12. Cannon-Albright LA, Skolnick MH, Bishop DT, Lee RG, Burt RW. Common inheritance of susceptibility to colonic adenomatous polyps and associated colorectal cancers. *N Engl J Med* 1988, **319**, 533–537.
13. Roscigno RF, Weiner M, Garcia-Blanco MA. A mutational analysis of the polypyrimidine tract of introns. *J Biol Chem* 1993, **268**, 11222–11229.
14. Hall NR, Murday VA, Chapman P, *et al.* Genetic linkage in Muir–Torre syndrome to the same chromosomal site as cancer family syndrome. *Eur J Cancer* 1994, **30A**, 180–182.
15. Newton CR, Graham A, Heptinstall LE, *et al.* Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucl Acids Res* 1989, **17**, 2503–2516.

Acknowledgement—This work was supported by the Imperial Cancer Research Fund.

European Journal of Cancer Vol. 30A, No. 10, pp. 1552–1558, 1994

0959-8049/94 $7.00 + 0.00

0959-8049(94)00289-4

Tissue-specific Expression of Neural Cell Adhesion Molecule (NCAM) May Allow Differential Diagnosis of Neuroblastoma From Embryonal Rhabdomyosarcoma

E.G. Phimister, A. Culverwell, K. Patel and J.T. Kemshead

The MSD1 region of neural cell adhesion molecule (NCAM) was originally described as being spliced into the 120-kDa isoform of NCAM isolated from muscle. The 105 bp region is inserted between exons 12 and 13 and actually consists of three separate exons, MSD1a, MSD1b and MSD1c of 15, 48, 42 bp, respectively. In addition, a further exon consisting of a single triplet has been designated MSD1d, making the full insert size 108 bp. As the MSD1 region was originally described as being selectively expressed in muscle tissue, we have investigated whether it is also present on tumours of rhabdoid origins and whether its presence can be used as the diagnostic marker to distinguish other small round cell tumours of childhood, such as neuroblastoma. Using a variety of human tumour cell lines, we demonstrated the presence of the MSD1 region on all rhabdomyosarcomas investigated. However, neuroblastoma cell lines only expressed subcompartments of the MSD1 region. The MSD1c exon was not spliced into the NCAM molecules isolated from any of the neuroblastoma cell lines investigated. On the basis of this finding, it appears that neuroblastoma and rhabdomyosarcoma can be distinguished by the expression of the MSD1c mini-exon. Further studies are underway to attempt to define a monoclonal antibody that recognises the region, using mice immunised with synthetic peptides, and to confirm the finding using fresh biopsy material.
Eur J Cancer, Vol. 30A, No. 10, pp. 1552–1558, 1994

INTRODUCTION

NEUROBLASTOMA IS a malignancy of childhood with an incidence of approximately one child in 10 000 between the ages of birth and 14 years [1]. Along with Wilms' tumour, Ewing's sarcoma, rhabdomyosarcoma and lymphoblastic leukaemia/lymphoma, it represents one of the small round cell tumours of childhood. Whilst these can often be differentiated on the basis of clinical presentation, pathology and a variety of biochemical investigations, it can prove difficult to distinguish one tumour type from another, particularly when the cells are highly anaplastic in nature.

As the therapy for the small round cell tumours of childhood differs for each malignancy, a concreted effort has been made to ensure an accurate diagnosis of tumour type. Various biochemical studies can be undertaken as an aid to identifying a particular tumour, such as the presence of catecholamine breakdown products in the urine of patients with neuroblastoma [2]. The fact that these cells often synthesise catecholamines can also be exploited in diagnostic imaging through the use of *meta*-iodobenzylguanidine (*m*IBG), a synthetic analogue of adrenaline and nor-adrenaline. This can be radiolabelled with an isotope of iodine, and it will accumulate *in vivo* in the majority of neuroblastomas investigated, allowing their identification by scintigraphy [3].

Correspondence to J.T. Kemshead.
All authors are at the Imperial Cancer Research Fund, Paediatric & Neuro-Oncology Group, Frenchay Hospital, Bristol BS16 1LE, U.K.
Revised 11 May 1994; accepted 9 June 1994.

Another tool extensively used in the differential diagnosis of this group of tumours is immunocytochemistry, aimed at identifying either cytoskeletal elements or cell membrane antigens associated with a particular tumour type. For example, desmin has been used as a marker to identify cells of rhabdoid origin [4], while the CD45 common leucocyte antigen (CD45) is expressed on leukaemias and lymphomas [5].

Although these diagnositic tests are applicable to the vast majority of tumours, there are cases where this is not so. For example, some patients with neuroblastoma do not excrete catecholamine breakdown products and equally some tumours do not take up *m*IBG. Whilst the expression of desmin has been generally accepted as being associated with rhabdoid tumours, cases of neuroblastomas containing this cytoskeletal protein have also been observed. Similarly, cases of neuroblastoma have been noted to express the common acute lymphoblastic leukaemia antigen (cALLA), CD10.

Other membrane antigens useful in the detection of tumour cells in bone marrow have a broader expression. Although antibodies to these determinants do not recognise cells in the haemopoietic compartment, they cross-react with several of the 'small round cell tumours of childhood'. Examples of these reagents include antibodies to the neural cell adhesion molecule (NCAM) which binds to Wilms' tumours, rhabdomyosarcomas, a subset of Ewing's sarcomas and neuroblastomas [6].

NCAM represents a highly complex family of glycoproteins encoded by a gene of approximately 70 kb found on chromosome 11 at q23. It consists of at least 25 different exons and some of these are subject to differential splicing. At the biochemical level, four different isoforms of the molecule have been identified in human brain of 180, 170, 140 and 120 kDa [7]. These either have a cytoplasmic C-terminal region or are linked to the membrane via a phosphoinositol bridge. In addition, a secreted form of NCAM has been identified. A different pattern of NCAM isoforms have been found in human muscle tissue. The difference in isoform expression between rhabdoid and neuroectodermal tissues cannot be exploited to differentiate tumours, as previous data has revealed expression of the 140 and 120 kDa isoforms in most samples of neuroblastoma and rhabdomyosarcomas analysed [8].

At a molecular level, differential splicing of exons into the NCAM molecule has been observed and described as occurring in a tissue specific fashion. The VASE exon spliced into the molecule between exons 7 and 8 was originally thought to occur only in neural cell NCAM, although this has now been shown to have a much broader pattern of distribution [9]. The 105-bp MSD1 region, inserted between exons 12 and 13, has been described as occurring only in muscle NCAM. In fact, this region can be subdivided into four separate exons, MSD1a, MSD1b and MSD1c of 15, 48, 42 bp, respectively [10]. In addition, a further exon consisting of a single triplet has been designated MSD1d, making the full insert a 108 bp.

Here, we investigate whether rhabdoid and neuroblastic tumours can be differentiated on the basis of the splicing pattern of the MSD1 domain. The results indicate that whilst the full MSD1 region was observed in all of the rhabdomyosarcoma lines studied, only MSD1a and MSD1b were identified in neuroblastomas. The lack of expression of the MSD1c region in neuroblastoma could form the basis of a diagnostic text to distinguish these tumour types.

MATERIALS AND METHODS

Cell lines and cDNA

The human rhabdomyosarcoma cell lines RD618, Rhab-1, SMS-CRT, TE671 and JR1, the neuroblastoma cell lines GOTO, IMR-32, Kelly, SK-N-BE, SK-N-SH, NB1 and PCF and the human neuroepithelioma cell line CHP100 were grown at 37°C in RPMI 1640 containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. When approaching confluency, cells were harvested from the dishes using trypsin/versene, washed and replated to allow exponential growth. The human T-cell leukaemic line GH1 was grown in suspension in the above medium and also maintained in an exponential growth phase. All cell lines were routinely tested for mycoplasma contamination. The risk of cross-contamination of lines was reduced to a minimum by handling one cell line at a time and by returning to frozen seed stocks at regular intervals.

The NCAM cDNA clone designated λ9.5 was a generous gift from Professor F. Walsh [10]. This cDNA codes for a region of the NCAM molecule which includes exons 12 and 13 as well as the MSD1 region.

Indirect immunofluorescence and fluorescent-activated cell sorting

Cells were incubated with the anti-NCAM monoclonal antibodies (MAbs) UJ13A and ERIC-1 and fluorescein-conjugated rabbit antimouse Ig, as previously described [11]. Staining was visualised by a Zeiss fluorescent microscope and a FACS scan (Becton Dickson) counting 10 000 events per sample. For staining with an antiDesmin MAb (Amersham International, Bucks, U.K.), cells were cytocentrifuged onto glass slides, fixed in cold acetone (−20°C) for 1 min, and incubated with primary and secondary antibodies as described previously [11].

RNA extraction

For each lineage, 2×10^7 cells were harvested in exponential growth, washed twice in PBS and lysed in 1.0 ml of buffer containing 5 M guanidine isothiocyanate, 50 mM trishydroxymethylamine (Tris) and 10 mM ethylene diamine tetra-acetic acid (EDTA) at 4°C. DNA was sheared by passing the lysate through a succession of syringe needles (1.1–0.6 mm inner diameter) and RNA precipitated overnight following the addition of 7 ml of cold 4 M lithium chloride. After centrifugation for 90 min at 14 000 *g* at 4°C, the RNA was re-precipitated with lithium chloride, centrifuged and solubilised in 2 ml of 10 mM Tris pH 7.5, containing 0.1% SDS and 1 mM EDTA. An equal volume of phenol equilibrated with 25 mM Tris and 10 mM EDTA (TE buffer) was added and the upper aqueous layer extracted. This procedure was repeated and a third extraction performed using chloroform 4% isoamylalcohol. RNA was precipitated at −20°C overnight following the addition of 50 μl of saturated ammonia acetate and two volumes of cold ethanol. The precipitate was centrifuged at 10 000 *g* at 4°C for 10 mins, washed three times in 90% alcohol, dried in a vacuum dryer and resuspended in TE buffer. The purity of the RNA was determined by spectroscopy at 260 and 280 nm. Only samples with an A_{260}:A_{280} ratio of greater than 1.8 were used for reverse transcription (RT) and PCR.

Synthesis of cDNA

Complementary DNA was generated by reverse transcription of RNA templates using a 21 bp oligonucleotide primer complementary to a region in exon 13, beginning 172 base pairs from the exon 12/13 junction (5′TCACTGCCAGACGGGA-

GCCTG3'). The primer was designed to maximise the probability of efficient and specific hybridisation with template RNA [12]. The reaction mixture contained 2 μg of RNA, 1 mM dNTP mix (Pharmacia), 18 U avian myeloid leukaemia reverse transcriptase, 1 μM of the above 3' primer, 37 U RNA guard (Pharmacia) and 2 μl of reverse transcriptase ×10 buffer (0.5 M Tris pH 8.3, 0.1 M $MgCl_2$ and 0.4 M KCl). The final volume of 20 μl was incubated at 42°C for 60 min and the cDNA stored on ice prior to amplification by PCR.

Synthetic PCR amplification

Eighteen microlitres of cDNA was amplified in a final volume of 100 μl containing 20 pmol 3' primer (as above) 20 pmol 5' primer, 200 μm dNTP, reaction buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$ and 1% Triton ×100) and 2.5 U of Taq polymerase (NBL). The 5' primer used was a 21 bp sequence beginning 80 bp 5' of the exon 12/13 border (5'CGCCGTAAGGCTGGCGGCGCT3'). Samples were layered with 100 μl of mineral oil and heated at 94°C for 3 min followed by 30 cycles consisting of three segments at 94°C for 1 min, 60°C for 1 min and 72°C for 90 s. This was followed by a final cycle of 94°C for 1 min, 60°C for 1 min and 72°C for 7 min. Samples were extracted with 4% isoamylalcohol in chloroform and visualised by electrophoresis.

Southern blot analysis of PCR products

Ten microlitres of PCR products were electrophoresed through a 2% agarose gel and the bands obtained passively transferred to a nylon membrane. The DNA on the filter was denatured in 0.4 M NaOH, neutralised with 2× SCC (0.15 m NaCl and 0.15 M sodium citrate) and allowed to dry naturally.

Radiolabelling of oligonucleotides

For each oligonucleotide, 0.5 μg was labelled at the 5' end following incubation with 7 U of polynucleotide kinase (NBL), 50 μCi ^{32}P dATP and 1 μl of ×10 labelling buffer (0.5 M Tris pH 6.0, 1 M $MgCl_2$, 50 mM dithiotheitol, 1 mM spermidine and 1 mM EDTA) in a final volume of 10 μl for 45 min at 37°C. Radiolabelled oligonucleotide was purified by column chromatography using a NAP-5 column (Pharmacia) pre-equilibrated with TE buffer.

Hybridisation and washing

Prior to hybridisation, dried blots were soaked in 2× SSC for 15 min. Blots were prehybridised at 65°C with 1.0 mg of denatured salmon sperm for a minimum of 1.5 h in 10 ml of hybridisation buffer (10% dextran sulphate, 1 M NaCl and 1% SDS) using a Hybaid dual hybridisation oven. Subsequently, 10^7 cpm of denatured oligonucleotide were added and the blot hybridised overnight at a temperature determined by the specific probe used [13]. Blots were washed twice in SSC ×2 containing 1% SDS for 5 min at room temperature, 2× SSC containing 1% SDS at the temperature of hybridisation and 2× SSC containing 0.1% SDS for 15 min, again at the hybridisation temperature. The blots were wrapped in cling-film and exposed to X-ray film in the presence of an intensifying screen.

For rehybridisation, oligonucleotide was removed from the blot by incubation in 0.4 M NaOH at 42°C for 30 min followed by neutralisation with 0.1× SSC containing 0.1% SDS and 0.2 M Tris pH 7.5. This wash cycle was repeated and the blots autoradiographed to ensure the removal of ^{32}P.

RESULTS

Cell lines

All the embryonal rhabdomyosarcoma and neuroblastoma cell lines studied were found to express NCAM, as determined by the binding of anti-NCAM MAbs UJ13A and ERIC-1 [6]. However, there were marked differences in the expression of the molecule as determined by the intensity of signals observed. For example, with similar background readings for autofluorescence and signal from non-specific binding of fluorescein-labelled antimouse Ig, the neuroblastoma lines Kelly and IMR-32 gave a median fluorescence signal of 364 and 245 nm, respectively, following incubation with MAb UJ13A and fluorescence-activated cell sorter (FACS) analysis. The PCF line was only weakly positive when stained with both ERIC-1 and UJ13A; a median fluorescence of less than 200 being observed by FACS analysis.

Immunocytochemistry demonstrated that all the lines of rhabdoid origin were found to express the intracellular intermediate filament protein desmin. In contrast, no signal was detected in any of the neuroblastoma lines. Similarly, only the rhabdomyosarcoma lines bound the MAb FMG25, a reagent previously described as capable of distinguishing the two tumour types [14].

RT PCR of RNA from human embryonal rhabdomyosarcoma cell lines

Two bands of approximately 360 and 250 bp were observed in all of the rhabdomyosarcoma cell lines investigated when they were probed with randomly primed oligonucleotides complementary to the λ9.5 clone, containing both the MSD1 region and those flanking the exon 12/13 border (Figure 1). The predicted size for a PCR product generated if the MSD1 domain is absent is 252 bp, while the theoretical size of a PCR product containing MSD1 is 357 bp (Table 1). The data, therefore, suggests that rhabdomyosarcoma cells alternatively splice the MSD1 domain, giving rise to mRNA which contains, and mRNA which lacks, the sequence. With the exception of the JR1 cell line, all the rhabdomyosarcoma lines revealed a weaker hybridisation signal of 360 bp as compared with the 252 bp band. This could imply that, in general, mRNA species lacking the MSD1 domain exceed those which contain it, although the PCR is not quantitative and the finding may be an artefact arising from the hybridisation conditions. Furthermore, there was marked variation in the intensity of signal seen between cell lines, with TE671 and JR1 repeatedly yielding weak and intense bands, respectively.

No signal was detected in either the water control or in the lane containing PCR products from the T-cell line GH1, known not to express NCAM. Although no fresh rhabdomyosarcomas were available for the study, where intact RNA could be isolated, human fetal muscle was shown to maintain a pattern of MSD1 expression similar to that found in rhabdomyosarcomas.

When this blot was stripped and re-probed with an oligonucleotide complementary to the MSD1 region, only the 360 bp band was observed in tracks containing PCR products from rhabdoid material (Figure 2). Once again, the intensity of the bands varied between cell lines, and no signal was detected in the negative controls.

To confirm that the 360 bp PCR product contained the MSD1 region, the PCR products from the JR1 cell line were size separated on a 2% agarose gel, eluted and subjected to two cycles of asymmetric PCR. Sequencing the PCR product revealed the expected full MSD1 sequence as reported previously (Figure 3).

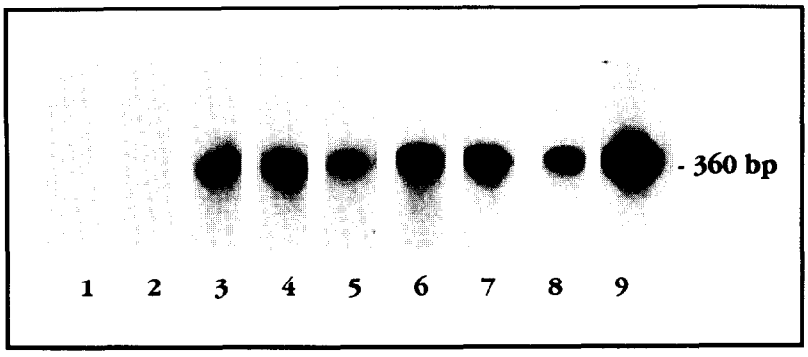

Figure 1. Expression of the MSD1 domains in human embryonal rhabdomyosarcoma cell lines. Following RNA isolation and RT PCR, products of the PCR reaction were size separated by agarose gel electrophoresis and blotted onto a nylon membrane. The blot was hybridised overnight with randomly primed oligonucleotides complementary to the λ9.5 clone. After washing to a stringency of 0.1× SSC, 0.1% SDS at 70°C, the blot was autoradiographed in the presence of an intensifying screen. The experiment was repeated using fresh RNA isolates and the data presented is representative of all the data accrued. Lane 1, negative control GH1 T-cell line; 2, water control for PCR reaction; 3, rhabdomyosarcoma RD618; 4, rhabdomyosarcoma RHAB1; 5, rhabdomyosarcoma SMS-CTR; 6, rhabdomyosarcoma TE671; 7, rhabdomyosarcoma JR1; 8, human fetal muscle; 9, positive control λ9.5.

The triplet mini-exon MSD1d was also found in the material analysed.

RT PCR of RNA from human neuroblastoma cell lines

All of the PCR products analysed from the human neuroblastoma cell lines exhibited a different pattern to those from rhabdomyosarcoma when the blot was probed with randomly primed oligonucleotides complementary to the λ9.5 clone (Figure 4). Although no band of 360 bp was observed in any of the neuroblastoma samples, weak bands of approximately 300 bp were observed in all samples apart from those obtained from the cell lines CHP100 and PCF. In addition, all samples contained a 250-bp band, presumably resulting from NCAM mRNA lacking the MSD1 sequence, as well as a band or bands of between 250 and 280 bp that could not be easily resolved. This suggests that the whole MSD1 domain is not spliced into NCAM mRNA isolated from neuroblastoma cells, but constituent exons of MSD1 are spliced into the molecule. The different combinations of exons that can theoretically be variably spliced between exons 12 and 13 and give rise to different PCR products are illustrated in Table 1.

Table 1. Predicted PCR fragment size

Exons	Predicted PCR fragment size (bp)
12–13	252
MSD1a	267
MSD1b	300
MSD1c	294
MSD1a–MSD1b	315
MSD1a–MSD1c–13	309
MSD1b–MSD1c–13	342
MSD1a–MSD1b–MSD1c–13	357

The complexity of isoform expression is markedly compounded if exon MSD1d is included in the above table. This triplet exon can be expressed alone or possible in combination with any of the mini-exon constrictions listed above. This would add 3 bp to the size of the predicted fragment and clearly such small changes cannot be resolved by South blot analysis.

Figure 2. Inclusion of the MSD1 domain in NCAM mRNA isolated from human embryonal cell lines. Following stripping the signal obtained with randomly primed oligonucleotides complementary to λ9.5, blots were rehybridised with an oligonucleotide complementary to the whole of the MSD1 sequence. Hybridisation and working conditions were those described in Figure 1. Lanes as legend in Figure 1.

The positive control (λ9.5 clone) and PCR products from the JR1 cell line gave signals similar to those identified in blots of other RMS cell lines (Figure 2). As well as the major 360 and 250 bp bands in the JR1 lane, a weaker band of approximately 300 bp was observed. This was apparent when the gels separating neuroblastoma RT PCR products were run for a longer period of time. Again, no signal was identified in either the water control or the PCR products isolated from the GH1 cell line.

When the blot of neuroblastoma PCR products was stripped and re-probed with the oligonucleotide complementary to MSD1a, a different binding pattern was observed. Weak bands in excess of 300 bp were identified in lanes containing RT-PCR products from IMR-32 and NB1. This could arise from NCAM mRNA containing exons MSD1a and b (Table 1, Figure 5). In the PCR products derived from GOTO, NB1 and IMR-32, a band of 270 bp was also observed. This may arise from NCAM mRNA species containing only MSD1a between exons 12 and 13 (Table 1). The appearance of the MSD1a 270 bp band in only three of the neuroblastoma samples does not concur with the 270 bp band seen in all the samples when blots were probed with either λ9.5 or a probe/oligonucleotide complementary to the MSD1 sequence (data not presented). This presumably reflects the different hybridisation conditions and the binding characteristics of the different oligonucleotides as the data was reproducible even when fresh RNA was isolated from all the cell lines.

In contrast with these findings, when blots were stripped and re-probed with an oligonucleotide complementary to MSD1c, no signal was seen in any of the neuroblastoma samples (Figure

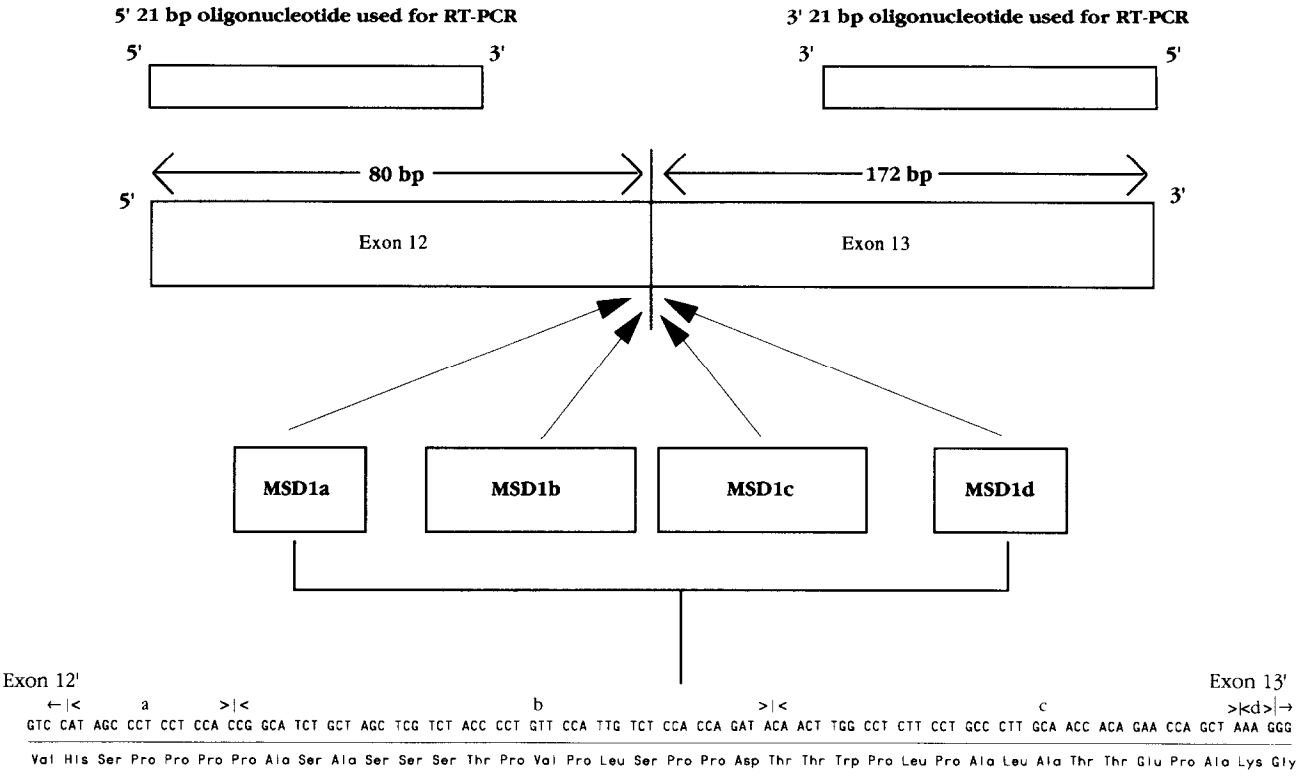

Figure 3. The MSD1 sequence and the exon 12/13 flanking regions, as determined by sequencing the 360 bp PCR products from the JR1 rhabdomyosarcoma cell line. Approximately 400 ng of PCR product from the JR1 cell line was electrophoresed through an 8% polyacrylamide gel and the 360 bp band identified by ethidium bromide staining. The band was cut from the gel and DNA eluted by the crush and soak method [21]. Twenty nanograms of DNA was subjected to asymmetric PCR as above with the exception that 50 pmol of the 3′ primer and 1 pmol of the 5′ primer were used. The amplified product was concentrated using an Isogene™ kit and the product sequenced according to the method of Winship [22].

6). The fact that MSD1c appears not to be spliced into NCAM mRNA accounts for the lack of a 360-bp signal on the blots. As a control to ensure efficient hybridisation, the λ9.5 clone and JR1 samples gave the expected 360-bp product following hybridisation with MSD1c (Figure 6).

DISCUSSION

The MSD1 region was first identified in human muscle NCAM, inserted between exons 12 and 13 in the isoform of NCAM linked into the membrane via a phosphoinositol bridge [10]. The insertion site lies between two fibronectin-type repeats close to the cell membrane. As well as the MSD1a, b and c exons, there is a further series of three nucleotides that can be spliced into the region, this being known as either the 'AAG triplet' or MSD1d exon [15]. Whilst the MSD1 region, including MSD1d, was first described as a complete entity, individual exons or groups of exons have been observed in a variety of tissues. For example, in rat heart and developing heart muscle, MSD1a and MAD1d have been found alone, along with combinations of a and d, b and d, bcd, abd, abc and abcd [16].

Although MSD1 was originally thought to be only spliced into the extracellular isoform of muscle NCAM, recent studies have demonstrated a broader distribution. MSD1a and d have been found in rat brain [17] and MSD1d has been found in small

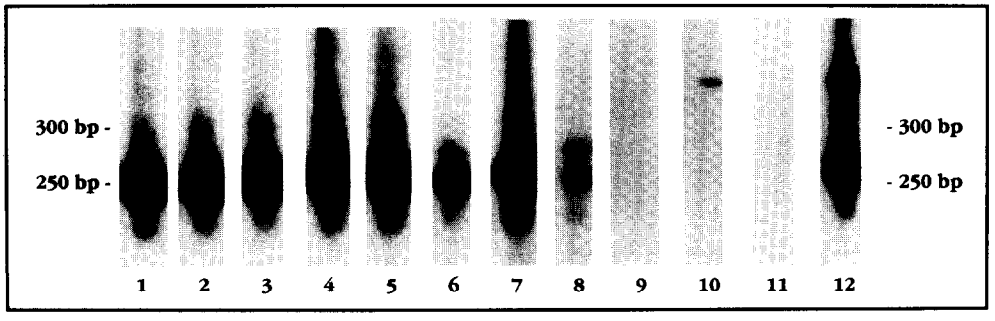

Figure 4. Southern blot of PCR products spanning the exon 12/13 borders, obtained from human neuroblastoma cell lines. Blot hybridised with the MSD1 oligonucleotide. PCR products obtained from RT PCR of RNA isolated from human neuroblastoma cell lines was hybridised with an oligonucleotide complementary to the MSD1 sequence, as described in Figure 1. Lane 1, NBL line SK-N-BE; 2, NBL line GOTO; 3, NBL line IMR-32; 4, NBL line Kelly; 5, NBL line NB1; 6, NBL line CHP100; 7, NBL line SK-N-SH; 8, NBL line PCF; 9, T-cell line GH1 negative control; 10, λ9.5 cDNA; 11, water control; 12, rhabdomyosarcoma cell line JR1.

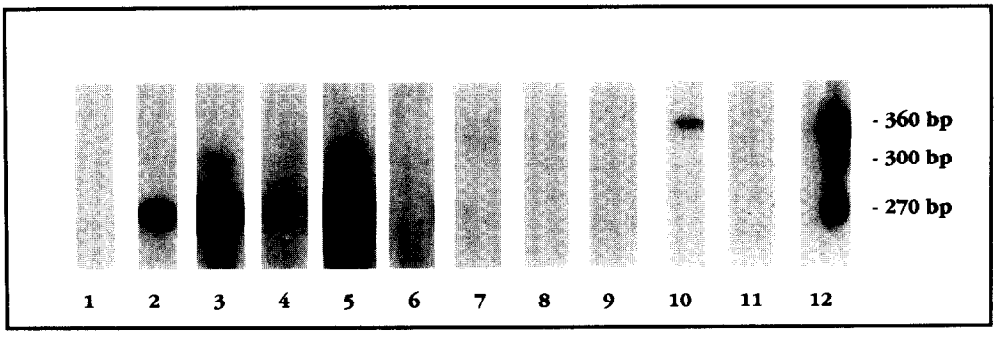

Figure 5. Rehybridisation of the PCR products obtained from neuroblastoma cell lines using an oligonucleotide complementary to MSD1a. The blot containing PCR products from human neuroblastoma cell lines and hybridised with MSD1 (Figure 4) was stripped, checked to ensure that no signal remained and re-hybridised with an oligonucleotide complementary to the MSD1a region (Figure 3). An overnight hybridisation at 45°C was used [13] and the blot was washed to a final stringency of 0.1% SDS 0.1× SSC at 45°C. Signal on the blots was detected by autoradiography and the experiment was repeated as indicted in Figure 4. Lanes as legend in Figure 4.

cell lung carcinomas [18], gliomas and schwannomas [19]. Furthermore, in chicken muscle NCAM the MSD1 region has been identified in a transmembrane isoform of NCAM.

Alternative splicing of the MSD1 exons into NCAM increases markedly the number of different isoforms of the molecule that can be synthesised. The biological rationale behind the generation of such diversity remains unclear. The MSD1a region encodes three proline residues alone and four when in tandem with MSD1b. This may produce a 'hinge' in the molecule, affecting its tertiary structure. The MSD1 sequence also encodes a region rich in serine and theonine residues. These may be O-glycosylated, but the functional consequences of this remain unclear [20]. Presumably, the diversity in NCAM isoforms generated by differential splicing, along with differential glycosylation, affects the 'adhesive' nature of the molecule in ways that remain to be fully investigated.

The data presented here suggests that the full MSD1 region is spliced into the NCAM RNA of rhabdomyosarcoma, but not neuroblastoma cell lines, and that the exon lacking in the latter group of tumour cell lines is MSD1c. Again, the functional significance of this finding is not completely understood. From the current data, it is not clear as to which combinations of MSD1a, MSD1b and MSD1d are found in neuroblastoma NCAM.

Probing the RT-PCR products with an oligonucleotide complementary to MSD1a suggests that the exon is expressed in some neuroblastomas and not in others. However, when the blots were stripped and probed with an oligonucleotide mixture complementary to the λ9.5 clone, a band of 270 bp was observed in the majority of samples, although this did not separate well from the 250 bp PCR product representing the exon 12/13 borders without the MSD1 exons included. This anomaly needs to be resolved, and to do this we have turned to cloning the PCR products.

Unfortunately, screening over 400 clones has revealed sequences representative of the exon 12/13 border or, in some cases, irrelevant sequences often containing repetitive sequences complementary to the oligonucleotide primers. Our inability to identify MSD1 exons in the RT-PCR products examined by TA cloning may reflect the proportion of NCAM molecules carrying these spliced exons. Consistent with this is the intensity of the 250 bp band as compared with the larger products seen after probing RT-PCR products with oligonucleotides complementary to the λ9.5 clone. Alternatively, differences in the band intensity may arise from the hybridisation conditions instead of the relative proportions of differentially spliced RNA species, as the quantitative aspects of the PCR reaction used in these studies has not been investigated.

In a separate series of studies, we have demonstrated that the embryonic, highly glycosylated form of NCAM is found on the majority of neuroblastomas and rhabdomyosarcomas investigated [8]. Neuraminidase treatment reveals a predominance of the 140-kDa isoform, although the 120-kDa isoform of the molecule was also identified. Whether the MSD1 exons are found associated with the transmembrane or extracellular isoform of the molecule remains to be resolved.

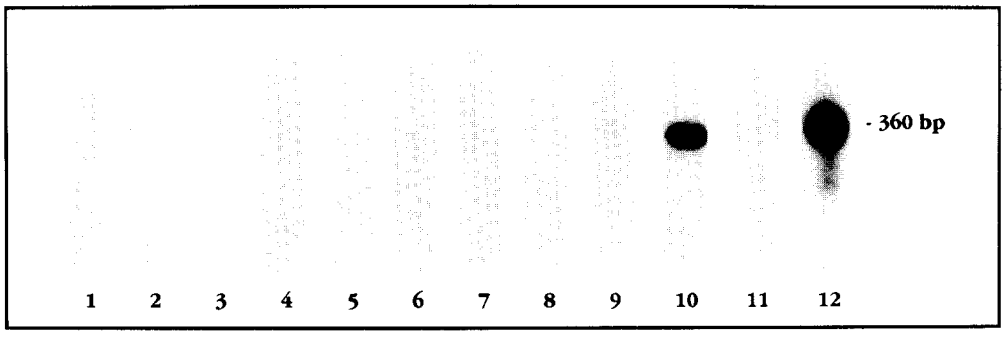

Figure 6. Re-hybridisation of the PCR products obtained from neuroblastoma cell lines using an oligonucleotide to MSD1c. The blot used in Figures 4 and 5 was stripped to ensure that no signal remained and subsequently hybridised with an oligonucleotide complementary to MSD1c (Figure 3). Hybridisation was carried out overnight at 60°C and the blot washed to a final stringency of 0.1% SDS, 0.1× SSC at 45°C. The blot was autoradiographed and the experiment repeated as indicated above. Lanes as legend in Figure 4.

The lack of inclusion of MSD1c in neuroblastoma NCAM mRNA as compared to rhabdomyosarcomas could prove useful in the differential diagnosis of these tumour types. Although it is possible to use RT PCR to make this differential diagnosis on cell lines, it is probable that tissues will prove more difficult to analyse. Not only is there the problem of maintaining the integrity of RNA isolated from tissues, there is also the difficulty of obtaining pure tumour tissue that is not contaminated with normal tissues such as blood, smooth muscle and components of the autonomic nervous system. The presence of these elements within tissue extracts could give rise to PCR not originating from tumour mRNA.

An immunocytochemical approach to the differential diagnosis of these tumour types would, therefore, probably prove more useful. To this end, we are currently attempting to raise monoclonal antibodies (MAbs) to a synthetic peptide representative of the MSD1c region and linked to Keyhole Limpet Haemocyanin.

These studies illustrate how a detailed investigation of the expression of a particular molecule on the membrane of a cell may lead to the generation of highly specific reagents for tumour diagnosis. Alternative splicing appears to occur in many different situations and, therefore, this approach to the differential diagnosis of tumours may be applicable to a variety of molecules and different tumour types.

1. Innocent G. An investigation of the epidemiology of neuroblastoma in children under the age of 15 years in England, Scotland and Wales. *Comput Appl Biosci* 1992, **8**, 97–106.
2. Bertani-Dziedzic L, Dziedzic SW, Gitlow SE. Catecholamine metabolism in neuroblastoma. In Pochedly C ed. *Neuroblastoma: Tumour Biology and Therapy*. CRC Press Inc, Boca Raton, 1990, 69–92.
3. Hoefnagel CA, Voute PA, De-Kraker J, Marcuse HR. Radionuclide diagnosis and therapy of neural crest tumours using iodine-131 *meta*-iodobenzylguanidine. *J Nucl Med* 1987, **28**(3), 308–314.
4. Osborn M, Hill C, Altmannsberge M, Weber K. Monoclonal antibodies to titin in conjunction with antibodies to desmin separate rhabdomyosarcomas from other tumour types. *Lab Invest* 1986, **55**, 101–108.
5. Schmidt RE. Non-lineage/natural killer section report: new and previously defined clusters. In Knapp W, Dorken B, Gilks W, Rieber EP, Schmidt RE, Stein H, Kr von dem Borne AE eds. *Leucocyte Typing IV: White Cell Differentiation Antigens*. Oxford University Press, Oxford, 1989, 517–551.
6. Patel K, Moore SE, Dickson G, Rossell RJ, Beverley PC, Kemshead JT, Walsh FS. Neural cell adhesion molecule (NCAM) is the antigen recognized by monoclonal antibodies of similar specificity in small cell lung carcinoma and neuroblastoma. *Int J Cancer* 1989, **44**, 573–578.
7. Frost G, Patel K, Bourne S, Coakham HB, Kemshead JT. Expression of alternative isoforms of the neutral cell adhesion molecule (NCAM) on normal brain and a variety of brain tumours. *Neuropath Applied Neurobiol* 1991, **17**, 207–217.
8. Phimister E, Kiely F, Kemshead JT, Patel K. Expression of neural cell adhesion molecule (NCAM) isoforms in neuroblastoma. *J Clin Pathol* 1991, **44**, 580–585.
9. Patel K, Culverwell A, Rossell RJ, Kemshead JT, Phimister E. VASE mini-exon usage by NCAM is not restricted to tumours of neuroectodermal origin. *Int J Cancer* 1993, **54**, 772–777.
10. Dickson G, Gower HJ, Barton CH, *et al.* Human muscle neural cell adhesion molecule (NCAM): identification of a muscle-specific sequence in the extracellular domain. *Cell* 1987, **50**(7), 1119–1130.
11. Allan P, Garson J, Harper E, Asser U, Coakham HB, Brownell B, Kemshead JT. Biological characterisation and clinical application of a monoconal antibody recognising an antigen restricted to neuroectodermal tissues. *Int J Cancer* 1983, **31**, 591–598.
12. Innis MA, Gelfand DH. Optimisation of PCRs. In Innis MA, Gelfand DH, Sninsky JJ, White TJ eds. *PCR Protocols*. Academic Press Inc, San Diego, 1990, 3–13.
13. Wahl GM, Berger SL, Kimmel AR. Molecular hybridisation of immobilised nucleic acids: theoretical concepts and practical considerations. *Methods Enzymol* 1987, **152**, 399–407.
14. Gibson F, Clarke J, Katz F, Kemshead JT. A monoclonal antibody (FMG25) that can differentiate neuroblastoma from other small round cell tumours of childhood. *Int J Cancer* 1987, **39**, 554–559.
15. Prediger EA, Hoffman S, Edelman GM, Cunningham BA. Four exons encode a 93-base-pair insert in three neural cell adhesion molecule mRNAs specific for chicken heart and skeletal muscle. *Proc Natl Acad Sci USA* 1988, **85**, 9616–9620.
16. Reyes AA, Small SJ, Akeson R. At least 27 alternatively spliced forms of the neural cell adhesion molecule mRNA are expressed during rat heart development. *Mol Cell Biol* 1991, **11**, 1654–1661.
17. Reyes AA, Schulte SV, Small S, Akeson R. Distinct NCAM splicing events are differentially regulated during rat brain development. *Mol Brian Res* 1993, **17**, 201–211.
18. Carbone DP, Koros AM, Linnoila I, Jewett P, Gazdar AF. Neural cell adhesion molecule expression and messenger RNA splicing patterns in lung cancer cell lines are correlated with neuroendocrine phenotype and growth morphology. *Cancer Res* 1991, **51**, 6142–6149.
19. Hamshere M, Dickson G, Eperon I. The muscle specific domain of mouse NCAM: structure and alternative splicing patterns. *Nucl Acids Res* 1991, **19**, 4709–4716.
20. Walsh FS, Parekh RB, Moore SE, Dickson G, Barton CH, Gower HJ, Dwek RA, Rademacher TW. Tissue specific O-linked glycosylation of the neural cell adhesion molecule (N-CAM). *Development* 1989, **105**, 803–811.
21. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Laboratory Press, New York, 1989, 646.
22. Winship PR. An improved method for directly sequencing PCR amplified material using dimethyl-sulphoxide. *Nucl Acids Res* 1989, **17**, 1266–1269.