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Specific Detection of CD56 (NCAM) Isoforms for the Identification of Aggressive Malignant Neoplasms with Progressive Development

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Alternative splicing of transcripts from many cancer-associated genes is believed to play a major role in carcinogenesis as well as in tumor progression. Alternative splicing of one such gene, the neural cell adhesion molecule CD56 (NCAM), impacts the progression, inadequate therapeutic response, and reduced total survival of patients who suffer from numerous malignant neoplasms. Although previous investigations have determined that CD56 exists in three major isoforms (CD56^{120kD}, CD56^{140kD}, and CD56^{180kD}) with individual structural and functional properties, neither the expression profiles nor the functional relevance of these isoforms in malignant tumors have been consistently investigated. Using new quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) strategies and novel CD56 isoform-specific antibodies, CD56^{140kD} was shown to be exclusively expressed in a number of highly malignant CD56⁺ neoplasms and was associated with the progression of CD56⁺ precursor lesions of unclear malignant potential. Moreover, only CD56^{140kD} induced antiapoptotic/proliferative pathways and specifically phosphorylated calcium-dependent kinases that are relevant for tumorigenesis. We conclude, therefore, that the specific detection of CD56 isoforms will help to elucidate their individual functions in the pathogenesis and progression of malignant neoplasms and may have a positive impact on the development of CD56-based immuno-

therapeutic strategies. (Am J Pathol 2009, 174:1160–1171; DOI: 10.2353/ajpath.2009.080647)

The neural cell adhesion molecule CD56 (NCAM) is a founding member of a large family of cell surface glycoproteins that share structural motifs related to immunoglobulin and fibronectin type III domains.^{1,2} Human CD56 is encoded by a single-copy gene on chromosome 11 that spans more than 314 kb and contains 19 major exons as well as 6 additional smaller exons.^{2–4} Alternative splicing results in the expression of three major isoforms that differ in their membrane association and their intracellular domains: the isoform CD56^{120kD}, which is linked to the plasma membrane by a glycosylphosphatidylinositol anchor, and the isoforms CD56^{140kD}/CD56^{180kD}, which both have a transmembrane domain and cytoplasmic tails of different lengths.²

Originally, CD56 was characterized as a mediator of cell-cell adhesion, but now it is also considered to be a signaling receptor that impacts cellular adhesion, migration, proliferation, apoptosis, differentiation, survival, and synaptic plasticity.^{5–10} CD56-mediated signaling can be activated after homophilic interaction or via heterophilic dimerization to a broad range of other molecules including the closely related adhesion molecule L1, fibroblast growth factor 1 (FGFR 1), the glial cell line-derived neurotrophic factor, and sulfate proteoglycans (CSPG and HSPGs).^{11–23}

Physiologically, CD56 is abundantly expressed in the developing as well as in the adult human brain and plays a pivotal role in neurogenesis, neuronal migration, and neurite outgrowth,^{19,24–26} on natural killer (NK) cells, a subset of T lymphocytes,^{27,28} as well as on neuroendocrine cells.²⁹ In human diseases, CD56 is a specific histological immune marker for the diagnosis of malignant ner-

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vous tumors (eg, medulloblastoma and astrocytoma),^{29,30} malignant NK/T-cell lymphomas (NK/T-NHLs),^{31,32} and neuroendocrine carcinomas (NECs).^{33–36} Moreover, increased serum levels of CD56 are associated with the progression of dementia of Alzheimer's type³⁷ as well as multiple myeloma (MM).^{38–42} Its overexpression in malignant neoplasms is associated with an aggressive tumor type, inadequate therapeutic response, and a reduced total survival time in a broad range of malignancies including lymphoblastic and myeloid leukemias (ALLs/AMLs),^{43–45} malignant melanomas,^{46,47} and numerous carcinomas.^{48–53}

Despite the correlation between CD56 expression and the progression of degenerative and neoplastic diseases, no reports of consistent investigations concerning the expression of different CD56 isoforms have been published. However, these data appear relevant as i) the different CD56 isoforms exhibit varying intramembrane localizations, mobility, and interaction partners²; ii) alternative splice products of many cancer genes that impact tumorigenesis are known to occur during tumor progression^{54,55}; and iii) CD56 transfected cardiomyocytes with stable overexpression of CD56 isoforms revealed strongly different, isoform-specific, gene expression profiles (S.G., unpublished data). Finally, because it has been determined that CD56 induces increased proliferation and decreased apoptosis in acute myeloid leukemias (AMLs) via the nuclear factor (NF)- κ B/*bcl2* pathway,⁵⁶ an effect that can be inhibited using the NF- κ B inhibitor wedelolactone,⁵⁶ the specific detection of CD56 isoforms may further elucidate their different functions in human malignant and degenerative diseases and therefore be the basis for novel CD56-related immunotherapeutic strategies.

Materials and Methods

Cell Lines and Human Tissue Samples

The human lymphoma/leukemia cell lines K562, U937, HL-60, Jurkat, Karpas, MEG01, Mo7e, SU-DH-L1, THP1, and MUTZ-2 were provided by the American Type Culture Collection (Manassas, VA) and by the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Plasmacytoma cell lines INA6, AMO-1, MOLP-8, RPM-8226, U266, KMS-12-BM, and MMIS were made available by Dr. T. Stühmer (University of Würzburg, Würzburg, Germany). Normal human tissue samples and tumor specimens were obtained from biopsy and autopsy material as described.^{56,57}

Specific Detection of CD56 Isoforms by Quantitative RT-PCR (qRT-PCR)

For qRT-PCR, RNA was extracted as previously described and 5 μ g of total RNA was reverse-transcribed adding 1 μ Ci of ³²P-dCTP. Adjustment to equal amount and quantification by radioactive RT-PCR were performed as described⁵⁶ using 0.1 μ Ci ³²P-dCTP per 25 μ l of reaction mix and GAPDH control RT-PCR. The primers for amplification of CD56 isoforms were as follows: A: CD56com-UP, 5'-ATGCTGCAAACCTAAGGATCTCA-3';

B: CD56^{120kD}-LP, 5'-CTAACAGAGCAAAAAGAAGAGTC-3'; C: CD56^{140kD/180kD}-LP, 5'-TCATGCTTTGCTCTCGT-TCTCC-3'; D: CD56^{180kD}-UP, 5'-CGGACCCGGAGC-CCACCCAGCC-3'. Amplifications were performed to detect CD56^{120kD/125kD} [primers A + B, size of PCR products 2173 bp (120 kDa) and 2283 bp (125 kDa)], CD56^{140kD/180kD} [primers A + C, size of PCR products 2544 bp (140 kDa) and 3354 bp (180 kDa)] and CD56^{180kD} (primers C + D, size of PCR product 399 bp) at 60°C annealing temperature and for 35 cycles each.

Cloning and Sequencing of Human CD56 Isoforms

Amplificates from CD56 isoform-specific qRT-PCRs (CD56^{120kD}, CD56^{140kD}, and CD56^{180kD}) were isolated from agarose gels and cloned into the pGEM T-vector (Promega, Heidelberg, Germany), transformed in DH5 α *Escherichia coli* competent cells (Invitrogen, Heidelberg, Germany) and DNA from recombinant colonies was isolated by miniprep and sequenced by the cycle-sequencing method as previously described.⁵⁶ Homology to known CD56 isoforms was confirmed by comparison with CD56 isoform-specific sequences using Blast search (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD).

Recombinant Protein Expression of CD56 Isoforms

CD56 isoforms (CD56^{120kD}, CD56^{140kD}, and CD56^{180kD}) were subcloned into the pTNT vector (Promega) and the corresponding proteins were expressed in the eukaryotic TNT reticulocyte lysate system using SP6 RNA polymerase and methionine S³⁵ (following protocols from Promega). For bacterial protein expression and epitope mapping, overlapping isoform-specific subclones were generated by cloning corresponding fragments from the common N-terminal regions and specific C-terminal regions into the pGEX4T2 vector. After transformation of *E. coli* BL21 competent cells (Stratagene, Heidelberg, Germany), recombinant GST-fusion proteins were expressed and purified using glutathione Sepharose 4B following the instructions of the manufacturer (Amersham Biosciences, Heidelberg, Germany) and detected by Western blot using an anti-GST monoclonal mouse antibody (Calbiochem, Heidelberg, Germany).

Production and Testing of Novel CD56 Antibodies

Unmodified peptides and N-terminally acetylated and/or C-terminally amidated derivatives, respectively, were coupled to keyhole limpet hemocyanin via an additional terminal cysteine residue using sulfosuccinimidyl-4-[(N-maleimidomethyl)cyclohexane-1]-carboxylate (Pierce, Rockford, IL) or via primary amino groups using glutaraldehyde (Sigma-Aldrich, St. Louis, MO). Rabbits were immunized intradermally with peptide conjugates emul-

sified in complete Freund's adjuvant (primary injections) and Montanide ISA 206 (boosts). All sera were routinely affinity purified on peptide affinity matrices (Immuno-globe, Himmelstadt, Germany). Matrices for coupling through cysteine and primary amino groups were Ultra-Link iodoacetyl supported (Pierce) and NHS-activated Sepharose 4 Fast Flow (GE Healthcare, Freiburg, Germany), respectively. The CD56 sequence information used for peptide design was as follows: anti-CD56^{120kD} antibody IG-656: peptides specific for CD56^{120kD} appeared not to be suitable as antigens. Therefore, a CD56^{120kD}-derived sequence (SAQPTAIPATLGGNSAS) was chosen despite an extended region of sequence identity with the CD56^{140kD} and CD56^{180kD} isoforms. CD56^{120kD} specificity was obtained in a second step by purification on a peptide matrix based on a CD56^{120kD}-specific sequence (PATLGGNSAS). Anti-CD56^{180kD} antibodies IG-640, IG-658: PSTADSSVSPAPA (CD56^{180kD}-specific sequence both for immunization and purification). Extraction of whole protein and detection of CD56 isoforms/controls by Western blotting were performed as described.⁵⁶

Immunohistochemistry and Clonality Analysis

Immunohistochemical and immunofluorescence stainings as well as molecular detection of clonal plasma cell populations by PCR amplification of the immunoglobulin heavy chain gene locus (FR3A and FR2A) with subsequent fragment analysis were performed as previously described.^{56,58}

Cell Transfection and Microarray Analysis

For eukaryotic cell transfections, the CD56 isoforms (CD56^{120kD}, CD56^{140kD}, and CD56^{180kD}) were cloned into the mammalian expression MIDGE vector pMCV1.2 (Mologen, Berlin, Germany). Cells (10⁶) of the CD56-negative plasmacytoma cell line INA6 (provided by T. Stühmer, University of Würzburg), were transfected by electroporation with each 20 μ g/ml of pMCV1.2 clones corresponding to individual CD56 isoforms, 4 μ g/ml pEGFP (enhanced green fluorescent protein), and 4 μ g/ml of the expression plasmid for human truncated CD4 (pCD4 Δ) for subsequent enrichment of transfected cells after 6 days of cell culture as previously described.⁵⁹ Purified cells highly enriched (~98%) for GFP/pCD4 Δ were collected and the overexpression of CD56 isoforms was confirmed by CD56 isoform-specific RT-PCR (see above). For microarray experiments, total RNA was extracted with TriPure isolation reagent (Roche, Mannheim, Germany). The samples were analyzed for gene expression using Affymetrix (Santa Clara, CA) human HG U133 plus 2.0 arrays. Expression data were sorted by difference in expression and visualized using the Chester and Treeview software from Michael Eisen (Berkeley, CA). Overexpression of up-regulated genes was confirmed by qRT-PCR using the following gene-specific primers: keratin 8, forward 5'-CTGGTGGAGGACTTCAA-GAAC-3'; reverse 5'-GACCTCAGCAATGATGCTGTC-3';

HSP70, forward 5'-TTCCAGCCCCCAATCTCAGAGCCG-3', reverse 5'-GTCTCCGTCGTTGATCACCTGGAA-3'; RhoA GTPase, forward 5'-TTCGGAATGACGAGCACACG-3', reverse 5'-GTCTAGCTTGAGAGCAGCT-3'; thrombospondin 1, forward 5'-ACCGCATTCAGAGTCTG-3', reverse 5'-GACGTCCAACTCAGCATT-3'; NF-YB, forward 5'-GCCATACCTCAAACGGGAAAG-3', reverse 5'-TCTCCTTTCATAGCCTCTCTG-3'; Malat 1, forward 5'-TAGTTGGCAGTGGCGTGTAC-3', reverse 5'-GTTTCC-CCTCCCTCATCACA-3'; neuroligin 1, forward 5'-GAA-TAACGAGGGAGGGGAGGTGG-3', reverse 5'-CCG-CTGGAGAAAGATGGAATGGTG-3'; NPPA, forward 5'-CAGTGAGCCGAATGAAGAAGC-3', reverse 5'-CTCC-AATCCTGTCCATCCTGC-3'; NFkBIB, forward 5'-CTTG-TACCCGATTCCGACTT-3', reverse 5'-GCTTCTTG-CCTGCCGGTCTAT-3'; tankyrase 2, forward 5'-GCT-GAGCCAACCATCCGAAAT-3', reverse 5'-GTAGGCG-GCAGGTTTGTAGAT-3'; GAPDH, forward 5'-CAACAGC-GACACCCACTCCTC-3', reverse 5'-CATGTGGGCCAT-GAGGTCCAC-3'.

Detection of Apoptosis and Phospho-Kinases

Presence of phosphatidylserine in the outer leaflet of the plasma membrane was detected by flow cytometry using fluorescein isothiocyanate-coupled annexin V (annexin V; BD Biosciences, Heidelberg, Germany) as described.⁵⁶ For further analysis of apoptotic cells, the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) assay was used (Apo-BRDU kit; BD, Pharmingen, Heidelberg, Germany) following the instructions of the manufacturer. For the detection of Ca²⁺-dependent phosphorylated kinases, whole protein extracts from INA6 cells transfected with individual CD56 isoforms (see above) were tested by Western blotting⁵⁶ using phospho-antibody sampler kits (Cell Signaling, Danvers, MA) specific for the Akt-pathway, the erk1/2 pathway, the p38 MAP kinase pathway, the SAPK/JNK pathway, as well as for CamKII, phospho-CamKII, and phospho-PKA C. The detection of intracellular calcium was performed by loading CD56-transfected INA-6 cells with the calcium indicator Fluo-4 following the instructions of the manufacturer (Molecular Probes, Invitrogen, Heidelberg, Germany).

Statistical Analysis

Data are expressed as means $\pm$ SD. Comparisons were made using the Mann-Whitney *U*-test and the SPSS for Windows software package (SPSS Inc., Chicago, IL). Differences were considered significant when *P* < 0.05.

Results

Detection of CD56 Isoforms in Normal Human Tissues, Cell Lines, and Malignant Human Tumors by Isoform-Specific qRT-PCR

For the specific amplification of CD56 isoforms, an isoform-specific qRT-PCR strategy with one oligonucleotide

Figure 1. CD56 isoform-specific quantitative RT-PCRs⁵⁶ using cDNAs from normal tissues and malignant neoplasms. **A:** Detection of CD56 isoform RNA in normal tissues (lane 1 = duodenum, lane 2 = salivary gland, lane 3 = thymus, lane 4 = lymph node, lane 5 = spleen, lane 6 = liver, lane 7 = testis, lane 8 = lung, lane 9 = kidney, lane 10 = pancreas, lane 11 = tonsil, lane 12 = placenta, lane 13 = cerebral cortex, lane 14 = basal ganglia, lane 15 = cerebellum) in five different samples from individual patients in comparison with GAPDH transcripts (loading control). In all extraneural normal human tissues (lanes 1 to 12) a ubiquitous expression of CD56^{120kD} as well as CD56^{140kD} and only few CD56^{180kD} transcripts were detected, whereas CD56^{140kD} and CD56^{180kD} were the predominantly expressed isoforms in tissue samples from the central nervous system (lanes 13 to 15). **B:** Detection of CD56 isoform RNA in human *ex vivo* samples from CD56⁺ malignancies (*n* = 153) of different type [multiple myeloma (MM, *n* = 22), acute myeloid leukemia (AML, *n* = 36), malignant T-NHLs and NK/T cell lymphomas (T-NHL, *n* = 28), small lung cell carcinomas (SLCC, *n* = 31), malignant melanomas (Mal. Mel, *n* = 14), and rhabdomyosarcomas (RMS, *n* = 22)] as well as in CD56⁺ tumor cell lines (cell lines, *n* = 10). In contrast to normal tissue, CD56^{140kD} was the only CD56 isoform found in human *ex vivo* samples from CD56⁺ malignancies and tumor cell lines.

primer from the homologous N-terminus of CD56 combined with isoform-specific C-terminal primers was used. As shown in Figure 1a, a consistently strong expression of the CD56^{120kD} and CD56^{140kD} isoforms (except the muscle-specific CD56^{125kD} version that is exclusively expressed in skeletal muscle instead of the CD56^{120kD} isoform) and only small amounts of CD56^{180kD} mRNA were detected in all extraneural normal human tissues tested, whereas in samples from the central nervous system a predominant expression of CD56^{140kD} and CD56^{180kD} was found. In contrast, all human CD56⁺ tumor cell lines (*n* = 10) as well as all *ex vivo* samples from CD56⁺ human malignancies (*n* = 153), including multiple myelomas (*n* = 22), acute myeloid leukemias (*n* = 36), malignant T-NHLs and NK/T cell lymphomas (*n* = 28), small lung cell carcinomas (*n* = 31), malignant melanomas (*n* = 14), and rhabdomyosarcomas (*n* = 22), exhibited an exclusive expression of CD56^{140kD}, the level of which is significantly higher than in normal tissues (Figure 1b).

Generation of CD56 Isoform-Specific Antibodies

To test the specificity of commercial and novel CD56 antibodies, we cloned the human CD56 isoforms CD56^{120kD}, CD56^{140kD}, and CD56^{180kD} (Figure 2a) and

expressed them recombinantly either as full-length proteins in reticulocyte lysates (Figure 2b) or as glutathione S-transferase fusion proteins coding for the common N-termini as well as the isoform-specific C-terminal regions (Figure 2c). Among 12 commercial anti-CD56 antibodies, 7 (Table 1) revealed a strong detection of CD56 and its isoforms. In particular, clones 1B6 (Novocastra, Newcastle upon Tyne, UK) and H-300 (Santa Cruz Biotechnology, Santa Cruz, CA) bind within the common N-terminus of CD56 and recognize all isoforms (Figure 2d), clone 123C3 (Santa Cruz; Abcam, Cambridge, MA) and N-CAM 13 (BD; Fitzgerald Industries, Concord, MA) bind within exon 11/12 and also recognize CD56^{120kD}, CD56^{140kD}, and CD56^{180kD} (Figure 2d). Interestingly, clone C-20 (Santa Cruz) was shown to bind in part at the end of C-terminus of exon 14 common to all CD56 isoforms and to the 5'-end of exon 15, which is specific for CD56^{120kD}. Finally, clone 12F11 (BD) and NCAM-OB11 (Sigma) were shown to detect the C-terminus-specific for both CD56^{140kD} and CD56^{180kD} (Figure 2d). Because none of the antibodies tested showed specificity for CD56^{120kD} or CD56^{180kD}, we generated specific polyclonal rabbit antisera directed against the specific C-termini of CD56^{120kD} (IG-656, Immunoglobine) and CD56^{180kD} (IG-658, Immunoglobine), respectively. As shown in Figure 2d, all of these antisera bound specifically to their corresponding isoforms and their binding was shown to be directed against the corresponding isoform-specific C-terminal regions used for immunization (Figure 2d).

Immunohistochemical Detection of CD56 Isoforms in Normal Human Tissues and Malignant Tumors Using Isoform-Specific Antibodies

The specificity of the CD56 isoform-specific antibodies was tested by Western blot analysis (not shown) as well as immunostaining on normal human tissue samples with high mRNA levels of individual CD56 isoforms. As shown in Figure 3, a and b, all isoform-specific antibodies detected CD56 isoform protein levels that correlated to the mRNA amounts of each respective isoform detected in the corresponding human formalin-fixed and paraffin-embedded tissue [eg, pancreas (Figure 3a) and cerebral cortex (Figure 3b)]. As expected after the qRT-PCR experiments, significantly stronger immunostaining than in normal tissues was observed in CD56-positive aggressive malignant neoplasms [eg, NK/T cell lymphoma (Figure 3c) and multiple myeloma (Figure 3d)] using the CD56^{pan} antibody (clone 1B6, Novocastra) as well as the CD56^{140/180kD}-specific antibody (clone NCAM-OB11, Sigma), whereas the reactions with antibodies specific for CD56^{180kD} (IG-658, Immunoglobine) and for CD56^{120kD} (IG-656, Immunoglobine) revealed negative results. Furthermore, as was to be expected for CD56, which is a cell membrane protein with transmembrane and intracellular domains, staining was located on the cell membrane as well as within the cytoplasm.

Figure 2. The exon structure of CD56 isoforms and identification of CD56 isoform-specific antibodies. **A:** Schematic presentation of the CD56 gene locus showing exons 1 to 19 constituting CD56 isoforms (*1 indicates the insertion point of the small VASE- or p-exon located between exons 7 and 8; *2 indicates the locus of the SEC-exon as well as the locus of four additional small exons located between exon 12 and 13).^{83–86} IgI–V indicates immunoglobulin homology modules; F3-I and -II correspond to fibronectin type III homology modules. **B:** Exon composition of the predominant human CD56 isoforms CD56^{120kD}, CD56^{140kD}, and CD56^{180kD}. Corresponding cDNAs were expressed as full-length proteins in reticulocyte lysates (see **D**). **C:** CD56-GST fusion proteins representing the N-terminus containing the immunoglobulin domains (GST-CD56 Ig domains) and fibronectin domains (GST-CD56 F3 domains) common to all CD56 isoforms as well as the isoform-specific C-terminal regions of individual CD56 isoforms (GST-CD56^{120kD} C-ter, GST-CD56^{140kD} C-ter, GST-CD56^{180kD} C-ter). **D:** Identification of CD56 isoform-specific antibodies by Western blotting using recombinantly expressed full-length CD56 isoforms (**left: a to c**) and CD56-GST fusion proteins (**right: 1 to 5**). As indicated in Table 1, clone 1B6 and clone 123C3 recognize all CD56 isoforms by binding either within the N-terminal immunoglobulin domain region (clone 1B6) or the fibronectin domain region (clone 123C3). In contrast, clone NCAM-OB-11 binds to an epitope in the common C-terminus of the CD56^{140kD} and CD56^{180kD} isoforms, whereas the novel antibodies IG 656 and IG 658 specifically detect CD56^{120kD} and CD56^{180kD}, respectively. As control (ctr), full-length CD56 isoforms were expressed in the presence of methionine S³⁵ (**left**) and GST-fusion proteins were detected by an anti-GST antibody.

CD56 Isoform-Specific Gene Expression Profiles and Phosphorylation of CamKII

To investigate CD56 isoform-specific gene expression profiles in malignant neoplasms, the CD56-negative plasmacytoma cell line INA6 was transfected with the human CD56 isoforms CD56^{120kD} and CD56^{140kD}. Using co-transfection of pEGFP (enhanced green fluorescent protein) and truncated human CD4 (pCD4Δ), transfected cells were purified to >95% transfected cells using CD4

affinity chromatography (Figure 4a).⁵⁹ CD56^{120kD} and CD56^{140kD} isoform-specific gene expression profiles were generated using the Affymetrix whole genome chip U133-2.0. In comparison with the mock control, 99 differentially expressed genes (55 up-regulated, 31 down-regulated; not shown) were detected in INA6 cells transfected with CD56^{120kD}, whereas INA6 cells transfected with CD56^{140kD} showed 82 differentially regulated genes (41 up-regulated, 41 down-regulated; not shown). In direct comparison between the expression profiles of

Table 1. Determination of the Isoform Specificity of CD56 Antibodies by Western Blot Epitope Mapping

Clone number/company/source	CD56 isoform specificity	Binding region
1B6, Novocastra (mouse mc)	120 kDa, 140 kDa, 180 kDa	N-terminus
H-300, Santa Cruz (rabbit pc)	120 kDa, 140 kDa, 180 kDa	N-terminus
123C3, Santa Cruz, Abcam (mouse mc)	120 kDa, 140 kDa, 180 kDa	Exon 11/12 (F3-1)
N-CAM 13, BD, Fitzgerald (mouse mc)	120 kDa, 140 kDa, 180 kDa	Exon 11/12 (F3-1)
C-20,* Santa Cruz (goat pc)	120 kDa, 140 kDa, 180 kDa	C-terminus
IG-656, immunoglobulin (rabbit pc)	120 kDa	C-terminus
12F11, BD (rat mc)	140 kDa, 180 kDa	C-terminus
NCAM-OB11, Sigma (mouse mc)	140 kDa, 180 kDa	C-terminus
IG-658, immunoglobulin (rabbit pc)	180 kDa	C-terminus

*This antibody was mapped to react with the specific C-terminus of CD56^{120kD/125kD} (exon 15) and the C-terminal region of exon 14 common to all CD56 isoforms (not shown).

No specific binding was achieved for the following antibodies (clone, company): UJ13A, Abcam; NKI-nbl-1, Abcam; MEM-188, Abcam; 12F8, BD; 56-C04, oncogene.

The bold indicated antibodies were produced and tested in cooperation with immunoglobulin (see also Material and Methods). mc, monoclonal, pc, polyclonal.

Figure 3. **A** and **B:** Detection of CD56 isoforms using immunohistochemistry in formalin-fixed, paraffin-embedded normal human pancreas (**A:** PN = peripheral nerve, GA = ganglion, EP = exocrine pancreatic gland, LI = Langerhans island) and brain (**B:** WM = white matter, GM = gray matter) from different individuals ($n = 15$) using anti-CD56 antibodies (**top row:** H&E; **second row:** pan CD56 antibody 1B6 immunostain; **third row:** CD56^{120kD}-specific antibody IG656 immunostain; **fourth row:** CD56^{140kD/180kD}-specific antibody NCAM-OB-11 immunostain; **bottom row:** CD56^{180kD}-specific antibody IG658 immunostain). **A:** In normal pancreatic tissue, a co-expression of CD56^{120kD} and CD56^{180kD} was found in exocrine pancreatic glands, peripheral nerves, and ganglion cells, whereas a weak but exclusive expression of CD56^{120kD} was demonstrated in Langerhans islands. In contrast, CD56^{180kD} is neither expressed in exocrine/endocrine pancreatic tissue nor in peripheral nerves. No CD56 at all was detected in the perineural pancreatic stroma. **B:** In the normal cerebral cortex, CD56^{140kD} was found in the gray matter as well as in the white matter, whereas CD56^{180kD} is expressed exclusively in the gray matter and no CD56^{120kD} was detectable in either of the regions. **C:** Tissue microarray (TMA) of samples from 83 individual CD56⁺ or CD56⁻ T-cell lymphoma cases [**top:** excerpt of sample overview; **bottom:** enlargements from two representative cases (**left:** CD56⁺; **right:** CD56⁻)] using anti-CD56 antibodies [**top enlargement:** 1B6 (pan CD56); **second enlargement:** IG656 (specific for CD56^{120kD}); **third enlargement:** NCAM-OB-11 (specific for CD56^{140kD/180kD}); **bottom enlargement:** IG658 (specific for CD56^{180kD})]. In all CD56-positive peripheral T-cell lymphomas examined ($n = 28$), CD56^{140kD} was the only detectable isoform (negative immunostaining using antibodies IG656 and IG658). **D:** Detection of CD56 isoforms in bone marrow trephines from 72 cases of multiple myeloma (MM) using conventional staining (May-Grünwald stain, **top**) as well as immunostaining with anti-CD56 antibodies [**second panel:** 1B6 (pan CD56); **third panel:** IG656 (specific for CD56^{120kD}); **fourth panel:** NCAM-OB-11 (specific for CD56^{140kD/180kD}); **bottom panel:** IG658 (specific for CD56^{180kD})]. CD56^{140kD} (positive staining with 1B6 and NCAM-OB-11) was found in every sample examined, whereas none of the samples contained CD56^{120kD} (negative staining using IG656) or CD56^{180kD} (negative staining using IG658). One representative sample is shown.

CD56^{140kD} and CD56^{120kD}, 85 probesets showed at least a fourfold difference in gene expression (36 up-regulated and 49 down-regulated for CD56^{140kD}; Figure 4b). Interestingly, most of the genes induced by CD56^{140kD} in comparison with CD56^{120kD} that have previously been functionally characterized exhibit an association to multiple myeloma (RhoGTPase,⁶⁰ HSP70,⁶¹ thrombospondin,⁶² NPPA,⁶³ keratin 8,⁶⁴ tankyrase 2⁶⁵) or to procarcinogenic, antiapoptotic signaling pathways (NFYB,⁶⁶ NF- κ B,⁵⁶ neuroligin,⁶⁷ and Malat1^{68,69}). The validity of the gene expression data were verified by mea-

suring the expression levels of selected genes by quantitative RT-PCR⁵⁶ using gene-specific primers (Figure 4c). Moreover, the functional relevance of the up-regulation of antiapoptotic pathways was shown by annexin V staining as well as using a TUNEL assay. Here, it was determined that CD56^{140kD} transfection in INA6 cells induces a 2.5-fold decrease in apoptosis in comparison with mock controls and CD56^{120kD}-transfected cells (Figure 4d).

In addition to these gene expression analyses, we used the purified INA6 cells expressing individual

Figure 4. CD56 isoform-specific gene expression profiles and induction of antiapoptotic/proliferative pathways. **A:** After co-transfection of the CD56-negative MM cell line INA6 with Δ CD4, GFP, and either CD56^{120KD} or CD56^{140KD}, transfected cells were enriched by affinity chromatography. FACS analyses for GFP expression (**top**) and CD56 isoform-specific RT-PCRs (**bottom**) show highly enriched INA 6 cells with specific overexpression of individual CD56 isoforms. **B:** Eighty-five probesets representing genes with at least a fourfold difference in expression between the isoforms CD56^{140KD} and CD56^{120KD}. Red color depicts increased expression and green color decreased expression. **C:** Analysis of MM-associated procarcinogenic/antiapoptotic gene induction by CD56^{140KD} in INA6 cells using quantitative RT-PCR.⁵⁶ In comparison with INA6 cells transfected with CD56^{120KD} and INA6 cells alone, INA6 cells transfected with CD56^{140KD} had elevated transcript levels of Malat 1, thrombospondin, HSP70, neurologin, NFYB, and NFKBIB. **D:** Annexin V FACS analysis (**left**) and TUNEL assay (**right**) demonstrate a 2.5-fold decrease in apoptotic rate in INA6 cells transfected with CD56^{140KD} in comparison with CD56^{120KD} and controls. **E:** Western blot analyses using antibodies specific for phosphorylated CD56-dependent kinases² show an increased phosphorylation of CamKII in CD56^{140KD}-overexpressing INA6 cells, whereas no changes were seen for other CD56-related kinases such as Akt and erk. The presence of similar amounts of total unphosphorylated CamKII in all samples was shown by Western blotting using a CamKII-specific antibody (Cell Signaling, Danvers, MA). **F:** Detection of intracellular calcium using the green fluorescent chromogen fluo-4 show increased calcium content in the cytoplasm of INA6 cells transfected with CD56^{140KD} cells in comparison with INA6 cells transfected with CD56^{120KD} cells or INA6 cells alone.

Figure 5. Identification of CD56^{140kD} as a prognostic marker in MGUS, an example of a CD56-positive precursor neoplasia of uncertain malignant potential. **A (top):** Scattered (5 to 10%) atypical plasma cells (**top left**, May-Grünwald stain) with immunohistochemical staining for the plasma cell marker CD138 (**top right**) in conventional bone marrow smears and trephines from cases of CD56⁺ (main panel) and CD56⁻ (**inset**) MGUS show positive results irrespective of CD56 expression. **A (bottom):** Trephines from CD56⁺ MGUS cases show specific CD56 staining (antibody NCAM-OB11, main panel), whereas no specific staining is visible in samples from CD56⁻ MGUS cases (**inset**) using the same antibody. **A (bottom right):** Predominant staining of λ and weak staining of κ indicate immunoglobulin light chain restriction for λ in conventional bone marrow smears and trephines from patients with MGUS. **B:** Confirmation of the coexistence of CD138 (Cy3, red) and CD56 (Cy2, green) in clonal plasma cells in MGUS by immunofluorescence showing expression of both markers in identical plasma cell populations (merged yellow). The clonality was confirmed by the detection of a clonal rearrangement of the framework 3a (FR3a) region of the immunoglobulin heavy chain (**bottom right**). **C:** Of 26 CD56⁺ cases of MGUS (36% of total, first column), 22 progressed to morphologically and clinically manifest multiple myeloma within 1.5 years after initial diagnosis (31% of total, second column), whereas this was the case for a significantly smaller proportion of CD56⁻ MGUS patients [9 (13% of total, fifth column) of 46 (64% of total, fourth column)]. Eighty-five percent of CD56⁺ MGUS cases were also diagnosed with other known risk factors^{70,71} for the progression of MGUS to multiple myeloma (third column), whereas this was only the case for 25% of CD56⁻ MGUS cases (sixth column). Significant correlations ($P < 0.001$) between CD56 expression and the progression of MGUS to multiple myeloma as well as established risk predictors for this progression were confirmed using the Mann-Whitney *U*-test.

CD56 isoforms to investigate their effect on the activation of CD56-dependent kinase pathways.² To do this, we used phospho-specific anti kinase antibody sampler kits and were able to show an increase in the phosphorylation of the calcium-dependent calmodulin kinase II (CamKII, Figure 4e) for CD56^{140kD}, but not for CD56^{120kD} or controls. Using immunofluorescence with the fluo calcium indicator fluo 4, we found that this effect is accompanied by an increase in intracellular calcium (Figure 4f).

CD56 Expression and Isoform Pattern in the Differential Diagnosis of Neoplasms with Uncertain Malignant Potential

As the clinical course of CD56 neoplasms with low/uncertain malignant potential is often unclear, there is need for indicators to better predict the progression of these tumor types. Based on the evidence for the tumorigenic potential of CD56^{140kD} we had found, we examined the predictive value of the expression of CD56 isoforms in monoclonal gammopathies of uncertain significance (MGUS), which is an example for such a precursor le-

sions of CD56-positive malignant neoplasms. To this end, we performed CD56 isoform-specific immunohistochemical stainings on bone marrow trephines that were infiltrated by MGUS, a plasma cell disorder that evolves to symptomatic multiple myeloma in 30 to 40% of cases.⁷⁰ As shown in Figure 5a, ~30% of the 72 MGUS patients examined ($n = 72$) exhibited CD56-positive clonal plasma cells in bone marrow trephines with exclusive expression of CD56^{140kD}. Coexistence of CD138 and CD56 in clonal plasma cells was confirmed by immunofluorescence (Figure 5b). More interestingly however, in longitudinal retrospective analyses (8 to 12 years), ~85% of CD56-positive MGUS cases showed progression to multiple myeloma characterized by clinical progression and nodular clonal plasma cell infiltrates in the bone marrow (>20%, Figure 5c). Not surprisingly, the attributes of these patients were in good correlation with the known risk factors for the progression of MGUS to MM.^{70,71} In contrast, 75% of the patients among the 70% MGUS cases with CD56-negative clonal plasma cells remained histologically and clinically stable and exhibited no increase of clonal plasma cell infiltrates during the time of the analysis.

Discussion

In this study, we present the specific molecular genetic and immunohistochemical evidence of the predominant CD56 isoforms CD56^{120kD}, CD56^{140kD}, and CD56^{180kD} present in the human using a novel qRT-PCR strategy as well as identification/generation of novel CD56 isoform-specific antibodies. Using these techniques, we were able to show that i) CD56^{140kD} is the only CD56 isoform expressed in numerous aggressive CD56-positive malignant neoplasms ($n = 155$) and cell lines ($n = 10$); that ii) gene expression profiles specific for CD56^{140kD} include the induction of tumor-specific genes as well as anti-apoptotic signaling pathways and increased calcium influx with subsequent activation/phosphorylation of Ca²⁺-dependent kinases; and iii) in MGUS, an example of a CD56-positive precursor lesion with uncertain dignity and malignant potential, the expression of CD56^{140kD} is a specific marker for progression of a clonal plasma cell population and for transition to symptomatic MM that is correlated with known risk factors for disease progression.

The production of several protein isoforms from one pre-mRNA by alternative splicing contributes to proteomic diversity and controlled switching of many cancer genes to specific splicing alternatives that occurs during tumor progression and plays a major role in carcinogenesis.^{54,55} Therefore, the detection of CD56 isoforms using the isoform-specific qRT-PCR strategy and antibodies described in this report may prove to be of significant relevance because the CD56 isoforms predominantly expressed in humans exhibit large structural and functional variations. In contrast to the membrane-associated CD56^{120kD} isoform present in various healthy tissues, the predominant CD56^{140kD} isoform in aggressive malignant neoplasms exhibits a transmembrane domain with a cytoplasmic portion and dependent regulatory tyrosine and nonreceptor tyrosine kinases.² Moreover, various studies have demonstrated that CD56 isoforms harbor different intramembrane microdomain localizations and mobility. These differences may be related to interactions with intracellular molecules and components of the cytoskeleton because individual CD56 isoforms have been demonstrated to have a number of direct or indirect differential interactions with various intracellular molecules such as Fyn (CD56^{120kD}), GAP (CD56^{120kD} and CD56^{140kD}), FAK and spectrin (CD56^{140kD}).^{72,76} According to our data, these structural variations also appear to be of major functional importance in tumorigenesis because we were able to show an overexpression of MM-associated and antiapoptotic/proliferative antigens as well as a 2.5-fold decreased rate of apoptosis in annexin V stainings and TUNEL assays after transfection of the CD56-negative MM cell line INA-6 with CD56^{140kD}, but not with CD56^{120kD} or mock controls. Moreover, as inhibition of Cam kinases augments cell death⁷⁷ and down-regulation of Cam kinase activator results in cell cycle arrest with impact for cancer therapy,^{78,79} the CD56^{140kD}-dependent calcium influx and increased phosphorylation/activation of the calcium-dependent kinase CamKII could be a trigger of CD56^{140kD}-dependent tumor growth and may therefore be the basis for further

investigations addressing the therapeutical potential of CD56-dependent pathways. Finally, the CD56-dependent phosphorylation/activation of CamKII also appears functionally regulated by CD56^{140kD} because CD56 is known to increase the Ca²⁺ influx via phosphatidylinositol 4,5-bisphosphate (PIP2), diacylglycerol (DAG), and arachidonic acid (AA),² leading to a subsequent increase in the phosphorylation of Ca²⁺-dependent kinases such as CamKII.

Concerning the obvious tumorigenic potential of CD56^{140kD} and its relation to the progression of malignant neoplasms, we finally investigated MGUS as an example for a CD56 neoplasm with low/uncertain malignant potential and could demonstrate the predictive value of CD56^{140kD} in a CD56-positive precursor lesion of unknown dignity and malignant potential. Although the clinical relevance of CD56 in MM has been the subject of extensive investigations^{40,41,71} and CD56^{140kD} was shown to be the exclusively expressed isoform in MM (see Figures 1b and 3d), the prognostic value of the expression of CD56 and its isoforms in MGUS remained to be clarified.^{70,80,81} In the retrospective analysis presented here, which is, to the best of our knowledge, the first of its kind concerning the expression of CD56 isoforms in a larger number of MGUS patients ($n = 72$, 8- to 12-year time span), a significant correlation between CD56^{140kD} expression and clinical as well as morphological transition of MGUS to MM was found, whereby the attributes of the patients in the group showing progressive MGUS were in good correlation with the known risk factors for the progression of MGUS to MM.^{70,71} These results are in agreement with previous reports in which CD56 was already described as progression marker in other neoplasms of unknown dignity (eg, the transition of histiocytosis X to Langerhans cell sarcoma⁸²), but further investigations will be needed to clarify the CD56 isoform specificity and its functional relevance in such neoplasms.

We therefore conclude that the specific detection of CD56 and its isoforms as well as the identification of CD56 isoform-dependent signaling cascades may contribute to the further elucidation of the progression and pathogenesis of CD56 malignant neoplasms. With regard to the predominantly inadequate response of CD56-positive aggressive neoplasms to conventional radio- and chemotherapy, our data may be the basis of alternative CD56-associated immune therapeutic approaches in analogy to the CD56/NF- κ B/bcl2 signaling pathway in AMLs described by us previously.⁵⁶

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