

Papers

Isolation and characterisation of antibodies which specifically recognise the peptide encoded by exon 7 (v2) of the human CD44 gene

A Borgya, A Woodman, M Sugiyama, F Donié, E Kopetzki, Y Matsumura, D Tarin

Abstract

Aims—Exon 7 of the human CD44 gene is overexpressed in many commonly occurring carcinomas. The aim of the study was to explore the diagnostic and therapeutic potential of this frequent abnormality.

Methods—A new monoclonal antibody (mAb, M-23.6.1) and a polyclonal antibody (pAb, S-6127) to the corresponding antigen were raised by immunising mice and sheep, respectively, with a specially constructed fusion protein HIV2 (gp32)-CD44 exon 7.

Results—Characterisation of mAb, M-23.6.1 by ELISA, western blotting, immunocytochemistry, and FACS analysis confirmed that it specifically recognises an epitope in the region between amino acids 19 and 33 of the peptide encoded by this exon. Western blotting experiments with two cell lines, RT112 and ZR75-1, known from RT-PCR data to be overtranscribing the exon, yielded a monospecific band of approximately 220 kDa, and immunocytochemistry showed discrete membrane staining on the same cell lines. Fluorescent antibody cell sorting (FACS) revealed binding to greater than 90% of the cells of each of these lines. Specificity of recognition of the antigen was shown by inhibition of the precise immunoreactivity typically seen in ELISA and Western blots, by pre-incubation with synthetic exon 7 peptide or fragments of it.

Conclusions—The new antibodies will be useful tools for the further analysis of abnormal CD44 isoforms and their clinical implications.

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There is now much evidence, reviewed below, that the expression of the CD44 gene is severely disturbed in several common types of human cancer. The repertoire of this gene includes a widely distributed family¹ of highly glycosylated transmembranous glycoproteins showing a di-

verse range of functions. The genomic structure of the human CD44 locus shows a 60 kilobase (kb) sequence composed of at least 20 exons.² Nine of these encode the almost ubiquitously expressed "standard" form (exons 1-5 and 16-19), while in humans at least a further 10 exons, numbered 7-15 and 20, are subject to inclusion through alternative splicing. This results in a large array of protein isoforms which are differentially expressed in various tissues and at various stages in development.

CD44 was originally described as an antigen on red blood cells and platelets and later work identified it as a lymphocyte homing receptor responsible for facilitating migration through lymph nodes.^{3,4} However, it is now clear that the various isoforms produced by this gene are involved in a wide variety of cell-cell interactions, which include acting as receptors for hyaluronic acid,^{5,6} collagen, and fibronectin,^{7,8} and more recent work has implicated them in tumour development and metastasis.⁹⁻¹³ However, the marked increase in the level of activity of the gene in cancer cells and the disorder, including the retention of introns, in the assembly of transcripts¹⁴ make it difficult to identify which, if any, of the inappropriately expressed elements are the key ones specifically associated with the neoplastic process. Certainly, expression of individual variant exons is altered in several malignancies; for example, that of exon 11 (v6) is increased in colon and breast carcinomas,^{11,13} of exon 7 in bladder, breast, and colon,¹⁵ and of exons 12/13 in cervix.¹⁶ Despite this evidence of grossly disturbed CD44 gene function, there is insufficient information at present to know whether imbalanced expression of an individual exon or of a particular combination of exons is specifically linked with induction and progression of malignancy. While much of this work has relied upon the use of reverse transcription-polymerase chain reaction (RT-PCR) to visualise abnormal gene transcription, several studies investigating CD44 protein expression have reported similar abnormal findings^{17,18} in tumours compared with normal tissues.

On the basis of such new observations the notion that abnormalities of CD44 expression could be useful as a diagnostic or prognostic

Boehringer Mannheim GmbH,
Nonnewald,
D-82372 Penzberg,
Germany
A Borgya
F Donié
E Kopetzki

Nuffield Department
of Pathology and
Bacteriology,
University of Oxford,
John Radcliffe
Hospital,
Oxford,
OX3 9DU, UK
A Woodman
M Sugiyama
Y Matsumura
D D Tarin

Correspondence to:
Dr Tarin.

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marker for malignancy is attracting much clinical interest. As with all new markers, several important issues, such as the availability of appropriate reagents and the reproducibility of the results must be examined and evaluated before they can be introduced into routine practice. Additionally in the case of CD44, which produces many isoforms, the most informative isoforms or component exons must be determined. Currently, it is believed that protein based assays such as enzyme linked immunosorbent assay (ELISA) and immunohistochemistry are the simplest and most flexible for clinical use. Thus the development of specific antibodies to CD44 gene products is vital for the evaluation of the potential role of this protein in routine tumour diagnosis. Various antibodies are available, either commercially or by donation, to the products of several of the standard form exons (for example, F10-44-2, exon 1 (Boehringer Mannheim); Hermes 3, exon 5 (Dr EC Butcher)), as well as to variant exons (for example, 3G5, exon 8 (R and D Systems); VFF8, exon 10 (Bender Medisystems); 2F10, exon 11 (R and D Systems); 2G1, exon 15 (Dr DL Cooper)). However, there was until recently no antibody available to the peptide encoded by exon 7.

Our previous RT-PCR studies had shown that one of the disturbances characteristically seen in many commonly occurring types of carcinoma (for example, breast, colon, and bladder) is the assembly of large transcripts containing the products of all exons, including the recently identified exon 7.^{11,15} The formulation of methods for simple rapid detection of large CD44 protein isoforms containing a segment encoded by this exon was therefore identified as an important priority. To satisfy this need, we constructed a fusion protein (HIV2gp32-CD44 exon 7) which was successfully used to raise monoclonal and polyclonal antibodies specifically recognising this component of human CD44, as described below.

Methods

PREPARATION OF SYNTHETIC PEPTIDES AND FUSION PROTEINS FOR CD44 EXON 7

Peptide synthesis

The sequence of CD44 exon 7 was obtained from Matsumura *et al.*¹⁵ Synthetic peptides corresponding to amino acids 1-43, 1-13, 9-23, 19-33, and 29-43 encoded by this sequence were synthesised to use in screening mono-

clonal antibodies against fusion proteins (see below). This was accomplished by 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry solid phase peptide synthesis on an Applied Biosystems model 431A peptide synthesizer, using the 0.25 mmol scale. Briefly, a 4 molar excess of amino acid derivatives was dissolved in dimethylformamide (DMF) and activated by the addition of *N*-hydroxybenzotriazole (HOBt) in *N*-methylpyrrolidinone (NMP) and *N,N*-dicyclocarbodiimide (DCC) in NMP. Twenty minute couplings in DMF were followed by deprotection of the *N*-termini by 3 and 10 min treatments with 20% piperidine in DMF. The peptide chain was extended through repetition of the activation/coupling/deprotection cycles. The *N*-terminus contained three γ -aminobutyric acid moieties, lysine, and biotin (attached to ϵ -amino group of lysine). The peptides were removed from the resin using trifluoroacetic acid (TFA) and scavengers. They were then precipitated in di-isopropylether, filtered, washed in di-isopropylether dissolved in 50% acetic acid, and freeze dried. Peptide purity was determined by reverse phase HPLC and identity verified by plasma desorption mass spectrometry. The sequences of the five synthetic peptides are shown in fig 1.

Fusion protein synthesis

For immunisation, biologically synthesised exon 7 proteins were used in an attempt to produce an antigen corresponding as closely as possible to the natural configuration in cells. As CD44 exon 7 encodes a peptide of only 43 amino acids, it is not suitable for cytoplasmic expression in a micro-organism. Therefore a duplicated fusion gene expressible in *E coli*, comprising of a part of the envelope protein gp32 of the HIV2 retrovirus ligated to two copies of the CD44 exon 7-DNA sequence in head to tail tandem repeat orientation, was constructed.

Construction of HIV2 (gp32)-partial gene (plasmid pUC18-HIV2-gp32) and E coli expression vector pDS56-6HIS-HIV2-gp32

The coding section of amino acids 48-162 of the HIV2-gp32 gene¹⁹ was synthesised by overlapping chemical gene synthesis and subsequent subcloning in the pUC18 plasmid. Then a fusion gene was constructed which codes for the *N*-terminus of the amino acid sequence MRGSHHHHHHTDPEF (poly-

CD44 exon 7 (1-13aa)-TLJSTSATATETA-NH₂
 CD44 exon 7 (9-23aa)-ATETATKRQETWDWF-NH₂
 CD44 exon 7 (19-33aa)-TWDWFSWLFLPSESK-NH₂
 CD44 exon 7 (29-43aa)-PSESKNHLHTTTQJA-NH₂
 CD44 exon 7 (1-43aa) TLJSTSATATETAKRQETWDWFSWLFLPSESKNHLHTTTQJA-NH₂

Figure 1 Sequences of CD44 exon 7 synthetic peptides. Standard one letter code except J = norleucine.

His tail) and the selected HIV2-gp32 antigen. For this, the vector pQE-10 (Viagen, Germany)²⁰ was digested with the restriction endonucleases BamHI and HindIII, and the resultant 3.4 kbp vector fragment isolated by agarose gel electrophoresis. Subsequently, the plasmid pUC18-HIV2-gp32 was digested by the restriction endonucleases BamHI and HindIII, and the 400 bp fragment isolated and ligated into the 3.4 kbp BamHI/HindIII-pQE-10 vector fragment. The desired plasmid was identified by restriction mapping and designated pDS56-6HIS-HIV2-gp32.

Construction of E coli expression vector pDS56-HIV2-CD44 exon 7

A fusion gene was constructed which encodes the N-terminus for the amino acid sequence MRGSHHHHHHTDPEF (poly-His tail), the selected HIV2-gp32 antigen and two copies of the CD44 exon 7 antigen. Two CD44 exon 7 gene fragments were produced by two polymerase chain reactions (PCR).²¹ In the first PCR, CD44 exon 7 DNA sequence was amplified from a template of subcloned CD44 cDNA (exon 5 to 11) incorporating the singular restriction endonuclease cleavage sites BamHI and HaeIII at the 5' and 3' end respectively. The PCR product was digested with BamHI and HaeIII and the 150 bp BamHI/HaeIII-CD44 exon 7 fragment purified by agarose gel electrophoresis. In the second PCR, the CD44 exon 7 DNA sequence was amplified from subcloned CD44 (exon 5 to 11) as template and provided with the singular restriction endonuclease cleavage sites HaeIII and HindIII at the 3' and 5' end respectively. After digestion with HaeIII and HindIII the 140 bp BamHI/HaeIII-CD44 exon 7 fragment was isolated by agarose gel electrophoresis.

The BamHI/HaeIII-CD44 exon 7 fragment from the first PCR reaction and the HaeIII/HindIII-CD44 exon 7 from the second PCR reaction were ligated by three-fragment ligation into the 3.8 kbp Bgl II/HindIII-pDS56-6HIS-HIV2-gp32 vector fragment. The plasmid was identified by restriction mapping and the PCR synthesised DNA regions checked by DNA sequencing.

Expression of HIV2(gp32)-CD44 exon 7 antigen in E coli

The *E coli* K12 strain RM82 (a methionine revertant of ED8654²²) was transformed with the expression plasmid pDS56-HIV2-CD44 exon 7 (ampicillin resistance) described above and the lacI repressor plasmid pUHA1 (kanamycin resistance).²⁰ The transformed cells were cultured in DYT medium (1% (wt/vol) yeast extract, 1% (wt/vol) Bacto Tryptone, Difco, and 0.5% NaCl) + ampicillin and kanamycin (50 mg/l) up to an absorbance of 0.6–0.9 at 550 nm. Cells were then induced with IPTG (1–5 mmol/l) for 4–8 h. The cells were harvested, washed with 10 mmol/l phosphate buffer (pH 6.8) and stored at –20°C until required.

Preparation of HIV2(gp32)-CD44 exon 7 antigen from E coli

To extract the expressed proteins from the cells, 20 g (wet weight) of RM82/pUHA1/pDS56-HIV2-CD44 exon 7 cells were resuspended in 100 ml 0.1 M Tris-HCl, pH 7, at 0°C; 30 mg lysozyme were added and the mixture was incubated for 20 min at 0°C, before the cells were lysed completely by mechanical dispersion (French press) and the DNA digested by the addition of 2 mmol/l MgCl₂ and 1 mg DNAase at 25°C for 30 min. Then 50 ml of 60 mmol/l EDTA, 6% Triton X100, and 1.5 M NaCl, pH 7.0, were added to the digestion mixture and incubated for a further 30 min at 0°C. The insoluble components (cell debris and inclusion bodies) were pelleted, resuspended in 100 ml phosphate buffer (0.1 M; pH 8.5), and incubated for 30 min at 25°C before the inclusion body products were isolated by centrifugation.

Purification of HIV2(gp32)-CD44 exon 7 antigen using metal chelate affinity chromatography

The ability of accessible neighbouring histidine residues (poly-His tail) to form tightly associated complexes with immobilised/chelated nickel ions was used for affinity purification of the HIV2(gp32)-CD44 exon 7 protein. Affinity purification of the HIV2(gp32)-CD44 exon 7 antigen was according to the method reviewed by Stueber *et al.*²⁰ Briefly, the inclusion body pellet (2.5 g wet weight) was suspended in 25 ml 6 M guanidine-HCl, 0.1 M phosphate buffer, pH 8.5, and stirred for 2 h at 25°C. The insoluble components were separated by centrifugation and the clear supernatant applied to an Ni-NTA-agarose resin column (ligand: NTA, nitrilotriacetic acid²³) equilibrated with 6 M guanidine-HCl, 0.1 M phosphate buffer, pH 8.5 (column volume: 50 ml, Ni-NTA agarose from Diagen). The column was washed with five volumes of 8 M urea, 10 mmol/l Tris-HCl, and 0.1 mmol/l phosphate buffer pH 8.5. The HIV2(gp32)-CD44 exon 7 antigen which had been bound to the nickel by the poly-His tail, was eluted with 8 M urea, 0.1 M phosphate buffer, pH 4.0. The full amino acid sequence of the resultant HIV2(gp32)-CD44 exon 7 fusion protein is shown in fig 2.

PREPARATION AND CHARACTERISATION OF MURINE MONOCLONAL ANTI-CD44 EXON 7 ANTIBODIES

Pathogen-free Balb/c mice, approx 12 weeks old, were primed by intraperitoneal (ip) injection of 100 µg of purified CD44 exon 7 fusion protein (HIV2(gp32)-CD44 exon 7) in Freund's complete adjuvant. The immune response was boosted by reinjecting a similar dose of antigen in Freund's incomplete adjuvant every four weeks for 12 weeks. A final course of injections of 100 µg antigen/mouse in phosphate buffered saline (PBS) was given intravenously (iv) 72, 48, and 24 h before their spleen cells were harvested for fusion with myeloma cells to generate hybridomas.

Splenocytes were harvested from immunised

MRGSHHHHHTDPEF **QQQQLLDVVKRQQLLRLTVWGTKNLQARVTAIE**
 (poly-HIS)

KYLQDQARLNSWGCAPRQVCHTTVPWVNS LAPDWDNMTWQEWKQ
 HIV2 (gp32)-117aa

VRYLEANISKSLEQAQIQQEKNMYELQKLSWD IRS *PAT* TLMST
 Exon 5;3 aa

SATATETATKROETWDWFSWFLPSESKNHLHTTTOMA *PAT* TLMSTSA
 Exon 7;43aa Exon 5;3aa

TATETATKROETWDWFSWFLPSESKNHLHTTTOMA
 Exon 7;43aa

One letter code; aa = amino acid. Bold capitals = 117aa section of HIV2(gp32). Bold capitals italic = 3aa section of CD44 exon 5. Bold capitals underlined = 43aa of full CD44 exon 7.

Figure 2 Protein sequence of HIV2 (gp32)-CD44 exon 7 fusion protein.

mice and fused with mouse myeloma cells according to Kohler and Milstein.²⁴ Briefly, splenocytes harvested by physical disruption were mixed in a 5:1 ratio with P3 × 63Ag8-653 mouse myeloma cells. Fusion was induced by incubation of the cell pellet with 50% polyethylene glycol 1500 (Boehringer Mannheim) for 1 min at 37°C. The fused cells were plated out in flat bottomed 96-well plates (Nunc) at 2 × 10⁴ cells per well in RPMI 1640 containing 10% fetal calf serum (FCS), azaserine-hypoxanthine (Sigma), and 100 U/ml interleukin-6 (Boehringer Mannheim) as a cytokine to replace feeder cells.

Supernatants were taken for screening at day 10 from wells with more than 30% confluent hybridoma cells. Reactivity was determined against the CD44 exon 7 fusion protein and against the CD44 exon 7 (1-43aa) peptide by an ELISA (see below). The positive hybridomas were further selected by testing their reactivity to natural CD44 exon 7 on cell lines in a cell ELISA (see below) and in western blotting. Further cloning of cells producing antibodies which specifically bind to natural CD44 exon 7 was undertaken using a fluorescent antibody cell sorter (FACS; Becton Dickinson). Supernatants of growing monoclonal antibodies were tested in the same way at the primary supernatants.

Reactive epitopes were mapped by preincubation of supernatants with 5 µg/ml of the five synthetic peptides (described above) (1-43aa, 1-13aa, 9-23aa, 19-33aa, 29-43aa) for 1 h at 37°C. Immunoreactivity was then determined in the peptide ELISA.

Three CD44 exon 7 specific hybridomas were selected for bulk production and purification. The class of these antibodies was determined by an iso-strip antibody isotyping kit (Boehringer Mannheim). Bulk production was undertaken by two methods: in vitro spinner flask culture (inoculum of 1 × 10⁵ cells/ml in RPMI 1640 + 10% FCS; stirred at 37°C for 7 d on a Techne biological stirrer) or in vivo in athymic nude mice, primed with pristane (Sigma) 10 d before an ip injection of 0.5 × 10⁶ hybridoma cells/mouse; ascites was harvested

after 7-14 d. The resultant IgG obtained by both methods was purified by absorption to protein A (using Bio-Rad MAPS II kit).

The purified mouse IgG samples thus obtained were characterised by ELISA, FACS scan, western blotting, and immunocytochemistry as follows.

ELISA for CD44 exon 7 fusion protein and synthetic peptide

For the fusion protein ELISA, 96-well microtitre plates were coated with 5 µg/ml fusion protein HIV2(gp32)-CD44 (exon 7) in carbonate coating buffer (Boehringer Mannheim) for 1 h at room temperature. Free binding sites were blocked for 30 min with blocking buffer containing 1% croctein C.

For the synthetic peptide ELISA, 96-well microtitre plates were coated with 200 µl/well streptavidin. After blocking with 1% croctein C, 2.5 µg/ml biotinylated peptide CD44 exon 7 (1-43aa) were bound to the plate in incubation buffer (sodium phosphate 40 mM, 0.5% croctein C) for 1 h at room temperature. After coating, both ELISAs were treated as follows.

The culture supernatants to be tested were added with or without the free peptides described above (1-43aa)(1-13aa)(9-23aa) (19-33) and (29-43) at 5 µg/ml and incubated for 1 h at room temperature. Wells were washed again before the addition of 20 mU/ml peroxidase labelled sheep anti-mouse *Fc*-gamma and *Fab* fragments (Boehringer Mannheim) and incubation for 1 h at room temperature. Reactivity was determined by incubating the wells with the colour substrate ABTS (Boehringer Mannheim) for 30 min at room temperature. The absorbance at 450/490 nm was measured on a Dynatech MR 700 microplate reader. Wells were washed with PBS containing 0.05% Tween 20 between each incubation step.

Cell ELISA for CD44 exon 7

For the screening by cell ELISA two cell lines were used: RT112, a human bladder carcinoma

line (ECACC No. 85061106) showing high exon 7 expression as determined by RT-PCR; and MDA-4A4, a subclone from MDA-MB-435 (donated by Dr Janet Price), a human breast carcinoma line, with no detectable exon 7 transcription.

The cells were grown in 96-well plates in Dulbecco's modified Eagle's medium (DMEM) + 10% FCS until 70–80% confluent. The culture medium was removed, cells washed with PBS, and allowed to air dry. Cells were fixed with cold methanol and stored at -20°C . Before use, plates were given 15 min to return to room temperature and non-specific sites were blocked with blocking buffer containing 1% croton C. Test supernatants/antibodies were incubated either for 2–4 h at room temperature or overnight at 4°C . Wells were then incubated with 20 mU/ml peroxidase labelled sheep anti-mouse Fab fragments (Boehringer Mannheim) for 1 h at room temperature. Reactivity was determined by the addition of the colour substrate BM-Blue (Boehringer Mannheim), and incubated for 30 min at room temperature before the reaction was stopped with 1M H_2SO_4 . The absorbance was measured at 450/690 nm in an SLT 340 ATTC microplate reader. Wells were washed between each step with PBS containing 0.1% Tween 20.

Western blotting

RT112, MDA 4A4, HT29 (human colon carcinoma), and ZR75 (human breast carcinoma; ATCC No CRL 1500) cell lines were grown to 70–80% confluence in DMEM + 10% FBS. The cells were removed from the flask using a flexible cell scraper (Gibco) and washed in PBS + 0.02 mmol/l amidinophenylmethane sulphonyl fluoride (APMSF) (Sigma) and 1 mmol/l EDTA as protease inhibitors. The cell pellet collected by centrifugation at 600 g for 15 min was lysed on ice for 15 min with 1:1 lysis buffer (composition in mmol/l: tris 20 (pH 8), NaCl 150, CHAPS 20, EDTA 2, APMSF 0.04, plus 20 $\mu\text{g}/\text{ml}$ aprotinin). The resultant lysate was clarified by centrifugation (10 000 g, 15 min), protein concentration determined (Bio-Rad protein assay kit), adjusted to 1 mg/ml by dilution with PBS, and mixed 1:1 (vol/vol) with gel loading buffer. Lysates and molecular weight standards were boiled for 5 min before separation by 6% SDS-PAGE under reduced conditions. The separated proteins were electroblotted (100 mA, 1 h) to immobilon-P membrane (Millipore) using transfer buffer 48 mmol/l tris, 39 mmol/l glycine, 0.1% SDS, 20% methanol, pH 9.2). Non-specific reactions were blocked with tris buffered saline (TBS) containing 5% skimmed milk before the membrane was incubated overnight at 4°C with either mAb Hermes-3 (anti-standard CD44 exon 5) (1/1000) as a positive control or test supernatants/antibodies. Reactivity was determined by the addition of peroxidase conjugated anti-mouse IgG (Sigma or Boehringer Mannheim, 1/1000) for 1 h at room temperature and the signals visualised using the enhanced chemiluminescence (ECL) detection

system (Amersham or Boehringer Mannheim). Hermes-3, ascites, and purified antibodies were diluted in 5% skimmed milk, the membrane was washed between each incubation step with TBS containing 0.1% Tween 20.

Immunocytochemistry

To determine the cellular localisation of the epitopes recognised by the test antibodies, the RT112, ZR75, MDA 4A4, and HT29 cell lines were grown to 75% confluence on slide flasks (Gibco) in DMEM containing 10% FBS. These cell lines were chosen for use in screening the antibodies because RT112 and ZR75-1 had been shown in earlier RT-PCR studies to show high levels of CD44 exon 7 transcription, while in HT29 and MDA 4A4 this activity was undetectable. It had to be assumed, for the purposes of this work, that the mRNA levels reflected the content of the corresponding protein in these cell lines, which later proved to be correct.

The base of each flask was removed and the adherent cells washed three times with PBS before fixation in cold methanol (10 min, 4°C). Non-specific reactions were blocked by pre-incubation with 20% normal rabbit serum diluted in TBS at 37°C for 45 min before incubation with Hermes-3 (1/50) or test supernatants/antibodies overnight at 4°C . Any endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min before the addition of biotinylated anti-mouse IgG (Dako, 1/400 dilution, room temperature, 2 h) and horseradish peroxidase conjugated ABCComplex (Dako, room temperature, 1 h). Immunostaining was visualised using diaminobenzidine/ H_2O_2 (Sigma) as substrate; cells were counterstained with haematoxylin. Between all antibody incubations, cells were washed by 3×5 min incubations with TBS on a shaking platform.

Immunofluorescent staining and flow cytometry

For one colour immunofluorescence staining, RT112/84, MDA 4A4, ZR 75-1 cell lines or unstimulated peripheral blood lymphocytes (5×10^5 cells) were incubated with hybridoma supernatant containing 10 $\mu\text{g}/\text{ml}$ of mAb (30 min, 4°C). Cells were washed once and resuspended in 100 μl of FITC labelled sheep anti-mouse Ig F(ab)₂ fragment (Boehringer Mannheim) for 30 min at 4°C . Subsequently, they were washed and resuspended in phosphate buffer (100 μl) before fixing in 1 ml of methanol (0.5%) and storage at 4°C . Before FACS analysis, the cells were washed again and resuspended in 250 μl phosphate buffer. FACS analysis was carried out in accordance with manufacturer's instructions.

Determination of immunoglobulin class and light chain

The mouse monoclonal antibody isotyping kit (Iso-Strip; Boehringer) was used. Culture supernatants (150 μl) diluted 1:100 (v/v) in PBS

Table 1 Reactivity of primary hybridoma supernatants with different CD44 v2 peptide regions shown by inhibition assay using specific synthetic peptides. Figures denote absorbance values at 450 nm

Primary culture	Without peptide	v2 (1-43) peptide	v2 (1-13) peptide	v2 (9-23) peptide	v2 (19-33) peptide
23.000.1	1.941	0.066	1.467	0.595	0.595
23.000.5	0.763	0.046	0.547	0.729	0.052
23.000.6	1.022	0.067	0.825	0.785	0.057
23.000.8	0.576	0.050	0.499	0.503	0.056
23.000.9	0.400	0.058	0.334	0.382	0.066
23.000.10	0.505	0.142	0.187	0.620	0.497
23.000.19	1.247	0.060	1.184	1.306	0.094
23.000.20	0.828	0.058	0.696	0.840	0.062

were added to the development tube and incubated at room temperature for 30 s. The tubes were agitated by a brief vortex to re-suspend the coloured latex before insertion of the iso-typing strip. The results were interpreted after 5 min by the appearance of a blue band in either the κ or λ section of the strip and in one of the class or subclass sections. The positive control bands on either side of the isotype strip must also appear indicating that the antibody coated latex beads were functional and had attached to the strip.

PREPARATION OF SHEEP ANTI-CD44 EXON 7 POLYCLONAL ANTIBODIES

To allow applications to be undertaken where polyvalent antibodies may be advantageous, sheep (Merino Suffolk) were immunised and boosted with 100 μ g HIV2(gp32)-CD44 (exon 7) fusion protein in Freund's adjuvant. After 12 weeks, animals were test bled and the serum screened for immunoreactivity in the CD44 exon 7 peptide ELISA and cell ELISAs.

Purification

Ammonium sulphate was added to the serum to a final concentration of 1.7 M. After dialysis the precipitate was applied to a DEAE-sepharose column (Pharmacia) and eluted by increasing the NaCl concentration. The CD44 exon specific IgG within the total IgG was prepared by immunosorption. CD44 exon 7 (1-43aa) peptide synthesised with an additional spacer and cysteine at the amino terminus was coupled to aminospherosil (Boehringer Mannheim) activated by maleimido-hexanol-*N*-hydroxysuccinimide. The IgG fraction of the DEAE column was applied to the CD44 exon 7 column in PBS at pH 7.4. The bound CD44 exon 7 specific IgG fraction was then eluted by 3 mmol/l HCl. Approximately 5% of the total IgG were specific to CD44 exon 7. Purified IgG was further characterised by western blotting and immunocytochemistry on RT112, MDA 4A4, and HT29 cell lines.

Results

SCREENING OF PRIMARY CULTURES

Ten mice immunised with the CD44 exon 7 fusion protein showed positive antibody titres (1:1000–1:2000) against the fusion protein and exon 7 synthetic peptides (1-43aa), (1-13aa), and (19-33aa) (data not shown).

To date, five cell fusion experiments have been performed, each with cells from the spleen

of a separate immunised mouse. The supernatants of all resulting clones were screened by ELISA for reactivity against CD44 exon 7 peptide (1-43) and natural CD44 exon 7 encoded epitopes on the RT112 cell line between 10–14 d postfusion. One fusion was more promising than the others and from this 480 clones yielded nine positive wells in the peptide ELISA (1-43aa) (table 1). Furthermore the immunoreactivities of seven of these nine primary cultures were inhibited with free (19-33aa) peptide and the remaining two showed inhibition with free peptide (1-13aa) (table 1). In cell ELISA, six of the nine primary cultures gave significant immunoreactivity against RT112, and none of them, as expected, showed reactivity to MDA 4A4 (negative control). RPMI 1640 was used as the background control and the monoclonal antibody F10-44-2 (anti-standard CD44 exon 1) was included as a positive control (table 2).

SCREENING AND EPITOPE MAPPING OF SUBCLONED CULTURES

The six primary hybridoma cultures producing antibodies reacting with CD44 exon 7 peptide

Table 2 Reactivity of CD44 v2 primary hybridoma culture supernatants with natural CD-44 v2 on cell lines shown by (cell ELISA). The values represent absorbance at 405 nm

Primary culture	RT112 cells	MDA 4A4 cells
23.000.1	0.850	0.237
23.000.2	0.733	0.243
23.000.6	0.830	0.262
23.000.10	0.348	0.250
23.000.19	0.689	0.252
23.000.20	1.010	0.254
RPMI-1640	0.210	0.273
MAK<CD44std>F10	1.536	1.357

Table 3 Reactivity of cloned hybridoma supernatants with natural CD44v2 on cell lines shown by (cell ELISA). Figures denote absorbance at 405 nm

Clone	RT112 cells	MDA 4A4 cells
23.6.1	0.892	0.263
23.9.1	0.878	0.265
23.10.1	0.900	0.275
23.11.2	0.684	0.263
23.12.2	0.929	0.286
23.22.6	0.884	0.217
23.26.6	0.896	0.212
23.32.10	0.372	0.255
23.34.10	0.388	0.286
23.45.19	0.748	0.252
23.46.19	0.749	0.274
23.48.19	0.685	0.242
23.51.20	1.064	0.276
23.57.20	0.999	0.297
RPMI-1640	0.210	0.273
MAK<CD-44std>F10	1.536	1.357

Table 4 Reactivity of the monoclonal antibody with different CD-44 v2 peptides, epitope mapping (inhibition test with free peptides)

Clone	v2 (1-43aa)	v2 (1-13aa)	v2 (9-23aa)	v2 (19-33aa)	v2 (29-43aa)
23.6.1	+	-	-	+	-
23.9.1	+	-	-	+	-
23.11.2	+	+	-	+	-
23.12.2	+	+	-	+	-
23.22.6	+	-	-	+	-
23.26.6	+	-	-	+	-
23.32.10	+	+	-	-	-
23.34.10	+	+	-	-	-
23.45.19	+	-	-	+	-
23.46.19	+	-	-	+	-
23.48.19	+	-	-	+	-
23.51.20	+	-	-	+	-
23.57.20	+	-	-	+	-
MAK<CD-44 std>F10	-	-	-	-	-

+, positive inhibition with the free peptide >70%; -, no inhibition with free peptide <10%.

(1-43aa) and native CD44 were subcloned and supernatants from 14 of the resulting hybridoma cell line were, as shown in table 3, found to show immunoreactivity to RT112 but not to MDA 4A4. The anti-CD44 standard form mAb F10-44-2 showed strong reactivity with MDA 4A4 cells, confirming that they produce mainly this isoform of the CD44 protein.

The reactive epitopes of the six hybridoma lines were mapped by an inhibition assay with overlapping CD44 exon 7 sequences in the peptide ELISA. Table 4 summarises the results. The reactivity of all supernatants was inhibited by the free peptide CD44 exon 7 (1-43aa). As the data shows, this inhibition was in all cases due to antibodies binding to epitopes either in the (1-13aa) or (19-33aa) regions of CD44

exon 7. No immunoreactivity was observed in the (9-23aa) or (29-43aa) regions. As expected, the F10-44-2 monoclonal antibody to the epitope encoded by standard CD44 exon 1 was not inhibited by any of the peptides.

WESTERN BLOT AND IMMUNOCYTOCHEMICAL ANALYSIS OF PRIMARY AND SUBCLONED CULTURES

Supernatants from the 14 subclones shown to be positive in the peptide and cell ELISAs were further characterised in western blot analysis of RT112 and ZR75 cell lysates under reduced conditions. Positivity in western blot preparations was defined as the presence of a monospecific band with a molecular weight greater than 200 kDa in lysates from both of these cell lines. All were confirmed to show such positivity in varying degree.

Additionally the subclones were characterised by their ability to detect CD44 exon 7 immunocytochemically on these cell lines. Not surprisingly, as these subclones had been found to be positive in cell ELISA, all 14 showed some degree of discrete membrane staining, but

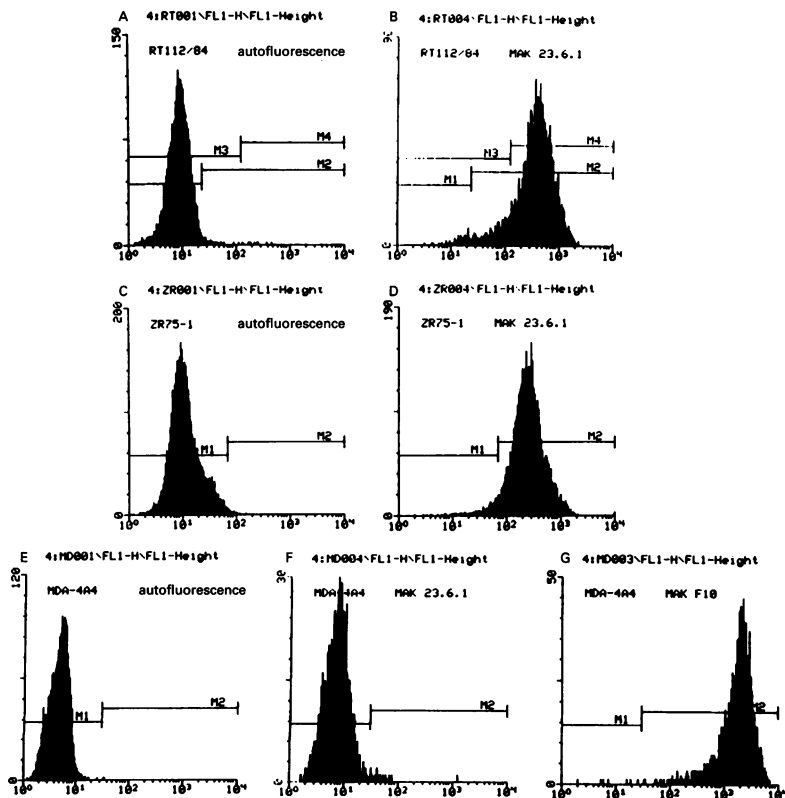


Figure 3 FACS analysis of cell lines stained with mAb M23.6.1 specific for CD44 exon 7 (v2) and mAb F1044-2 for the CD44 standard form (Boehringer Mannheim, Cat No 1441. 272). (A) RT112 cells: autofluorescence of the cells: 0.09%; (B) RT112 cells: binding with mAb 23.6.1: 90%; (C) ZR-75-1 cells: autofluorescence of the cells: 0.28%; (D) ZR-75-1 cells: binding with mAb 23.6.1: 97%; (E) MDA 4A4 cells: autofluorescence of the cells: 0.02%; (F) MDA 4A4 cells: binding with mAb M23.6.1: 1%; (G) MDA 4A4 cells: binding with mAb F1044-2: 100%.

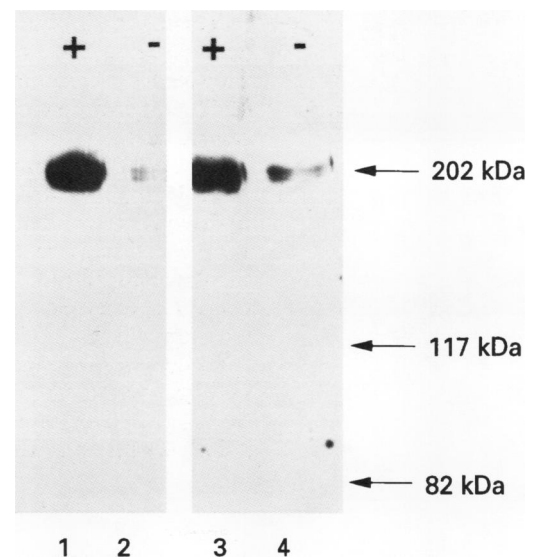


Figure 4 Western blot of RT112 (human bladder carcinoma cell line) (10 µg/well total protein) under non-reducing conditions using purified ascites derived monoclonal antibody mAb M-23.6.1 (10 µg/ml) (lane 1) and the same antibody preabsorbed with peptide CD44 exon 7 (1-43aa) (lane 2). Lanes 3 and 4 are under the same conditions using spinner produced antibody (10 µg/ml) (lane 3) and preabsorbed with peptide CD44 exon 7 (1-43aa) (lane 4).

Table 5 Immunocytochemical analysis of RT112, ZR75, MDA 4A4, and HT29 cell lines with crude ascites (10 µg/ml) and spinner produced antibodies (10 µg/ml)

Clone	Ascites				Spinner	
	RT112	ZR75	MDA	HT29	RT112	MDA
23.6.1	++++	+++	+/-	-	+++	-
23.46.19	++	++	-	-	+	-
23.57.20	+++	+	-	-	++	-

-, No staining; +, weak membrane staining on 0-10% cells; ++, weak membrane staining on 90%+ of cells; +++, moderate membrane staining on 90%+ cells; + + + +, intense membrane staining on 90%+ cells.

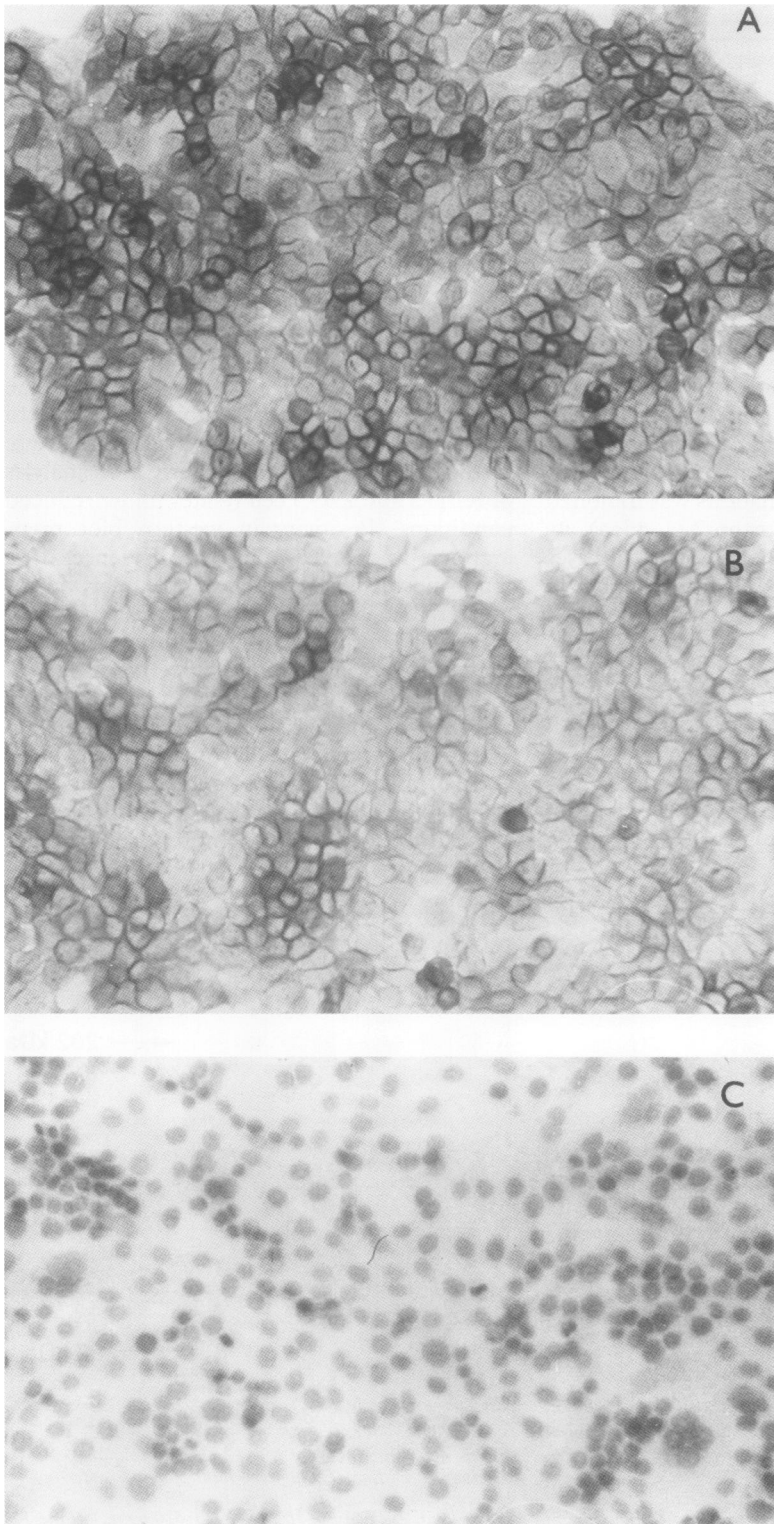


Figure 5 Immunocytochemical staining of RT112 human bladder carcinoma cell line with purified monoclonal antibody M-23.6.1 (10 µg/ml) obtained from ascites (A) and spinner culture (B), showing strong membrane reactivity. The negative staining obtained with MDA 4A4 is shown in (C). Magnification × 590.

the intensity of this reaction varied significantly between clones.

Consequently, from these data, three CD44 exon 7 specific hybridomas were selected for further study on the basis that they showed marked positivity in both western blotting and immunocytochemistry on RT112 and ZR75-1 cell lines but not on HT29 or MDA 4A4. They were named mAb<CD44 exon 7>M-23.6.1 (IgG1, λ), mAb<CD44 exon 7>M-23.46.19 (IgG1, κ) and mAb<CD44 exon 7>M-23.57.20 (IgG1, λ). The data in brackets refer to the immunoglobulin class.

FACS analysis of monoclonal antibodies 23.6.1, 23.46.19, and 23.57.20

FACS analysis of these three monoclonal antibodies shows positive binding with the CD44 exon 7 expressing cells RT112 (mAb 23.6.1 = 90%; mAb 23.46.19 = 72%; mAb 23.57.20 = 86%) and with ZR 75-1 (mAb 23.6.1 = 97%; mAb 23.46.19 = 97%; mAb 23.57.20 = 96%) (fig 3). With the CD44 exon 7 negative MDA 4A4 cells, all three monoclonal antibodies showed only about 1% binding. Anti standard from F-10-44-2 showed 100% binding with MDA 4A4 (fig 3). Unstimulated peripheral blood lymphocytes were found to be negative with all three monoclonal antibodies to products of exon 7 (data not shown).

Characterisation and evaluation of antibodies secreted by selected subclones

Following bulk production of the antibodies secreted by these three selected clones, as described above, ascites and spinner culture fluids from each were characterised by western blotting and immunocytochemistry. In western blots performed under non-reducing conditions, antibodies obtained from all three hybridomas by either production method and standardised to a concentration of 10 µg/ml defined a single high molecular weight band corresponding to a size of just over 200 kDa in both RT112 and ZR75-1. However, no bands were observed with MDA 4A4 and HT29.

Table 5 summarises the immunocytochemical data which showed differences both between clones and between production methods. In all cases staining was most intense on the cell membranes, but some cytoplasmic reactivity was seen.

As all three of these clones had previously been mapped to an epitope in the CD44 exon

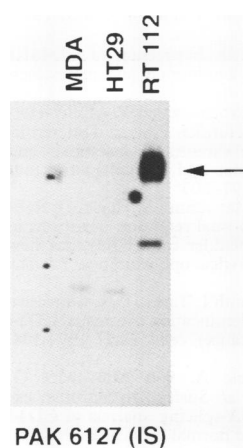


Figure 6 Western blot of RT112, MDA 4A4 and HT29 cell lines (10 µg/well total protein) under reducing conditions using purified polyclonal antiserum pAb S-6127. Arrow shows position of 220 kDa marker.

7 (19–33aa) region, we decided to purify only ascites and spinner supernatant of 23.6.1.

CHARACTERISATION OF PROTEIN A PURIFIED mAb <CD44 EXON 7>M-23.6.1

Western blotting with the purified monoclonal antibody identified a monospecific band of approximately 200 kDa in RT112 cell lysates, but not in those from control lines (HT29 and MDA 4A4) which are negative for exon 7 expression. Figure 4 shows a western blot on RT112 using pure ascites derived 23.6.1 (10 µg/ml) (lane 1) and the specific inhibition of immunoreactivity by peptide CD44 exon 7 (19–33aa) (lane 2). Similar immunoreactivity was observed with spinner produced antibody (10 µg/ml) (lane 3) and diminished after inhibition with peptide CD44 exon 7 (19–33aa) (lane 4).

Immunocytochemical staining of RT112 (fig 5) with pure 23.6.1 (10 µg/ml) obtained from ascites (A) and spinner (B) showed strong membrane reactivity. As with the crude preparations, more intense staining was observed with the ascites derived antibody. MDA 4A4 cells did not stain (C).

CHARACTERISATION OF POLYCLONAL ANTI-CD44 EXON 7 ANTIBODIES

Several of the sera screened for immunoreactivity proved to be positive in both fusion protein and cell ELISAs. CD44 exon 7 peptide (1–43aa) immunosorbed antibodies showed several immunoreactive bands on Western blotting. The blot obtained with the most promising polyclonal antibody termed pAb S-6127 is shown in fig 6. With RT112 cell lysates, a major band of immunoreactivity was seen around 200 kDa, with a second minor band at between 140 and 160 kDa. With MDA 4A4 and HT29, a single minor band was seen at around 90–100 kDa. Immunocytochemistry on RT112 cells showed similar staining, both in terms of intensity and localisation, to that observed with the monoclonals, while apart from some weak background cytoplasmic staining, MDA 4A4 and HT29 were negative.

Discussion

This work has resulted in the isolation and molecular characterisation of a new monoclonal antibody with an internal image corresponding to an epitope encoded by exon 7 (v2) of the human CD44 gene. The immunoreactivity of the antibody with the products of exon 7 has been demonstrated in western blots performed on lysates of several human tumour cell lines, in which it reproducibly recognises a 220 kDa mono-specific band and by peptide ELISA and cell ELISA techniques. We have evidence that the high molecular weight protein identified in western blots also contains the products of several other exons from the variably expressed region of the CD44 gene (data in preparation for publication). The specificity of this reactivity was further confirmed by competitive inhibition experiments using synthetic peptides and the fu-

sion peptide employed for raising the antibodies in mice. The distribution of the epitope in the tumour cells studied is confirmed to be typically in the cell membrane, as would be expected for an integral transmembrane glycoprotein, but reactivity was also seen to a lesser extent in the cell cytoplasm. This is interpreted to be the epitope on its way to insertion in the membrane, but may also represent excess abnormal protein accumulating in the cytoplasm of tumour cells, with disordered processing of this complex molecule.

Further studies using this new antibody on human tumour cell lines and on frozen and paraffin wax embedded sections of normal and tumour tissues have confirmed that the increased transcripts from the v2 exon previously seen in several human tumour tissues and in tumour cell lines (data to be published separately) are translated into proteins. This immunocytochemical and immunohistochemical work has been conducted on cells and tissues from many patients with carcinoma of the urinary bladder, colon, and breast. Weak cross reactivity with mucin droplets in colonic epithelial cells has been identified but this is not of any diagnostic consequence.

We have also described the production of the polyclonal antiserum (pAb S-6127) to CD44 exon 7. It appears to be useful for western blotting and immunocytochemistry. Furthermore, pAb S-6127 is currently undergoing evaluation in a sandwich-ELISA system, where the use of purified polyvalent antibodies may be beneficial.

Several previous studies,^{11–16} from this and other laboratories, have confirmed abnormal transcription and splicing of the CD44 gene in tumour cells relative to their normal counterparts, and the presence of abnormal large CD44 protein isoforms has recently been shown²⁵ in exfoliated cancer cells in the urine of patients with bladder cancer. The reproducibility and specificity of such differences between samples from the bladder cancer patients and from unaffected control subjects suggests that abnormal patterns of CD44 expression may be a useful marker for the early detection of some types of malignancy. The new antibodies described in this paper may be a helpful reagent in the analysis of CD44 gene expression in normal and neoplastic cells. It may also be useful in the search for new sensitive and specific tests for cancer cells in clinical specimens.

In this context, it is relevant to mention that the presence of exon 7 (v2) of this gene was first suspected from RT-PCR observations on the human tumour specimens¹¹ in which a PCR product (1650 bp), which was too large for the then known structure of the CD44 locus, was detected in all tumour specimens. This does not mean that the expression of this exon is the cause of the neoplasm, but it does indicate that large isoforms containing products of all coding regions of the CD44 gene are characteristically overexpressed in cancer cells. It is possible that unbalanced expression of one or of a group of exons is an important aetiological factor in, or a specific marker of, neoplasia.

The new antibody will be a useful tool for the further analysis of these abnormal CD44 isoforms and their clinical implications.

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