 (0.24-0.67)	0.0004	0.41 (0.18-0.93)	0.03
Metastasis	1.03 (0.59-1.81)	0.92		
Bone metastasis	1.22 (0.74-2.01)	0.44		
CD133 copies (<200; >200)	1.77 (1.06-2.94)	0.03	2.90 (1.33-6.32)	0.007

Variable	Multivariate Analysis			
	All patients		Bone metastasis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Hemoglobin	0.73 (0.54-1.00)	0.05	0.70 (0.36-1.37)	0.30
Leukocytes	1.01 (0.87-1.18)	0.86		
Lactate dehydrogenase*	1.07 (1.04-1.11)	<0.0001	1.61 (1.03-2.53)	0.04
Alkaline phosphatase*	1.16 (0.82-1.63)	0.40	0.96 (0.56-1.63)	0.87
Treatment-naïve	0.38 (0.18-0.79)	0.009	0.71 (0.12-4.14)	0.70
CD133 copies (<200; >200)	1.83 (0.80-4.18)	0.15	9.73 (1.08-87.49)	0.04

NOTE: All recorded clinical characteristics were entered in a univariate analysis together with CD133 mRNA expression, dichotomized as high (>200 copies) versus low (≤200 copies). All variables included in the multivariate model showed P < 0.10 in the univariate analysis. In patients with metastasis to the bone, high CD133 copies were identified as an independent predictor of overall survival.
Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.
*Variable divided by 100 units.

(P = 0.051). In patients with bone metastases, high CD133 mRNA expression (at least 200 copies mRNA per 10,000 copies U1A DNA) and lactate dehydrogenase were both significant and independently related to survival. In this group, the hazard ratio for death in patients with high CD133 mRNA expression was 9.73 (95% confidence interval, 1.08-87.50), whereas the hazard ratio for lactate dehydrogenase was 1.61 per 100 units (95% confidence interval, 1.03-2.53).

Discussion

Endothelial cells and endothelial precursor cells are considered hopeful candidates for monitoring angiogenesis, tumor growth, and treatment response in cancer patients. We therefore developed a real-time NASBA to quantitatively monitor the mRNAs of stem cell marker CD133 and the pan-endothelial cell marker CD146 in the mononuclear fraction of cancer patients. NASBA is a 96-well–based method of nucleic acid amplification highly receptive to standardization and currently a Food and Drug Administration–approved method to determine viral load in HIV-1–infected patients (35, 36, 40). We show that CD133 mRNA expression is increased in patients with metastasis, more specifically bone metastasis, independent of primary tumor type. Furthermore, an exploratory analysis showed that high levels of CD133 mRNA are associated with decreased survival in patients with bone metastasis. CD146 mRNA was not increased in this group of cancer patients nor showed a relationship with clinical variables tested.

Experimental data have clearly established a role for bone marrow–derived progenitors in tumor progression (12, 15, 17, 18). Recently, vascular endothelial growth factor receptor 1 (VEGFR1)–positive hematopoietic progenitor cells have also been linked to the regulation of metastasis (19). A subset of these VEGFR1-positive cells was CD133 positive. Clusters of these VEGFR1-positive cells in human tissues prepare for the arrival of metastatic tumor cells. These findings indicate that progenitor cells may play a role at different levels of disease progression: initiation of metastasis and disease progression. However, despite the convincing preclinical evidence, there are few clinical studies demonstrating a role for progenitor cells. Tumors of six patients who developed cancer after receiving bone marrow transplantation from donors of the opposite sex showed varying (1-12%) incorporation of endothelial progenitor cells in the tumor vasculature (14).

A large number of human studies have measured circulating endothelial cell and endothelial progenitor cell numbers in the circulation as a surrogate marker for tumor progression (1–4, 20, 24–26, 41, 42). Only few studies have investigated CD133 as a marker in cancer patients. Sussman et al. (43) measured with real-time PCR mRNA of CD133, VEGFR-2, Tie-2, and VE-cadherin in the peripheral blood cells of 19 patients with breast cancer and 11 controls, and only found a significant increase in Tie-2 mRNA in patients with infiltrating carcinomas as opposed to benign disease. Rabascio et al. (2) assessed the same variables as Sussman et al. in the blood of 84 cancer patients (comprising seven different types of cancer) and 14 controls. They found that VE-cadherin was the only marker that was increased in cancer patients. VE-cadherin was particularly increased in hematologic malignancies. Neither study shows a significant increase in CD133 mRNA. These findings are in agreement with our results for the total cancer group, but we did find a significantly increased CD133 mRNA in cancer patients with bone metastasis. A possible explanation for the negative results on progenitor cell detection in patient studies are probably caused by the assay detection limit. The amounts of circulating endothelial cells present in cancer patients by flow cytometry are reported to be between 3,900 and 100,000 cells/mL (1, 44–46). A PCR-based assay of circulating endothelial cells described the lowest level of detection to be ~500 cells/mL blood (4). Endothelial progenitor cell levels are two orders of magnitude lower than circulating endothelial cells, constituting between ~0 and 500 cells/mL, in untreated patients and controls (1, 45). Rabascio et al. and Sussman et al. did not mention the detection limit of CD133-positive cell number in their PCR-based assay. In our spike experiments, we could accurately quantify 50 spiked cells expressing CD133 or CD146 per milliliter of blood, and the lowest limit of detection of our NASBA was 25 and 33 cells, respectively.

We hypothesize that the close proximity of cancer to the bone marrow can directly recruit and mobilize CD133-positive endothelial and hematopoietic progenitors to facilitate tumor growth. Our findings are also in agreement with the novel concept that bone marrow–derived progenitors facilitate metastasis. In an exploratory analysis, we found that among patients with bone metastasis, those with high CD133 copy numbers had a significantly shorter median survival than patients with low CD133 copy numbers. Whether high CD133 copy number is correlated with enhanced growth of primary tumor or metastatic lesions, and/or enhanced tumor angiogenesis, needs to be established in large, prospective, and controlled clinical trials.

CD133 expression has also been shown on human cancer stem cells (47, 48). This subpopulation of cancer cells is present in cancer tissue and has not been shown in patient blood. It is not possible to ascertain which specific cell type is positive for CD133 of CD146, as real-time mRNA quantification is done on a mixed population of PBMCs and does not allow typing of individual cells. This is a limitation of real-time mRNA quantification assays compared with other quantification methods, such as flow cytometry and the magnetic bead assay. A recent flow cytometric study indicates that all bone marrow CD133-positive cells coexpress hematopoietic stem cell marker CD34 (49). Preliminary results from our laboratory showed that 85% of CD133-positive cells by flow cytometry also coexpressed hematopoietic marker CD34.[5] However, using mRNA amplification, it is not possible to exclude the possibility that CD133 mRNA detected in our study might be also expressed by a different cell type than endothelial or hematopoietic precursors.

In our study, CD146 mRNA expression did not correlate with presence of cancer, survival or clinical data. Recently, Fürstenberg et al. (4) published an increase of CD146 mRNA using real-time PCR in 11 patients with newly diagnosed breast cancer. Although sensitivity of both assays is in the same order of magnitude, the discrepancy between our study and their study could be explained by the difference in tumor type: breast cancer versus patients with cancers of the kidney, prostate, head and neck, colon, and rectum.

[5] N. Mehra et al., unpublished data.

In summary, we have shown that CD133 levels are increased in patients with metastases, specifically bone metastases. Exploratory analysis of the relation between levels of CD133 and survival provides a rationale to pursue CD133 as a potential marker of cancer spread and progression. Real-time mRNA amplification of CD133 is a reliable and easy method that may be used to monitor large patient groups. Further validation of these markers requires homogenous, large patient populations with access to clinical data and adequate follow-up.

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