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Variant CD44 expression by human fibroblasts

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Abstract
CD44 is a cell surface glycoprotein with roles in tumour invasion and metastasis. CD44 is variably spliced from ten variant exons and mis-splicing is a biomarker for detection of colon, urothelial and other carcinomas. Fibroblasts are normally considered to lack variant exons and thus should not generate false-positive signals. Transcription of variant exons by fibroblasts was investigated by exon-specific reverse transcription-polymerase chain reaction (RT-PCR) for variant exons v2–v10 using normal primary fibroblasts, immortalized and experimentally transformed fibroblasts. Flow cytometry, immunocytochemistry and Western blotting were used to determine expression. All types of fibroblasts, including normal primary culture fibroblasts, transcribed low levels of variant exon mRNA. Expression could not be detected by blotting or immunocytochemistry but flow cytometry revealed minor expression of some exons by all three types of cultured fibroblast. Fibroblasts do transcribe and express small amounts of variant exon CD44. This may need to be considered when using exon splicing as a biomarker for malignancy in clinical samples containing connective tissue.

Keywords: *CD44, mRNA splicing, variant exons, constitutive exons*

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Introduction

CD44 is a cell surface glycoprotein involved in cell adhesion and migration, wound healing, lymphocyte recirculation and metastasis (Haynes et al. 1989, Haynes et al. 1991). Most cells express the standard isoform (CD44s) coded by 'constitutive' exons (Naor et al. 1997). However, human epithelia also express variant isoforms in which ten variant exons (v1–10) are expressed by alternative splicing (Arch et al. 1992, Heider et al. 1993, Dall et al. 1994).

The process of CD44 variant exon mRNA splicing is known to be deranged in many cancers and has been a useful biomarker for diagnosis and prognostic prediction. Blotting, polymerase chain reaction (PCR) and immunocytochemistry analysis have revealed an impressive array of readily detectable abnormalities not seen in normal tissues (Matsumura & Tarin 1992, Heider et al. 1993, Tanabe et al. 1993, Finn et al. 1994). Breast carcinoma shows overexpression of variant exon v6 (Foekens et al. 1999, Herrera-Gayol & Jothy 1999), colon carcinoma of v3 and v4 exons

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(Ni et al. 2002) and urothelial carcinoma of v8–v10 (Miyake et al. 2002) together with evidence of mis-splicing and intron retention (Matsumura et al. 1995, Yoshida et al. 1995, Goodison et al. 1998).

These alterations in CD44 gene expression can be detected in both primary and metastatic neoplasm specimens and sensitive techniques such as PCR are capable of identifying changes in the small number of cells present in faecal and urine screening samples (Matsumura & Tarin 1992, Tarin & Matsumura 1993, Goodison & Tarin 1998, Miyake et al. 2002). It has been considered that variant exon mis-splicing detected in such samples would originate from epithelial cells. However, during investigation of splicing we noted that fibroblasts normally considered to express only constitutive exons (CD44s), can also transcribe and express variant exons.

This is not the first report of variant CD44 expression by fibroblasts. Fibroblasts isolated from the inflamed synovial joint of both rheumatoid (RA) and osteoarthritic (OA) patients, have been shown to express CD44 splice variants at both the mRNA and protein level (Croft et al. 1997). CD44 splice variants have also been previously detected in human gingival and periodontal fibroblasts (Hirano et al. 1997). The aims of the current study were therefore, to characterize this splicing in normal epidermal fibroblasts, immortalized and transformed epidermal fibroblast cell lines to assess any potential to interfere with the use of clinical CD44 splicing assays as biomarkers of cancer.

Materials and methods

Maintenance and culture of primary, immortalized and experimentally transformed fibroblasts

Primary normal human dermal fibroblasts (NHDF) (Clonetics, Lonza Wokingham Ltd, Wokingham, Berkshire, UK) were maintained in basal medium supplemented with singleQuots from FGM-2 Bulletkit (Clonetics). Spontaneously immortalized dermal fibroblasts (161BR, 142BR and 1BR3) (Arlett et al. 1988, Green et al. 1991) were grown in minimum essential medium (MEM; Sigma-Aldrich Ltd, Gillingham, Dorset, UK) supplemented with 15% fetal calf serum (FCS), 2 mM glutamine, 500 units ml^{-1} penicillin, 500 μg ml^{-1} streptomycin and 1% non-essential amino acids (Sigma). Experimentally transformed dermal fibroblast lines (1BR3N and 1BR3G) were immortalized by transfection of the parent cell line (IBR3) using pSV3neo and pSV3gpt, respectively (Arlett et al. 1988). These cells were grown in MEM supplemented with 10% FCS. Positive control normal human epidermal keratinocytes (NHEK) (Clonetics) were maintained in keratinocyte basal medium supplemented with singleQuots from KGM-2 Bulletkit (Clonetics). The primary cell lines NHDF and NHEK were not passaged more than five times. For immunocytochemistry, cells were removed with 0.1% trypsin/EDTA solution, resuspended in FCS, seeded onto polytetrafluoroethylene (PTFE)-coated microscope slides and grown to 80% confluence at 37°C in 5% CO_2.

Reverse transcription-polymerase chain reaction

For reverse transcription (RT)-PCR, RNA was extracted from cells using RNeasy spin columns (Qiagen, Crawley, West Sussex, UK) as previously described (Bloor et al. 2001). cDNA was synthesized from 5 μg of total RNA for 1 h at 37°C with 12.5 ng of

oligo dT primer and 10 units of Superscript II RT enzyme (Invitrogen Ltd, Paisley, Renfrewshire, UK). Hot start PCR was performed using 10% of RT product (cDNA) with primers for β-actin, total CD44 (primer P1, situated in exon 3 and primer P2, which is present at the boundary of exon 17 and intron 18) and individual variant exons v1 to v10 as previously described (Bloor et al. 2001, 2002). Briefly, 5 μl of cDNA was added to a reaction containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1 × Q solution (Qiagen), 200 μM of each dNTP, and 20 μM of each 5′ and 3′ primer. This was denatured for 5 min at 95°C followed by a 10-min elongation phase at 72°C during which time 0.25 units of Taq DNA polymerase (Qiagen) was added to the reaction. Amplification of the cDNA was performed with 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 57°C, and finally, elongation for 2 min at 72°C. The resulting reaction products were resolved by electrophoresis on a 1.5% agarose gel alongside a 100 bp marker ladder (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK).

RT-PCR controls and specificity

RT-PCR was performed on fibroblasts with P1 and P2 primers to confirm presence of CD44 mRNA and with β-actin for RNA quality/loading control. Variant exon-specific PCR was performed with all primers on NHEK as positive control and with a water-negative control. To confirm specificity of amplification, DNA bands of interest were excised from the gel and the PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were set up using the ABI PRISM automated sequencing kit (Applied Biosystems, Warrington, Cheshire, UK), sequencing was performed using the Perkin Elmer ABI 377, and identity of the PCR products was confirmed using BLAST software from NCBI (Zhang & Madden 1997).

Immunocytochemistry

Cell phenotype was confirmed by immunocytochemistry (ICC) for cytokeratin, vimentin and fibroblast marker prolyl 4-hydroxylase. CD44 variant exon staining was performed on acetone-fixed adherent cells grown on APES treated PTFE-coated microscope slides. The slides were blocked in 3% hydrogen peroxide in 50% methanol for 30 min to stop endogenous peroxide activity and in 20% normal rabbit serum (Dako UK Ltd, Ely, Cambridgeshire, UK) for 10 min to block non-specific binding of antibodies. All of the primary antibodies were reported to be suitable for ICC by the manufacturers, and a titration assay was performed for each antibody, using five different concentrations covering the recommended range of the manufacturer, to determine the optimum concentration producing highest contrast and minimal non-specific staining. After blocking, the slides were incubated for 60 min with the appropriate concentration of primary antibody (Table I), followed by 30 min in biotinylated rabbit anti-mouse secondary antibody (Dako Ltd, UK; 1:300) and 30 min in streptavidin–biotin horseradish peroxidase (Dako Ltd; 1:50). Between each reagent, cells were washed twice for 3 min each in PBS. Antibody binding was visualized with 3,3′ diaminobenzidine (DAB+, Dako Ltd) and counterstained with haematoxylin (VWR International Ltd, Lutterworth, Leicestershire, UK). Isotype-matched irrelevant antibodies were used as negative controls.

Table I. Details of primary antibodies.

Antibody	Company[a]	Isotype	Final concentration (μg ml^{-1})		
			ICC	WB	FC
β-actin	1	IgG	–	2	–
Cytokeratin, MNF116	2	IgG1	2	–	–
Vimentin	2	IgG1	5.8	–	–
Prolyl hydroxylase 5B5	2	IgG1	3.1	–	–
Total CD44, 2C5	3	IgG2A	1	2	16.7
Exon v3, 3G5	4	IgG2b	2	2	16.7
Exon v4, VFF11	4	IgG1	1	2	16.7
Exon v5, VFF8	4	IgG1	5	2	16.7
Exon v6, 2F10	4	IgG1	1	2	16.7
Exon v7, VFF9	4	IgG1	5	2	16.7
Exon v7/8, VFF17	4	IgG2b	2	0.5	16.7
Exon v9, 441V	5	IgG1	2	2	16.7
Exon v10, VFF14	4	IgG1	20	2	90

[a]1, Santa Cruz, USA; 2, Dako Ltd, UK; 3, R&D Systems, UK; 4, Serotec, UK; 5, AMS Biotech, Europe. ICC, immunocytochemistry; WB, Western blotting; FC, flow cytometry.

Flow cytometry

Approximately, 10^6 fibroblasts detached with 10 mM EDTA/PBS were washed and resuspended in 100 μl of PBS with 2.5% FCS and incubated for 1 h at 4°C with primary antibody against CD44 and specific variant exons at final concentrations determined by control titration assays (Table I). Following binding, cells were washed in PBS, incubated for 30 min at 4°C with FITC-conjugated goat Fab fragments to mouse IgG (1:5000; Cappel Laboratories, Malvern, PA, USA), washed again in PBS, and fixed in 2% paraformaldehyde. Flow cytometry (FC) was performed using the FACScan (Becton Dickinson UK Ltd, Littlemore, Oxford, UK) measuring 10 000 events per sample. Background/non-specific staining was subtracted using controls stained with irrelevant isotype-matched antibody at the same concentrations and keratinocytes were used as positive controls.

Preparation of cell lysates and Western blotting

Cell monolayers at 80% confluence were washed with ice-cold PBS, lysed for 10 min on ice with NP40 buffer (Patel et al. 1997) and the postnuclear supernatant was then stored at −70°C. Then, 25 μg protein (determined by Coomassie blue dye-binding procedure (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK), was boiled for 5 min with gel-loading buffer, and was then resolved in 8% polyacrylamide-SDS resolving gels. The protein was transferred to immobilon-P membrane (Millipore UK Ltd, Croxley Green, Watford, UK) using a semi-dry transfer method (Multiphor II, GE Healthcare, Amersham, Buckinghamshire, UK) at 20 V for 15 min. Non-specific binding sites were blocked by incubation with 5% dried skimmed milk or BSA in PBS containing 0.1% Tween 20 (PBS-T). Blots were then analyzed (Harlow & Lane 1989) using monoclonal antibodies against total CD44 and individual variant exons (Table I) using β-actin as loading control. All of the primary antibodies were titrated in the range of 2–10 μg ml^{-1} using test blots, to determine the optimum antibody concentrations. The blots were incubated with their primary

antibody in PBS-T for 1 h at room temperature. Antibody binding was then visualized using horseradish peroxidase-conjugated secondary antibody (1:5000 to 1:10 000; Amersham) in PBS-T for 45–60 min at room temperature followed by enhanced chemiluminescence (ECL, Amersham) according to the manufacturers protocol.

Results

Cell phenotype

All fibroblasts expressed vimentin and the fibroblast marker prolyl 4-hydroxylase but not the cytokeratin marker MNF116 confirming cell type. Keratinocytes showed the converse epithelial phenotype (data not shown).

Transcription of CD44 variant exons by fibroblasts

mRNA of suitable quality was extracted from all cells as shown by a 411-bp band with primers for β-actin (Figure 1A). All fibroblasts transcribed the standard form of CD44, represented by a band of 549 bp using primers P1/P2 (Figure 1B). Two fibroblast lines, 1BR3 and 1BR3G (lanes 3 and 4), showed an additional band at 700 bp suggesting the presence of variant exon transcripts. However, fibroblasts

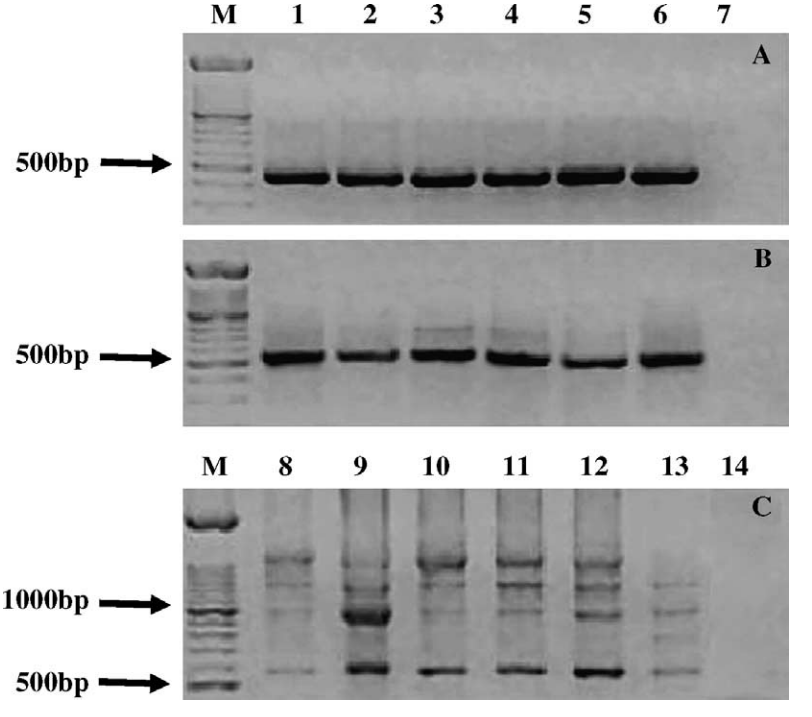

Figure 1. Amplification of β-actin and CD44 by fibroblasts and keratinocytes. RT-PCR for β-actin (A), CD44, using the P1 and P2 primers, (B and C) was performed, and resolved by electrophoresis on a 1.5% agarose gel with a 100-bp ladder (100 bp to 1 kb). Lanes 1–6 (A and B) were the fibroblast cell lines: NHDF, 161BR, 1BR3, 1BR3G, 1BR3N, and 142BR, respectively. Lanes 8–13 (C) were six different batches of NHEK cells. Lanes 7 and 14 were PCR no-template water controls.

Table II. Transcription and expression of CD44s and variant exons in normal human epidermal keratinocytes (NHEK) and fibroblasts.

	CD44 variant exons										
Cells	CD44s	v1	v2	v3	v4	v5	v6	v7	v8	v9	v10
NHEK											
RNA	●		●	●	●	●	●	●	●	●	●
Protein	+	NT	NT	+	+	+	+	+	+	+	+
NHDF											
RNA	●				●	●	●	●		●	●
Protein	+	NT	NT	−	+	+	+	−	−	+	−
161BR											
RNA	●			●	●	●	●	●	●	●	
Protein	+	NT	NT	−	+	+	+	−	+	+	+/−
142BR											
RNA	●					●	●	●	●		●
1BR3											
RNA	●					●	●	●	●	●	●
Protein	+	NT	NT	−	+/−	+/−	+	−	+	+	−
1BR3N											
RNA	●		●	●	●	●	●	●	●	●	●
Protein	+	NT	NT	+	+/−	+	+	−	+	+	−
1BR3G											
RNA	●		●	●	●	●	●	●	●	●	●

●, Transcription found; +, >40% cells positive; +/−, <30% cells positive; −, almost all cells were negative; NHDF, normal human dermal fibroblasts; NT, not tested. 142BR and 1BR3G were not tested by flow cytometry.

transcribed many fewer and less intense bands above 549 bp than positive control keratinocytes (Figure 1C).

RT-PCR with exon-specific primers showed that all fibroblasts transcribed variant exons, but in less well-defined transcripts than keratinocytes (Table II, Figure 2). Both primary (NHDF) and immortalized (161BR, 142BR and 1BR3) fibroblasts appeared to commence transcription from variant exon 4 or 5 with the exception of 161BR (Figure 2B). Similarly, the transformed lines (1BR3N and 1BR3G) transcribed all variant exon v2 to v10 in short transcripts (Figure 2E). Sequencing confirmed the specificity of amplification for v2–v10 bands except for the 700-bp band of 1BR3 and 1BR3N which was present in insufficient quantity for analysis. Exon v1 is not normally transcribed in human and was included as a negative control. Apparent amplification (Figure 2) proved negative on sequencing (showed constitutive region only) for the bands in 161BR, 1BR3N and 1BR3G.

Expression of CD44 variant exons by fibroblasts

ICC of fibroblasts showed membrane staining for only CD44s and not variant exons (data not shown) and this was confirmed by Western blotting (WB), which also detected only CD44s (data not shown). However, FC detected expression of several variant exons in all fibroblast cell lines tested (Table II, Figure 3A–H) with a few

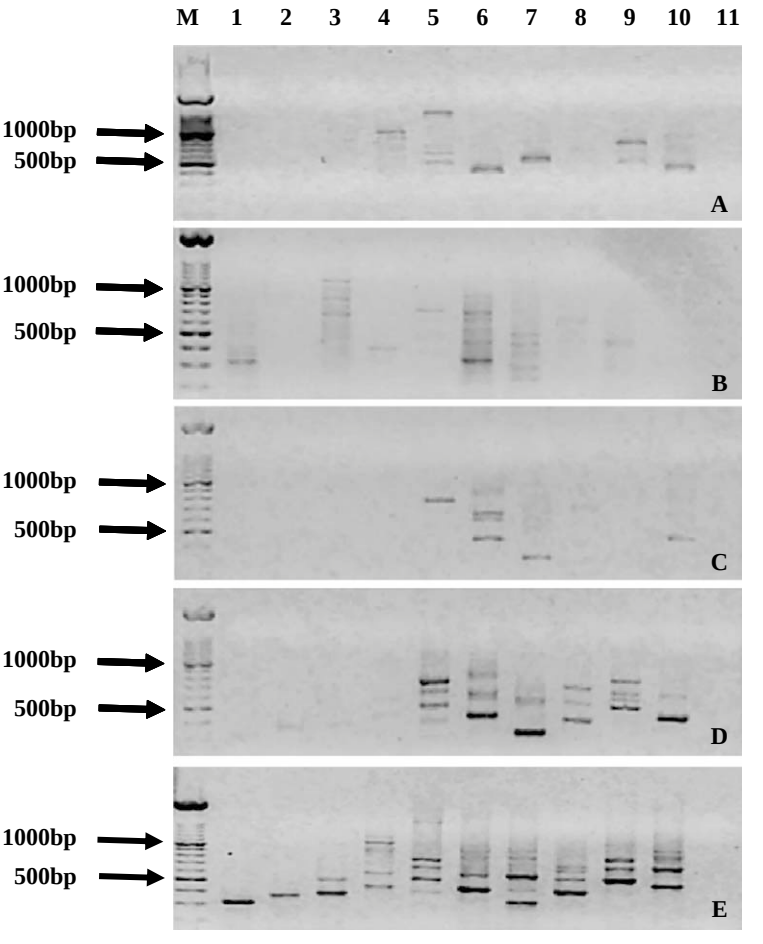

Figure 2. Exon-specific RT-PCR for CD44 variants in fibroblasts. RT-PCR using exon-specific primers (lanes 1–10 were exons v1 to v10, respectively, lane 11 was a PCR no-template water control) was performed, and resolved by electrophoresis on a 1.5% agarose gel with a 100-bp ladder (100 bp to 1 kb). Normal primary dermal fibroblasts; NHDF (A), spontaneously immortalized dermal fibroblasts – 161BR (B), 142BR (C), 1BR3 (D), and finally, experimentally transformed dermal fibroblasts, IBR3N (E) were assessed.

exceptions; variant exon 3 was found in greater than 40% of 1BR3N cells only, variant exon 10 in less than 30% of 161BR cells only, variant exon 8 was not detected in NHDF cells, and variant exon 7 was not detected in any of the fibroblast cell lines tested. Expression of all variant exons was detected in positive control keratinocytes by all three investigative methods for protein expression.

Discussion

The CD44 gene has been considered a promising biomarker for screening for malignancy of colon, bladder and cervix as well as a useful metastatic or prognostic marker for many other malignancies. It is generally accepted that variant exon expression and exon/intron mis-splicing are limited to the epithelial component of

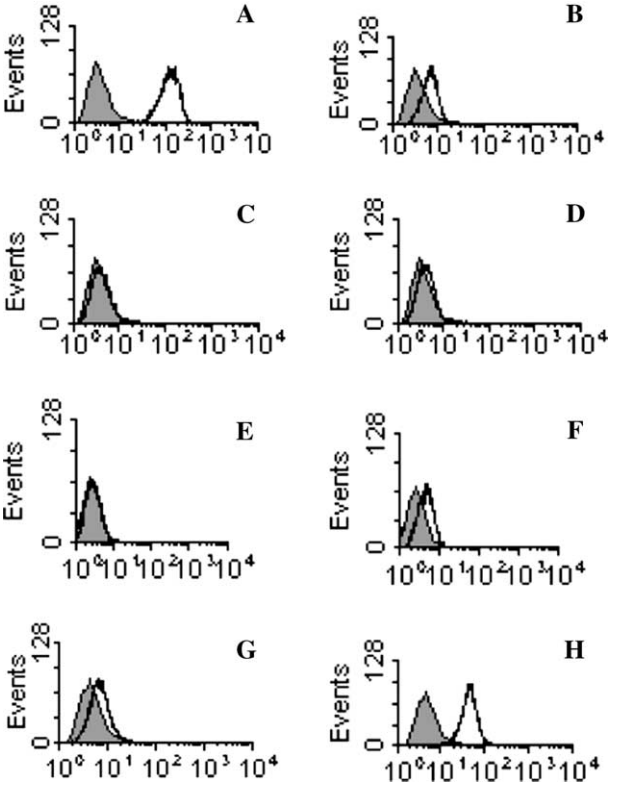

Figure 3. Flow cytometric analysis of CD44 expression in fibroblasts. Percentage cell staining was calculated by subtraction of relevant isotype-matched controls from the primary antibody measured in 10 000 events by flow cytometry. Representative results from three experiments show: normal human dermal fibroblasts cells (NHDF) analyzed with CD44s (A), CD44 v6 (B), CD44 v7 (C) and CD44 v7/8 (D), spontaneously immortalized dermal fibroblasts, 1BR3, analyzed with CD44 v3 (E) and CD44 v6 (F), and finally, experimentally transformed dermal fibroblasts, 1BR3N, analyzed with CD44 v4 (G) and CD44 v9 (H).

these malignant neoplasms but the screening techniques have used highly sensitive molecular methods with the theoretical disadvantage that very small amounts of signal from normal cell populations might be detected. For instance, lymphocytes express variant exon 6 at low levels during activation (Griffioen et al. 1994). In the present study we have also shown the transcription and expression of CD44 variant isoforms in normal (NHDF), immortalized (161BR, 142BR and 1BR3), and experimentally transformed (1BR3N and 1BR3G) fibroblasts and confirmed these findings with the appropriate positive and negative controls. This fibroblastic expression might have the potential to generate a false-positive signal in small clinical samples if these results reflect synthesis *in vivo*.

Fibroblasts have long been considered to express only CD44s (Hudson et al. 1995, 1996), without variant exons, and our results with P1/P2 RT-PCR, ICC and WB appear to support this contention. Our results also show that the P1/P2 primers, whilst detecting CD44s in all cell lines, failed to detect the low levels of variant transcription in fibroblasts in all but two of the fibroblastic cell lines (Figure 1B). However, the positive control keratinocytes (NHEK) demonstrated detection of many

CD44 isoforms using these primers (Figure 1C). Most other studies of fibroblasts have also analyzed CD44 transcription using RT-PCR with primers P1/P2 and so have detected only CD44s in their fibroblast populations (Hudson et al. 1995, 1996). However, the more sensitive exon-specific RT-PCR and flow cytometry have detected low levels of transcription and expression of many variant exons by the fibroblastic cell line samples in this study (Figure 2).

Another study using Northern blotting of normal human dermal fibroblasts in primary culture detected a 700-bp band and additional bands at 3.6 and 1.8 kb with a probe spanning the 5′ end of the constitutive region (Hudson et al. 1995). The authors ascribed these larger transcripts to the usage of different polyadenylation sites (Shtivelman & Bishop 1991) rather than variant exon inclusion. Our results with a more sensitive technique support the presence of at least six variant exons in both primary fibroblasts (NHDF) and cell lines (Figure 2), thus supporting the presence of variant exons in the 700-bp band detected in two of our fibroblastic cell lines with the general primers.

One criticism of the present work is that the results for 1BR3N and 1BR3G might be the result of the transformation procedure, transformed from the parental line, 1BR3, using different vectors (pSV2neo and pSV3gpt) (Arlett et al. 1988). However, the parent line also transcribes variant exons v5 to v10 and normal fibroblasts (NHDF) also show similar features. It is also possible that the culture conditions might contribute as they have been shown to increase expression (Zhou et al. 1999), and alter splicing in keratinocytes (Sugino et al. 1998), probably as a reflection of sustained growth and loss of cell–cell contact. We have reduced the culture periods to the minimum but, unfortunately, culture conditions can not be made identical, we cannot apply FC directly to fibroblasts *in vivo* and ICC and WB have proved incapable of detecting a low level of variant expression in tissue (unpublished data). While the current results are probably not a reflection of steady-state tissue fibroblasts they may well reflect active or dividing populations such as might be sampled in wound healing or from the stroma of malignant tumours. There are currently no sufficiently sensitive methods to detect expression and confirm the results of present work *in vivo* and no possibility of extracting a pure fibroblastic population from tissue samples without culture.

Given the small amounts of variant exon present it is not surprising that previous studies have not detected it. Indeed, we ourselves have struggled to identify CD44 variant isoforms at the protein level using WB and ICC. Both these methods involve denaturation or fixation prior to the immunodetection process and it is well documented that exon assortment and post-translational modifications of CD44 variant molecules, including, glycosylation can mask CD44 exon-specific epitopes and affect the detection of variant exons by monoclonal antibodies (Martegani et al. 1999). Another possibility for the lack of detection by WB could be the modification of the variant molecules by glycosaminoglycan moieties which could potentially exclude them from entering the acrylamide gel due to their high molecular weights. It has also been previously shown that in high-expressing tumour samples most of the variant mRNA does not detectably translate into proteins by direct comparison of Southern and Western blots (Woodman et al. 1996). Finally, other studies, may have failed to detect these variants simply because fibroblasts from different tissues or sites could have diverse CD44 variant isoform complements.

Further evidence to support our results comes from a multitechnique study which showed transcription of variant exons in gingival and periodontal fibroblasts and that transcription was unaffected by stimulation with interleukin (IL)-1β and tumour necrosis factor (TNF)-α (Hirano et al. 1997). Variant exons have also been detected in the synovial fibroblasts of arthritic joints (Croft et al. 1997). This same group later showed that antibody blockade of CD44 v7/8 inhibited the proliferation of RA synovial fibroblasts (Wibulswas et al. 2000), whereas anti-v3 and v6 reduced their invasive abilities, but had no effect on their migratory behaviour (Wibulswas et al. 2002). The authors suggested that the CD44 variant expression by these synovial fibroblasts might have functional implications in both survival and matrix degradation.

Fibroblasts in the tumour microenvironment are often thought to closely resemble healing wounds, and to be under the increased survival pressures of the growth factors and other factors produced by the tumour as part of tumour stroma interactions (Mueller & Fusenig 2002). Tumour stroma, has also been shown to produce proteases involved in matrix degradation as part of tumour progression and metastasis (Pedersen et al. 2005). The likelihood of fibroblast contamination causing difficulties in interpretation of clinical tests is unclear. The tumour stroma has been estimated to contribute between 20–90% of the mass of a solid tumour (Mersmann et al. 2001, Tahtis et al. 2003), and fibroblasts are the main cellular component, even in some cases outnumbering the cancer cells within a tumour mass (Li et al. 2007). It is clear that there is potential for contamination, but the effects would probably vary with the tissue and the specific CD44 signature being sought.

In conclusion, these data show that sensitive techniques detect transcription and expression of variant exons in fibroblasts, both normal and transformed, that were previously considered not to express variant exons. This requires further investigation if expression and splicing are to be exploited as biomarkers for screening and diagnosis.

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