



CD44v6, a target for novel antibody treatment approaches, is frequently expressed in multiple myeloma and associated with deletion of chromosome arm 13q

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Background and Objectives. Despite recent advances in the treatment of multiple myeloma (MM), this disease remains incurable in the majority of patients. Therefore, innovative treatment strategies are mandatory. Bivatuzumab mertansine is a novel cytotoxic immunoconjugate specifically targeting the CD44 splice variant CD44v6. To investigate the applicability of this compound to clonal plasma cell disorders, we analyzed CD44v6 expression on malignant plasma cells from patients with multiple myeloma.

Design and Methods. Bone marrow samples from 57 patients with monoclonal gammopathy of undetermined significance (MGUS), MM, and plasma cell leukemia (PCL) were examined for CD44v6 expression by using flow cytometry (FACS) analysis. In addition, all samples were investigated by fluorescence *in situ* hybridization (FISH) with a specific probe for the chromosomal band 13q14.

Results. In only 1 of 16 cases (6%) with MGUS and 1 out of 6 cases (17%) with stage I MM were plasma cells CD44v6 positive. In contrast, 43% of the cases with stage II/III MM or PCL expressed CD44v6. In these cases, CD44v6 expression was significantly correlated with chromosome 13q14 deletion as determined by FISH ($p=0.02$).

Interpretation and Conclusions. CD44v6 is frequently expressed in advanced, high-risk MM. CD44v6 expression correlates with chromosomal band 13q14 deletions, a well-known risk factor in MM. These results suggest that this epitope is a potential new target for monoclonal antibodies such as bivatuzumab mertansine.

Key words: CD44v6, multiple myeloma, bivatuzumab mertansine, deletion 13q14, flow cytometry.

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Despite the most recent advances in the treatment of multiple myeloma (MM), only a few patients with this disease are cured. Most patients relapse after primary treatment and require further therapy. Novel drugs, such as the proteasome inhibitor bortezomib, became available for these patients with relapsed or primary refractory disease, but are ineffective in a substantial proportion of cases.¹ Therefore, innovative treatment approaches are needed. Antibody-based treatment strategies are successfully applied to various hematologic cancers, such as the lymphomas.² However, monoclonal antibodies specifically targeting myeloma cells are not available yet but very recent publications indicate promising approaches are under way.³ Bivatuzumab mertansine (Boehringer Ingelheim Pharma, Germany) is a novel compound manufactured from mertansine,

a cytotoxic drug from the family of maytansines, and bivatuzumab, a humanized monoclonal antibody specifically recognizing CD44v6.⁴ CD44v6 is an isoform of the type 1 transmembrane glycoprotein CD44 generated by alternative splicing and has been implicated in cell adhesion and signaling, growth factor and cytokine presentation, as well as tumor dissemination. In contrast to the standard form of CD44, which is almost ubiquitously expressed in normal tissues, CD44v6 expression is highly restricted to a subset of epithelial tissues (e.g. skin, oral mucosa, esophagus, cervix). CD44v6 is expressed in the majority (>90%) of squamous cell carcinomas (SCC) of the respiratory, digestive, and genital tracts, as well as of the skin.⁴ Data on the incidence of CD44v6 in clonal plasma cell disorders are scarce. Bone marrow biopsies from a limited number of patients with

MM have been examined for expression of various CD44v isoforms employing immunohistochemistry, and the proportion of CD44v6 positive tumor samples has been reported to be in the range of 6% to 22%.⁵⁻⁷ Other isoforms (e.g. CD44v9) are also expressed in MM samples but specific antibodies that could be used for targeted therapies are not available so far. For a more detailed evaluation of the incidence of CD44v6 as a potential target for innovative immunoconjugates, a series of 57 patients with monoclonal gammopathy of undetermined significance (MGUS), MM, and plasma cell leukemia (PCL) was analyzed using flow-cytometry analysis.

Design and Methods

Patients and controls

From December 2002 to December 2003, bone marrow aspirates from 57 patients with MGUS (n=16), MM (Salmon-Durie stage I, n=6; stage II, n=4; stage III, n=28) and plasma cell leukemia (n=3) were obtained during diagnostic procedures. Informed consent was obtained from all patients. There were 24 females and 33 males. Ages ranged from 33 to 81 years (median, 56 years). Two MGUS patients were being treated with corticosteroids at the time of analysis, one because of renal amyloidosis, the other because of Sjögren's syndrome. Four MM patients had received cytotoxic therapy directly prior to analysis (high-dose dexamethasone, n=2; 4 cycles of vincristine/adriamycin/dexamethasone [VAD] n=1; 1 cycle of idarubicin/dexamethasone, n=1), but had significant tumor burden when included in the study. The remaining cases were newly diagnosed and untreated. To determine the threshold for CD44v6 positivity, bone marrow aspirates from 6 healthy volunteers and 7 patients without a monoclonal gammopathy served as controls.

Flow-cytometry analysis

Bone marrow specimens were analyzed within 1 to 4 hours after the samples had been drawn in order to prevent potential antigen internalization or shedding. After red cell lysis, plasma cells were identified by CD138 staining and the percentage of CD44v6-positive cells was determined by flow cytometry analysis using the monoclonal mouse-anti-human IgG1-CD44v6 antibody (clone VFF18; Bender MedSystems, Vienna, Austria) as described earlier.⁸ In brief, cells were first labeled with the CD44v6 antibody and subsequently in a second step with a goat-anti-mouse IgG1-FITC antibody. For all stainings, isotype-matched irrelevant antibodies were used as controls.

Flow cytometric acquisitions were performed with the *Epics XL* flow cytometer (Coulter) and analyzed using *Expo 32* software.

Fluorescence in situ hybridization (FISH)

Positive selection of plasma cells for FISH was performed using the *AutoMACS* system and CD138 coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) in 13 out of 16 cases with MGUS and 25 out of 41 MM/PCL samples. FISH and immunocytology were done as previously described.⁹ In brief, bone marrow samples were centrifuged on a Ficoll-hypaque gradient to enrich for mononuclear cells. Cytospin slides were prepared and dried overnight at room temperature. The slides were then dehydrated in an ethanol series and stored at -20°C after air drying. Slides were pretreated by 5 minutes shaking in 1 × phosphate-buffered saline (PBS) and then digested for 2.5 minutes in pepsin solution at 37°C, washed in 1×PBS for 5 minutes, fixed in 2% paraformaldehyde solution, and finally washed in 1×PBS for 5 minutes before dehydrating in an ethanol series. The FISH probe D13S272 for chromosome band directly labeled in red was prepared as recommended by the manufacturer's instructions. Five microliters of each probe were placed on a pretreated slide, denatured on a hot plate (75°C) for 7 minutes, and allowed to hybridize in a 37°C humidified chamber overnight. Following hybridization, the slides were washed in 0.5 × standard saline citrate (SSC) for 2 minutes at 72°C then for 5 minutes at room temperature. Excessive liquid was dropped off and slides were then stained with goat antihuman κ and λ light-chain antibodies conjugated with AMCA 7-amino-4-methylcoumarin-3-acetic acid (AMCA; Vector Lab, Burlingame, CA, USA); a cover-slip was added, sealed with rubber cement, and the slides were incubated overnight in a humidified chamber at 37°C. After the incubation, the slides were washed in 1×PBS for 5 minutes. Finally Vectashield (Vector Labs) was applied and the slides were again covered with cover-slips. At least 100 AMCA-positive plasma cells were scored for each patient.

Results

Threshold for CD44v6 positivity

The percentage of CD44v6⁺/CD138⁺ plasma cells in the 13 control samples ranged from 0% to 4%. The threshold for CD44v6 positivity was calculated to be 5% (mean percentage of CD44v6⁺/CD138⁺ cells plus 3 times the standard deviation).

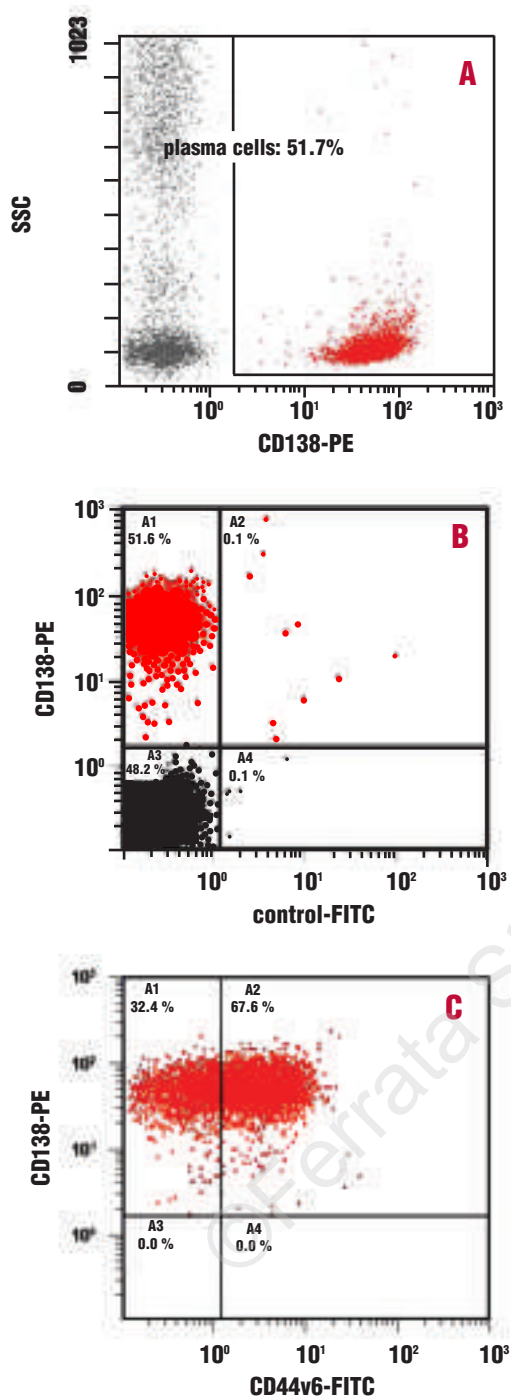


Figure 1. Detection of CD44v6 expression in CD138-positive plasma cells using flow cytometry (FACS) analysis in a patient with plasma cell leukemia. **A.** Human bone marrow cells were gated for CD138-positive cells. **B.** Negative control for the FITC-labeled monoclonal mouse anti-human CD44v6 antibody. **C.** Co-expression of CD138 and CD44v6 on the cell surface of malignant plasma cells.

Incidence of CD44v6 expression in MGUS, asymptomatic MM, symptomatic MM, and PCL

Since the cell surface antigen CD138 is more specific for plasma cells than CD38, bone marrow cells

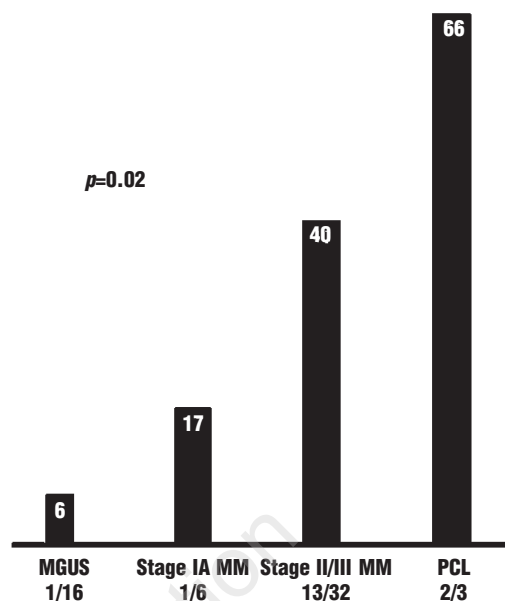


Figure 2. Incidence (in %) of CD44v6 expression in 57 patients with MGUS, indolent MM, symptomatic MM, and PCL.

were gated for CD138 positive cells (Figures 1A,B). This gate was used to identify CD44v6 co-expressing plasma cells (Figure 1C). The frequencies of CD44v6 positivity in patients with MGUS, asymptomatic MM (Salmon & Durie stage IA), symptomatic MM (Salmon & Durie stage IB to IIIB), and PCL in our series are given in Figure 2. The incidence of CD44v6-positive cases significantly increased with tumor stage ($p=0.02$; Fisher's exact test). Only one patient with MGUS and one patient with indolent MM were weakly CD44v6⁺ (6% and 17% CD44v6 positive cells, respectively). No increase of the monoclonal protein has been observed in these two patients during short follow-up periods of 12 and 7 months, respectively. In the CD44v6⁺ group of patients ($n=17$), the proportion of CD44v6⁺ plasma cells (CD44v6⁺/138⁺) ranged from 6% to 82% (median: 28%). In these cases, the ratio of CD44v6⁺/CD138⁺ to CD44v6⁻/CD138⁺ cells ranged from 0.1 to 82.0 (median: 2.0). The density of CD44v6 expression was classified as *low* and *high* by flow cytometry analysis. Eleven cases exhibited a low epitope density, while high CD44v6 expression was found in 6 cases.

Correlation of CD44v6 expression with major risk factors

CD44v6 expression was correlated with two important indicators of adverse prognosis in MM: elevated $\beta 2$ microglobulin (>3 mg/L) and presence of chromosome band 13q14 deletion, as detected by FISH. In the present series of 35 patients with stage

II/III MM or PCL, CD44v6 was not associated with a high tumor burden as reflected by elevated $\beta 2$ microglobulin levels ($p=1.0$). In contrast, CD44v6 was significantly correlated with 13q14 losses ($p=0.01$; Fisher's exact test).

Discussion

Immunohistochemical studies revealed almost universal expression of the CD44v6 in various types of squamous cell carcinomas while expression of this epitope was reported in only small subgroups of patients (6% to 22%) with MM. As monoclonal antibodies specifically recognizing CD44v6 have now been developed, we performed a flow cytometry analysis to study the expression of this epitope as a potential therapeutic target in clonal plasma cell disorders. Van Driel and colleagues published data regarding the expression of CD44v6 in MM; they found no difference in the expression of this isoform between normal plasma cells and malignant plasma cells from patients with MM.¹⁰ This seems to be in contrast to our data but because of different gating strategies (CD38 vs CD138, respectively), the different number of patients (31 vs 57) and the fact that more than 90% of our patients were untreated, the two data sets cannot be compared. In addition, our study describes a cytogenetically well defined MM population while some previous studies lack this information. Since flow cytometry is more sensitive than immunohistochemical staining, the incidence of CD44v6 among symptomatic tumors (Salmon & Durie stage II/III MM or PCL) was much higher in our series (43%) than in previous studies.^{5-7,10} The significantly lower incidence of CD44v6 expression in MGUS and indolent MM (Salmon & Durie stage I MM) suggests that CD44v6 expression might be associated with disease progression. In addition, we found that the expression of CD44v6 in monoclonal gammopathies is highly variable with respect to the density of the epitope as well as with respect to the proportion of tumor cells carrying this glycoprotein (6% to 82%, median: 28%). There are only scarce data on the prognostic significance of CD44 isoforms in MM, and published data referring to a correlation of CD44v isoforms with chromosomal aberrations are lacking. In two studies, the CD44v9 isoform, but not CD44v6, was associated with advanced Salmon & Durie stages and progressive disease.^{6,7} The authors concluded from these studies that CD44v9 might represent a novel prognostic parameter in MM. To our knowledge, the present study is the first demonstrating a significantly positive correlation between chromosome arm 13q losses and CD44v6 expression, suggesting an unfavorable prognosis in CD44v6⁺ tumors

and pointing to a potential pathogenetic link between this genomic abnormality and CD44v6 expression in MM. In contrast, CD44v6 was not correlated with elevated $\beta 2$ microglobulin levels. $\beta 2$ microglobulin levels are associated with high tumor burden but not with distinct biological features of the malignant cell. Since we believe that aberrant CD44v6 expression is associated with malignant properties of highly aggressive plasma cells rather than with tumor mass, it is not surprising that these parameters do not correlate.

Recently, several novel substances have been introduced for the treatment of multiple myeloma,^{11,12} but none of them directly targets the malignant cell. Based on our data, a therapeutic monoclonal antibody against CD44v6 could be the first approach towards a targeted therapy in CD44v6-positive MM. Clinical studies using bivatuzumab mertansine or ¹⁸⁶Re-labeled bivatuzumab in tumors of epithelial origin have been recently published^{13,14} and show excellent tumor targeting with low to moderate side effects.¹⁴ This is of special importance since CD44v6 is also expressed on normal epithelia and finding that the drug is well tolerated in effective doses makes it even more useful for new applications such as multiple myeloma.

In conclusion, plasma cells in MGUS and asymptomatic MM rarely exhibit CD44v6, whereas CD44v6 expression is frequently detectable by flow cytometry analysis in symptomatic MM and PCL and is positively correlated with chromosome 13q deletion. Therefore, immunoconjugates targeting CD44v6, such as bivatuzumab mertansine – currently being used to treat various solid tumors within clinical trials – should also be investigated in advanced plasma cell tumors, for instance high-risk MM.

PL: responsible for the conception and design of the study, genetic testing regarding the del13q14, wrote parts of the paper and critically reviewed and approved the final version; SE: responsible for the conception and design of the study for the and FACS analysis. Wrote parts of the paper and critically reviewed and approved the final version. CS: responsible for the design of the study and for the design of the FACS analysis, together with SE, critically reviewed and approved the final version; GM: responsible for the conception of the study, antibody preparation; critically reviewed and approved the final version; GS: responsible for the conception of the study, for the antibody preparation; critically reviewed and approved the final version; HD: responsible for the conception and design of the study, for the linkage between the methods, reviewed and approved the final version; MS: responsible for the conception and design of the study, and for the FACS analysis and the evaluation of the FACS data: wrote parts of the paper and critically reviewed and approved the final version.

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