Pharmacogenetic profiling of CD133 is associated with response rate (RR) and progression-free survival (PFS) in patients with metastatic colorectal cancer (mCRC), treated with bevacizumab-based chemotherapy

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Introduction

Recent studies suggest CD133, a surface protein widely used for isolation of colon cancer stem cells, to be associated with tumor angiogenesis and recurrence. We hypothesized that gene expression levels and germline variations in CD133 will predict clinical outcome in patients with metastatic colorectal cancer (mCRC), treated in first-line setting with 5-fluorouracil, oxaliplatin and bevacizumab (BV), and we investigated whether there is a correlation with gene expression levels of CD133, vascular endothelial growth factor (VEGF) and its receptors. We evaluated intra-tumoral gene expression levels by quantitative real-time (RT) PCR from 54 patients and three germline variants of the CD133 gene by PCR-restriction-fragment length polymorphism from 91 patients with genomic DNA. High gene expression levels of CD133 (>7.76) conferred a significantly greater tumor response (RR = 86%) than patients with low expression levels (<7.76, RR = 38%, adjusted P = 0.003), independent of VEGF or its receptor gene expression levels. Gene expression levels of CD133 were significantly associated with VEGF and its receptors messenger RNA levels (VEGFR-1 P <0.01), -2 and -3, P <0.05). Combined analyses of two polymorphisms showed a significant association with progression-free survival (PFS) (18.5 months vs 9.8 months, P = 0.004) in a multivariate analysis as an independent prognostic factor for PFS (adjusted P = 0.002). These results suggest that CD133 is a predictive marker for standard first-line BV-based treatment in mCRC.

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The cancer stem cell hypothesis proposes that only a subpopulation of cancer cells is responsible for tumor initiation, disease progression and chemoresistance. Cancer stem cells (CSC) have many shared characteristics with normal stem cells, including the ability to self-renew, the capacity for homeostatic control, thus sustaining tumor growth and the potential to produce more differentiated progenitors, albeit aberrant
differentiation in the case of CSC.\textsuperscript{1,2} Prospective identification of CSC was first reported in leukemia. They have subsequently been identified in a wide range of tumors including glioblastoma, liver, breast, prostate and colon cancer.\textsuperscript{3-8} Several cell surface markers have been proposed for the identification of these CSC. Among these, CD133, a pentaspan transmembrane protein, has been used by several groups to isolate CSC in colorectal cancer.\textsuperscript{6,7,9,10} CD133 has been associated both in vitro and in vivo with tumor growth and progression.\textsuperscript{6,7,9,10} In vitro studies demonstrated the ability of human CD133\textsuperscript{+} colon cancer cells to initiate and maintain tumourspheres, whereas CD133\textsuperscript{-} cells did not grow in cell culture. In xenograft-models, human CD133\textsuperscript{+} colon cancer cells have demonstrated a higher clonogenic capacity and engraftment rate for tumor growth in immunocompromized mice than CD133\textsuperscript{-} cells.\textsuperscript{6,7} Recent work by Zhu et al.\textsuperscript{11} highlighted the importance of CD133 in colorectal cancer. They demonstrated that the murine analog of CD133, Prominin-1, marks intestinal adult stem cells, which are susceptible to malignant transformation. However, there has been a significant controversy concerning CD133 expression as a marker of CSC. Shmelkov et al.\textsuperscript{12} proposed, on the basis of the use of a knock-in LacZ transgenic mouse model that in fact CD133 is mainly a marker of mature colonic epithelial cells. In human colons, they observed that expression of CD133 message overlapped with the expression of an epithelial cell adhesion molecule. Although this seems at first contradictory, in fact the issue is not only whether CD133 is transcriptionally active in colon cancer stem cells, but also which alternatively spliced and glycosylated epitopes of the protein can be recognized by specific monoclonal antibodies; in this case Prom1/CD133 antibodies. Additionally, CD133 messenger RNA levels may not originate from the colon CSC or more differentiated colon cancer epithelium, but from endothelial cells.

However, studies investigating characteristics of CD133\textsuperscript{+} cells suggest a subpopulation among these cells demonstrating attributes of endothelial progenitors, leading toward maturation into endothelial cells and thereby proving angiogenic potential.\textsuperscript{13,14} Bevacizumab (BV), a monoclonal human antibody targeting vascular endothelial growth factor (VEGF), has been proven its efficacy in colorectal cancer and is given as first-line chemotherapy in patients with metastatic colorectal cancer (mCRC).

The rationale for this study was to investigate whether gene expression levels and potentially functional germline variations (rs3130, rs2286455 and rs2240688) in CD133 could predict clinical outcome in patients with mCRC, treated in first-line setting with 5-fluorouracil, oxaliplatin and the anti-angiogenic agent BV. For further confirmation of our thesis, we investigated whether there is a correlation between gene expression levels of CD133, VEGF and its receptors (VEGFR-1, -2 and -3).

Patients and methods

Patients

Ninety-one patients with primary colorectal adenocarcinoma, either metastatic or recurrent, were eligible for this study (registered at http://www.clinicaltrials.gov, NCT00070122). The patients received first-line treatment with FOLFOX or XELOX and BV between April 2004 and January 2009 at the University of Southern California/Norris Comprehensive Cancer Center or the Los Angeles County/University of Southern California Medical Center. Primary tumor samples were available from 54 patients whereas whole blood samples for genotyping were available from all participating 91 patients. This study was conducted at the University of Southern California/Norris Comprehensive Cancer Center and approved by the Institutional Review Board of the University of Southern California for Medical Sciences. All patients signed an informed consent; follow-up information and clinical data were collected through a prospectively started database and in a retrospective attempt through chart review.

Tumor response evaluation

Baseline evaluations were conducted within 1 week before administration of study drug. Scans and X rays were conducted ≤4 weeks before start of therapy. The Response Evaluation Criteria In Solid Tumors was used. Tumor response was evaluated every 6 weeks. Response was defined as follows: complete response, disappearance of all target lesions; partial response, at least a 30% decrease in the sum of longest diameter (LD) of target lesions taking as reference the baseline sum LD; stable disease, neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progression of disease taking as references the smallest sum LD since the treatment started; progression of disease, at least a 20% increase in the sum of LD of target lesions taking as references the smallest sum LD recorded since the treatment started, or the appearance of one or more new lesions. Disease progression was also recorded by clinicians even without radiological assessment when patient symptoms deteriorated.

Microdissection

For the assessment of gene expression levels, formalin-fixed paraffin-embedded tissue samples from the primary tumor from 54 patients were available. After a representative review of hematoxylin and eosin-stained slides by a pathologist, 10\textmu m-thick sections were obtained for laser-captured microdissection (P.A.L.M. Microlaser Technologies AG, Munich, Germany) from the regions with the highest amount of tumor cells according to a standard procedure.

Isolation of RNA and complementary DNA synthesis

The sections were then transferred to a reaction tube containing 400\textmu l of RNA lysis buffer; RNA isolation from formalin-fixed paraffin-embedded samples was performed according to a patented procedure of Response Genetics (Los Angeles, CA, USA; Patent No 6,248,535). After RNA isolation, complementary DNA synthesis was performed as previously described.\textsuperscript{15}

Real-time PCR quantification of messenger RNA expression

The quantification of gene expression levels of CD133, VEGF and VEGFR1, -2 and -3 was performed using β-actin as
an internal housekeeping gene and the gene-set for above named genes in a fluorescence-based real-time detection method (ABI prism 7900 Sequence Detection System, TaqMan) Perkin-Elmer Applied Biosystem, Foster City, CA, USA). Real-time PCR was executed as previously described by Lord et al.;15 primers and probes are listed in Table 1. Quantification of gene expression levels is validated with the assessment of cycle threshold values. These cycle threshold values are inversely correlated with the amount of complementary DNA in each sample and imply number of PCR cycles, until the fluorescent signal exceeds the threshold, and is therefore detected.

Candidate polymorphisms
Candidate polymorphisms were picked with the assistance of the Ensembl homepage. Two main criteria were chosen for selecting the germline variations:

(1) That the polymorphism has some degree of likelihood to alter the function of the gene in a biologically relevant manner. All three polymorphisms are located in the 3'-untranslated region of the CD133 gene, where they might alter gene expression levels of CD133 that is, by influencing microRNA-binding sites.

(2) That the frequency of the polymorphism is sufficient enough that its impact in clinical outcome would be meaningful on a population level.16

Isolation of genomic DNA and genotyping
Peripheral blood was available from 91 patients. Genomic DNA was extracted from white blood cells using the QiaAmp kit (Qiagen, Valencia, CA, USA). Forward and reverse primers were used for PCR amplification. Samples were analyzed by PCR-restriction-fragment length polymorphism assays. Forward/reverse primers, digesting enzymes and annealing temperatures are listed in Table 1.

Table 1: Primers and probes for gene expression in CD133 and VEGF, VEGFR-1, -2, -3 and genotyping in CD133

| Gene Gen Bank accession | Forward (5’–3’) | Reverse (5’–3’) | Taqman probe (5’–3’) | Digesting enzyme |
|-------------------------|-----------------|-----------------|----------------------|-----------------|
| β-actin                 | GAGCCGGGCTACAGTT | TCCGTAATGTCAACAGCTT | ACCACACGGGCCGAGCCG | EcoR1           |
| CD133                   | CAAGGAACAGGCTTCACAG | GTGGGGTCAAGTGCGGTA | TTCCGCGCTTGAAGCTTGA | Mbo1            |
| VEGF                    | ACGTGCTCAAGGCTT | GCCGCGCGGGAGACTACGCTGG | ATGCGAGAAGGAGGAGGAATCA |              |
| VEGFR-1                 | CGATATGGACATGTCGGA | GCCATGGCTTGCCGGAATG | GTGTTCTGGACCCCTGAACCTAA |              |
| VEGFR-2                 | CTTCGTTGTCTCGCTGTA | CTGAGCCCGAGATCAAG | CACTAGGGAAAACACAGGCGGC |              |
| VEGFR-3                 | GGACACCCCTGGAAGATTTT | TCACGGCACTTGGGCATGA | CGGCGCCCGGACAGCTCATGG |              |

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptors.

Statistical analysis
Primary end points of this study were progression-free survival (PFS) and response rate (RR). The PFS was calculated from the date of the first treatment with FOLFOX/BV or XELOX/BV at the University of Southern California medical facilities, until the first observation of disease progression or death from any cause. If no disease progression occurred and the patient was still alive at the time of the last follow-up, PFS was censored at the date of the last follow-up.

The association between CD133 gene expression value and tumor response was assessed using maximal $\chi^2$ method.17,18 The optimal cut-off value of CD133 was used to separate patients into two groups in terms of likelihood of responding to the therapy. The $P$-value for the association was adjusted with 2000 bootstrap-like simulations that estimated the distribution of the maximal $\chi^2$ statistics. The maximal $\chi^2$ method had been used in our previous studies to examine the associations between the gene expression and clinical outcome.19–21 The differences in CD133 gene expression value by CD133 polymorphisms were tested using the Wilcoxon two-sample test.

Allelic distribution of CD133 polymorphisms by each race was tested for deviation from Hardy-Weinberg equilibrium using $\chi^2$-test. The associations of individual CD133 polymorphisms with PFS were analyzed using Kaplan-Meier curves and log-rank test assuming codominant, dominant or recessive genetic model. The associations between genomic polymorphisms and clinicodemographic parameters and tumor response were assessed using contingency tables and the Fisher’s exact test. The estimate of the hazard ratio with 95% confidence intervals was based on the log-rank test for the univariate analysis.22 The cumulative effect of
CD133 polymorphisms on clinical outcome was examined by combining \( \geq 2 \) CD133 alleles.

The Cox proportional hazards regression model was used to assess the association between CD133 polymorphisms and PFS when adjusting for gender, the number of metastatic sites and race. The Spearman correlation coefficient method was used to investigate the correlations between gene expression levels of CD133, VEGF and VEGF-receptor genes.

Finally, we performed leave-one-out cross-validation, in which one patient was removed from the analysis and the association between CD133 and PFS adjusted for covariates was assessed using the Cox proportional hazards conditional survival function in the remaining patients. The process was repeated with each patient left out at a time. This method provides an approximation of the unbiased estimate of the concordance probability, an index for discrimination and the predictive accuracy of models. \(^{21,23}\) Around 95% bootstrap confidence intervals of the concordance probability were calculated using the bias correct method with 1999 bootstrap samples. \(^{24}\) The concordance probability ranges from 0.5 (no discrimination) to 1.0 (perfect discrimination).

The level of significance was set to \( P < 0.05 \), and all tests were two-sided. Analyses were performed using the SAS statistical package version 9.2 (SAS Institute Cary, NC, USA) and SAS %MACRO (%cpe developed by Gönen & Heller).

### Results

A total of 91 patients (54 men and 37 women) with a median age of 56 (range 28–81) participated in this study. The racial/ethnic distributions of study participants were as follows: 37 whites, (41%), 21 Asians (23%), 28 Hispanics (31%) and 5 African American (5%). At a median follow-up of 28.7 months (range 3.3–53.8 months), the median PFS was 12.4 months (95% confidence intervals: 8.3–15.2) in these patients. One metastatic site was observed in 51 patients, whereas 40 patients had \( \geq 2 \) metastatic sites. Tumors of 67 patients showed moderate differentiation, 21 patients had poor differentiation and 3 patients had missing data on differentiation. Of the 91 patients, 31 patients died whereas the median overall survival has not been reached. Patient characteristics are described in Table 2.

### Gene expression levels of CD133 and tumor response and PFS

Gene expression levels of CD133 were quantifiable in 54 patients with a median messenger RNA level for CD133 of 6.31 (Table 3a). The gene expression levels of CD133 were significantly associated with tumor response (adjusted \( P = 0.003 \), maximal \( \chi^2 \) method). A cut-off value for CD133, \( 7.76 \), was determined as the optimum value to divide patients into poor- and good-prognosis subgroups in terms of response to treatment. Patients with high gene expression levels of CD133 (\( > 7.76 \), \( n = 22 \)) showed a significantly better tumor response (86%) than patients with low expression levels (\( \leq 7.76 \), \( n = 32 \), RR = 38%, Figure 1a).

### Table 2 Baseline characteristics among patients whose specimens were available for genotyping

| Patients with CD133 gene expression data (n = 54) | All patients (n = 91) |
|-----------------------------------------------|----------------------|
| Frequency | % | Frequency | % |
| Median age, year (range) | 57 (30–81) | 56 (28–81) |
| Sex | | |
| Female | 26 | 48 | 37 | 41 |
| Male | 28 | 52 | 54 | 59 |
| Race | | |
| Asian | 12 | 22 | 21 | 23 |
| Black | 1 | 2 | 5 | 5 |
| Caucasian | 25 | 46 | 37 | 41 |
| Hispanic | 16 | 30 | 28 | 31 |
| Primary site | | | | |
| Colon | 39 | 75 | 62 | 70 |
| Rectal | 12 | 23 | 24 | 27 |
| Recto-sigmoid | 1 | 2 | 3 | 3 |
| Differentiation | | | | |
| Moderate | 42 | 82 | 66 | 76 |
| Poor | 9 | 18 | 21 | 24 |
| No. of metastatic sites | | | | |
| 1 | 32 | 59 | 51 | 56 |
| \( \geq 2 \) | 22 | 41 | 40 | 44 |

### Table 3a Median mRNA levels of tested genes

| Variable | N  | Median | Range          |
|----------|----|--------|----------------|
| CD133    | 54 | 6.31   | (0.03–35.09)   |
| VEGF     | 53 | 9.70   | (3.63–28.70)   |
| VEGFR-1  | 52 | 7.27   | (1.32–52.68)   |
| VEGFR-2d | 53 | 1.86   | (0.45–14.25)   |
| VEGFR-3  | 52 | 0.32   | (0.001–1.56)   |

**Abbreviations:** VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptors.

### Table 3b Relationship (Spearman’s correlation coefficients) between gene expression levels of CD133 and genes in VEGF pathway

| Gene   | CD133 |
|--------|-------|
| VEGF   | 0.306*|
| VEGFR-1| 0.439**|
| VEGFR-2| 0.339*|
| VEGFR-3| 0.334*|

**Abbreviations:** VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptors.

\*\( P < 0.05 \).

**\( P < 0.01 \).
VEGFR-2 and -3
¼ VEGF or VEGFR-receptor gene expression levels (VEGF, tumoral CD133 gene expression levels also showed high VEGFR 1, -2 and -3 (Figures 2a–d). Patients with high intra-tumoral levels of CD133 were significantly associated with VEGF, VEGFR gene expression levels were not significantly associated in 53 and 52 patients, respectively (Table 3a). VEGF and Gene expression levels for VEGF and VEGFR1 were quantifiable in gene expression samples. In addition, high intra-tumoral CD133 gene expression levels were also significantly associated with the favorable alleles by the combination analysis of two CD133 polymorphisms (P = 0.044), implying that patients who respond to 5-fluorouracil/BV show an increased PFS.

**Genetic variants in CD133 and PFS**
A total of 91 patients were successfully genotyped for rs3130, rs2240688 or rs2286455. The allelic frequencies observed for rs2240688 and rs2286455 variants were within the probability limits of Hardy-Weinberg equilibrium (P > 0.05, χ²-test for Hardy-Weinberg equilibrium) in each race group. rs3130 allelic distribution in white patients significantly departed from Hardy-Weinberg equilibrium (P = 0.035 χ²-test). There were no significant differences in the distribution of three CD133 genetic variants by race (Table 4). None of the three CD133 polymorphisms were significantly associated with tumor response or PFS. In a combination analysis, we found rs3130 and rs2286455 significant for PFS. Patients who carried C/C in rs2286455 and rs3130 or the combination of C/T with either C/T or T/T showed a significantly increased PFS of 18.5 months, compared with 9.8 months PFS for patients with CC in one polymorphism and C/T or T/T in the other polymorphism (P = 0.004, log-rank test, Figure 1b). Allele frequencies were 15.3% for rs2286455 and 54.1% for rs3130 for each variant allele in the white study population. After adjustment for sex and number of metastatic sites, multivariate analysis showed the combination analysis of rs2286455 and rs3130 to be an independent prognostic factor for PFS (adjusted P = 0.002). The concordance probability (0.675, 95% bootstrap confidence intervals 0.653–0.726) estimated using the leave-one-out cross-validation indicated good discrimination and predictive accuracy of the multivariate model including CD133 and covariates (gender and number of metastatic sites).

The association between favorable CD133 genetic variants and tumor response was not significant (data not shown).

**Combination of gene expression and genetic variants**
Gene expression values of CD133 and genotypes in rs2286455 were significantly associated with each other (P = 0.041, the Wilcoxon two-sample test). Patients carrying C/C had a lower CD133 gene expression levels (median = 4.43, range: 0.03–31.17) compared with patients carrying C/T or T/T in rs3130 showed a significantly increased PFS, compared with a decreased PFS for patients with C/C in one polymorphism and C/T or T/T in the other polymorphism (unfavorable alleles).

However, there was no significant difference in PFS between high and low CD133 expression levels (data not shown).

**Gene expression levels of CD133 and VEGF-pathway genes**
Gene expression levels for VEGF and VEGFR1 were quantifiable in 53 and 52 patients, respectively (Table 3a). VEGF and VEGFR gene expression levels were not significantly associated with PFS or RR (data not shown). Gene expression levels of CD133 were significantly associated with VEGF, VEGF1, 1,-2 and -3 (Figures 2a–d). Patients with high intra-tumoral CD133 gene expression levels also showed high VEGF or VEGFR-receptor gene expression levels (VEGF, VEGFR-2 and -3 = P < 0.05, VEGFR1 = P < 0.01, Table 3b).

**Discussion**
Despite an understanding of its molecular function, the cell surface epitope defined by prominin1/ CD133 has garnered significant notoriety as a marker that is associated with stem cells, CSC and symmetric cell division. However, the expression of the CD133 gene is clearly not restricted to a stem/progenitor population, and CD133 gene expression is also associated with more differentiated epithelium. Our data demonstrate for the first time, to the best of our knowledge that patients with mCRC and high intra-tumoral CD133 gene expression levels benefit from treatment with the angiogenesis-inhibitor BV. Furthermore, we show that...
patients with high CD133 gene expression also express high levels of VEGF and its receptors. Additionally, the combination analysis of two polymorphisms in the CD133 gene (rs2286455 and rs3130) was associated with favorable benefit in PFS.

CD133 has been shown to have a role in postnatal physiological and pathological angiogenesis. Several studies have demonstrated that tumor growth and aggressiveness is directly correlated with the degree of neovascularization. 28 The angiogenic process is controlled by the release of pro-angiogenic factors such as VEGF and interleukin 8.

In a mouse model of diabetic ischemic ulcers, Barcelos et al.25 showed that CD133 positivity was associated with expedited wound healing. The authors associated this effect with stimulation of endothelial cell proliferation and migration. Further analysis revealed high gene expression levels of angiogenic factors such as VEGF-A and interleukin 8.

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Willett et al.29 demonstrated that the effect of a single infusion with BV decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure and circulating endothelial and progenitor cells. Patients with locally advanced rectal cancer received neoadjuvant treatment with BV 5 mg kg⁻¹ alone, followed 2 weeks later by concurrent administration of BV, 5-fluorouracil and external beam radiation to the pelvis before surgery.

Table 4 Allelic distribution of CD133 polymorphisms by race

| Polymorphisms | Total N | Race | P value<sup>a</sup> |
|---------------|---------|------|-------------------|
|               |         | African American | Asian | Hispanic | White |
| rs2286455     |         |                  |       |          |       |
| C/C           | 60      | 80.00            | 66.67 | 53.57    | 75.00 |
| %             |         |                   |       |          |       |
| C/T           | 27      | 20.00            | 28.57 | 46.43    | 19.44 |
| %             |         |                   |       |          |       |
| T/T           | 3       | 0.00             | 4.76  | 0.00     | 5.56  |
| %             |         |                   |       |          |       |
| T allele frequency | 0.100 | 0.190            | 0.232 | 0.153    |
| rs3130        |         |                  |       |          |       |
| C/C           | 24      | 50.00            | 14.29 | 29.63    | 29.73 |
| %             |         |                   |       |          |       |
| C/T           | 36      | 25.00            | 61.90 | 37.04    | 32.43 |
| %             |         |                   |       |          |       |
| T/T           | 29      | 25.00            | 23.81 | 33.33    | 37.84 |
| %             |         |                   |       |          |       |
| T allele frequency | 0.375 | 0.548            | 0.519 | 0.541    |
| rs2240688     |         |                  |       |          |       |
| T/T           | 51      | 80.00            | 65.00 | 61.54    | 60.00 |
| %             |         |                   |       |          |       |
| T/G           | 25      | 0.00             | 30.00 | 26.92    | 40.00 |
| %             |         |                   |       |          |       |
| G/G           | 5       | 20.00            | 5.00  | 11.54    | 0.00  |
| %             |         |                   |       |          |       |
| G allele frequency | 0.200 | 0.200            | 0.250 | 0.200    |

<sup>a</sup>Based on Fisher's exact test.
3 days after the initial BV treatment, the amount of viable CD133\(^+\) cells in peripheral blood was significantly decreased (\(P<0.05\), Wilcoxon-signed ranked test). In our study, patients with high intra-tumoral CD133 gene expression levels showed an increased response rate to treatment with BV, indicating a predictive role for CD133 to anti-VEGF treatment.

To our knowledge, there are no studies that describe a relationship with germline variations in CD133 in relation to clinical outcome. The fact that the polymorphisms that are significantly associated with a prolonged PFS are also linked with gene expression levels of CD133 might mirror the effect of an increased response rate to BV therapy and further support a functional significance for these polymorphisms. Germline variations may impact the expression not only in the tumor but also in the tumor environment and normal tissue such as endothelial cells.

Nonetheless, this study has several limitations. First, Patients were all treated with BV and FOLFOX/XELOX without a control arm receiving only FOLFOX/XELOX. Second, based on the fact that gene expression levels were significantly associated with polymorphisms in CD133, we assumed a functional significance for these polymorphisms. However, a function for the CD133 gene product, no less the polymorphisms. Germline variations may impact the expression not only in the tumor but also in the tumor environment and normal tissue such as endothelial cells.

Importantly however, these are the first data to show that patients with high intra-tumoral CD133 gene expression levels benefit from treatment with the angiogenesis-inhibitor BV, indicating on a preliminary level a predictive role for CD133 in anti-VEGF treatment, data that warrant validation in large prospective studies.

**Conflict of interest**

The authors declare no conflict of interest.

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