Pergamon

European Journal of Cancer, Vol. 34, No. 10, pp. 1607–1611, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00177-4

Original Paper

Decreased Expression of CD44 Splicing Variants in Advanced Colorectal Carcinomas

S. Weg-Remers,[1] M. Anders,[2] B. von Lampe,[2] E.O. Riecken,[2] G. Schüder,[3] G. Feifel,[3] M. Zeitz[1] and A. Stallmach[1]

[1]Department of Internal Medicine II, University of the Saarland, 66421 Homburg; [2]Department of Gastroenterology, Benjamin Franklin Hospital, Free University of Berlin, Berlin; and [3]Department of Surgery, University of the Saarland, Homburg, Germany

CD44v6 expression appears to be associated with adverse prognosis and propensity for metastasis in patients with colorectal cancer. However, expression of CD44 variants in different tumour stages has been poorly characterised. CD44 variant expression was investigated in normal colonic mucosa ($n = 36$), colorectal adenomas ($n = 15$), carcinomas ($n = 62$) and metastases ($n = 6$) by reverse transcriptase–polymerase chain reaction (RT-PCR) and Southern blotting with exon-specific probes. High frequencies of CD44standard (CD44s) and CD44 epithelial (CD44e) were observed in normal and neoplastic tissue. CD44v2 was seen predominantly in adenomas (27%) and UICCI carcinomas (29%). CD44v5 expression was low in normal mucosa (3%), higher in adenomas and carcinomas (29–33%), independent of tumour stage. CD44v6 expression was low in normal mucosa (6%) and higher in adenomas (47%) and carcinomas (42%). Surprisingly, a significant decrease of CD44v6 was observed in metastatic primary tumours (8%) and metastases (17%) (UICCIV) ($P \leq 0.05$). Therefore, the concept of CD44v6 conferring metastatic potential to malignant cells cannot be supported by our data.

Key words: CD44, colorectal carcinoma, tumour stage, metastasis, RT–PCR
Eur J Cancer, Vol. 34, No. 10, pp. 1607–1611, 1998

INTRODUCTION

COLORECTAL CARCINOMA is the second most frequent cause of cancer mortality in Western countries. The prognosis of the disease is largely dependent on tumour stage at the time of surgery and is poor when development of metastasis has occurred. There is increasing evidence that the expression of variants of the glycoprotein CD44 is related to the invasiveness and metastatic potential of tumour cells. CD44 is a transmembranous cell adhesion glycoprotein that plays a role in cell–matrix interactions [1]. It is known to be a major ligand for hyaluronate and to bind collagen, laminin and fibronectin. Various isoforms of CD44 are generated by alternative splicing of exons 6 (v1–2) to exon 14 (v10) [2]. The significance of variant CD44 expression for the metastatic capacity of tumour cells was first reported by Günthert and colleagues [3]. In transfection studies, they demonstrated that expression of a CD44 protein variant encoded by alternatively spliced CD44 exon v6 conferred metastatic potential to a rat pancreas carcinoma cell line. Overexpression of CD44v6 and other variant exons in colorectal cancer and other gastrointestinal tumours was demonstrated in immunohistochemical studies [4, 5] Northern blots or reverse transcriptase–polymerase chain reaction (RT–PCR) [6–8] and *in situ* hybridisation [9, 10]. Data about CD44v6 expression in colorectal neoplasms depending on expansion of the tumour have been inconsistent. In most of these studies, however, stratification according to stage of the tumours was not carried out.

From a clinical point of view, an association of CD44v6 expression with poor prognosis in colorectal cancer patients has recently been observed. Mulder and coworkers [11] determined the CD44v6 status of primary tumours of 68 patients with colorectal cancer by immunohistochemistry and correlated CD44v6 status with survival. They found a significantly worse prognosis for patients with CD44v6 positive tumours compared with patients with negative tumours. A critical point in this study is that patients were not stratified

Correspondence to A. Stallmach.
Received 8 Oct. 1997; revised 23 Feb. 1998; accepted 5 Mar. 1998.

according to stage of tumour. Thus, the question arose whether CD44v6 was an independent prognostic parameter in these patients. The aim of our study was to investigate the expression of CD44 variants in colorectal neoplasia in relation to tumour stage. CD44v5, another variant conferring metatatic potential to tumour cells in animal models and CD44v2, a variant first described in bladder cancer [12], were also investigated.

MATERIALS AND METHODS

Patients

67 patients with colorectal carcinomas or metastases of colorectal carcinomas and 15 patients with colorectal adenomas treated in 1996 in the University Hospital of the Saarland were consecutively included in this prospective study after patients gave their consent to participate. Tissue samples of colorectal adenomas, colorectal carcinomas and normal mucosa of at least 10 cm distance from neoplastic tissue were obtained during surgery or colonoscopy. Samples of metastatic tissue were taken during cryotherapy or surgery of the liver. Tissue specimens were snap-frozen in liquid nitrogen and stored at -70°C until use. Grading and staging of the tumours according to the UICC classification were carried out by standard surgical pathology techniques [13].

Histologically all the investigated tumours were either adenocarcinomas or adenomas with different grades of differentiation.

Preparation of cDNA and Southern blot analysis

The preparation of mRNA of tissue samples was conducted using a commercially available mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesised with mRNA as the template for an oligo-dT primed reaction catalysed by MMLV reverse transcriptase (Gibco BRL Karlsruhe, Germany). The quality of the mRNA preparation and cDNA synthesis of the tissue samples was checked by RT–PCR using (glyceraldehydephosphate dehydrogenase) GAPDH-specific primers as a internal control [14] to confirm the integrity of the template cDNA in CD44 negative samples.

The primer sequences (Table 1) were chosen after the GAPDH sequence of a full length genomic DNA clone [15]. The GAPDH sense primer corresponded to position 17–38 in exon 1, the GAPDH antisense primer to position 2468–2890 in exon 5 of the *GAPDH* gene. RT–PCR studies concerning CD44 were carried out using primers P1 + and P1 – (Table 1), sharing sequence homology with exons 5 and 15, localised up- and downstream from the variable exons of the *CD44* gene. After RT–PCR with primers P1 + and P1 –, DNA fragments were separated on agarose gels. Bands were visualised after Southern blotting and hybridisation with specific probes. The probes were produced by PCR with exon-specific primers (Table 1) and digoxigenin-labelled dNTPs (Boehringer, Mannheim, Germany). After Southern blotting, hybridisation and stringency washing, the detection of filter bound digoxigenin probes was conducted by a calorimetric reaction with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Boehringer).

Calibration studies were conducted to determine the sensitivity of our method. Using HT-29 as a CD44v6 positive cell line and human fibroblasts expressing only CD44standard (CD44s), we could show in mixture experiments (decreased ratio of epithelial to mesenchymal cells) that as few as one CD44v6 positive cell per 1,000–10 000 CD44v6 negative mesenchymal cells could be detected by this experimental approach.

Statistical analysis

Statistical differences were calculated using Fisher's exact test or the chi-square test, and were considered to be significant at the $P \leq 0.05$ level.

RESULTS

Expression of CD44 variants in normal mucosa, colorectal adenomas and carcinomas

Figure 1 illustrates a representative Southern blot. High levels of expression were observed for common CD44 splicing variants in normal mucosa, as well as in neoplastic tissue (Table 2). CD44s was expressed in 81% of normal mucosa samples, in 93% of adenomas and in 82% of colorectal carcinomas with no statistically significant difference between the study groups. CD44epithelial (CD44e) was observed in 47% of the normal colonic mucosa tissue samples, and in 87% of colorectal adenoma tissues ($P \leq 0.01$) and in 71% of carcinoma tissues ($P \leq 0.05$). In neoplastic tissues, CD44e bands were usually denser on ethidium bromide stained gels than in normal mucosa.

Expression frequencies of alternatively spliced variants CD44v2, CD44v5, and CD44v6 in normal mucosa were generally low (CD44v2: 6%, CD44v5: 3%, CD44v6: 6%) compared with expression frequencies of the common variants CD44s and CD44e. In colorectal adenomas, expression

Table 1. *Primer sequences used for amplification of CD44 in reverse transcriptase–polymerase chain reaction (RT–PCR) and for production of digoxigenin-labelled probes for Southern blots*

Specificity	Code	Sequence
Pan-CD44	P1 +	5′CCT ACT GAT GAT GAC GTG AGC AGC G
	P1 –	5′TCA GAT CCA TGA GTG GTA TGG GAC C
	P1* –	5′TGG TAG CAG GGA TTC TGT CTG TGC
CD44v2	P2 +	5′GAC AGC AAC CAA GAG GCA AGA AAC C
	P2 –	6′GAT CCA GCC ATT TGT GTT GTT GTG TGA AG
CD44v5	P5 +	5′TGT AGA CAG AAA TGG CAC C
	P5 –	5′CTT GTG CTT GTA GAA TGT GG
CD44v6	P6 +	5′GGC AAC TCC TAG TAG TAC AAC
	P6 –	5′CAG CTG TCC CTG TTG TCG AAT
GAPDH	GAPDH +	5′TTC GAC AGT CAG CCG GAT CTT C
	GAPDH –	5′CTT CTC CAT GGT GGT GAA GAC G

*Used together with P1 + for generation of a pan-CD44 probe.

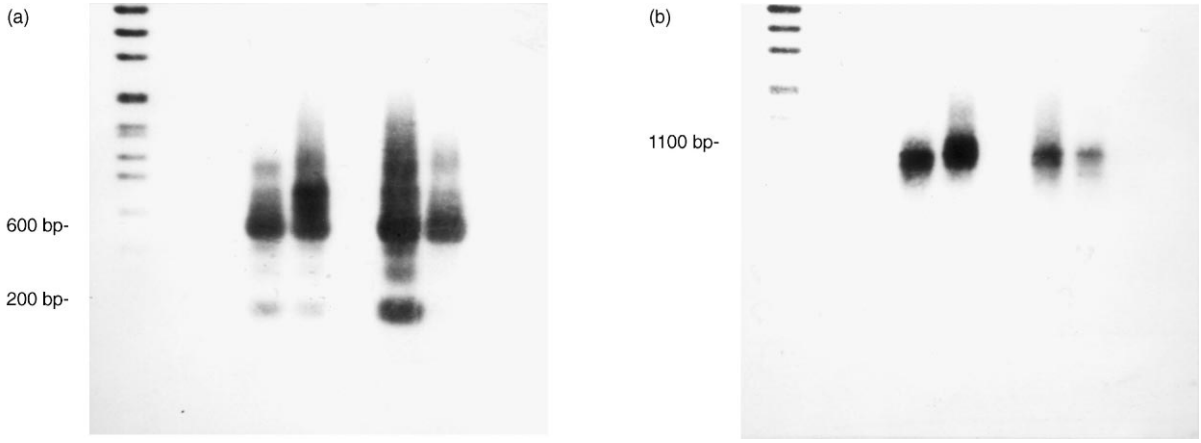

Figure 1. Southern blots of reverse transcriptase–polymerase chain reaction (RT–PCR) products of seven carcinomas. (a) pan CD44 probe: CD44standard (200 bp) and CD44epithelial (600 bp), larger bands of alternatively spliced variants. (b) CD44v5-specific probe: large splicing variants (1100 bp) containing CD44v5.

was significantly higher (CD44v2: 27%, CD44v5: 33%, CD44v6: 47%), compared with normal mucosa ($P \leq 0.05$). In colorectal carcinomas, expression was significantly higher for CD44v5 (29%) and CD44v6 (42%) compared with normal mucosa ($P \leq 0.001$), but not for CD44v2 (16%) ($P > 0.05$). Frequencies of CD44 variants in colorectal adenomas and carcinomas were similar with no significant differences.

Expression of CD44 variants in colorectal carcinomas in relation to UICC stages

Analysis of relative frequencies of CD44 splicing variants in relation to stage and grade of the tumour (Figure 2) revealed differences for CD44v2 and CD44v6 expression depending on pathological stage, but no difference with tumour grade.

No significant differences were identified between expression frequencies for CD44s (UICC I: 93%, UICC II: 70%, UICC III: 94%, UICC IV: 75%) in carcinomas of different stages. Similar observations were made for CD44e (UICC I: 79%, UICC II: 60%, UICC III: 88%, UICC IV: 58%).

For CD44v2, low frequencies were observed in normal mucosa (6%), and in advanced colorectal cancer (UICC II: 5%, UICC III: 18%, UICC IV: 17%). Only colorectal adenomas and early colorectal carcinomas of stage I (T1-2N0M0) had frequencies of 27% and 29% for CD44v2, significantly higher than in normal mucosa ($P \leq 0.05$). None of six metastatic tissue samples from primary colonic adenocarcinomas showed expression of CD44v2.

CD44v5 expression was significantly higher ($P \leq 0.05$) in colorectal adenomas (33%) and carcinomas (29%) than in normal colonic mucosa (3%). Statistical analysis revealed no difference in CD44v5 expression between carcinomas of different UICC stages (UICC I: 36%, UICC II: 30%, UICC III: 25%, UICC IV: 25%). In two of six metastatic tissue samples, CD44v5 expression was observed.

In colorectal adenomas and non-metastatic colorectal carcinomas of UICC stages I to III, significantly higher ($P \leq 0.05$) frequencies of CD44v6 expression ranging between 45 and 57% were observed compared with normal mucosa (6%). Surprisingly, in comparison with non-metastatic colorectal carcinomas, a decrease of CD44v6 expression was shown in metastatic tumours ($P \leq 0.05$). Of 12 metastatic UICC IV primaries, only one expressed CD44v6. In addition, only one of six metastases was CD44v6 positive.

Additionally, CD44 variant expression patterns were analysed in detail. Normal and neoplastic tissues always expressed variants CD44v2, v5 or v6 together with the common CD44s and CD44e. In normal colonic mucosa, two samples with only expression of CD44v2, one with only CD44v6 and one with coexpression of only CD44v5 and v6 were seen. In neoplastic tissues, coexpression of CD44v2, v5,

Table 2. Relative frequencies of expression of CD44 isoforms CD44standard (CD44s), CD44epithelial (CD44e), CD44v2, CD44v5, and CD44v6 in normal colonic mucosa, colorectal adenomas, and colorectal carcinomas

	Normal mucosa (%)	Colorectal adenomas (%)	Colorectal carcinomas (%)
CD44s	29/36 (81)	14/15 (93)	51/62 (82)
CD44e	17/36 (47)	13/15 (87)†	44/62 (71)⋆
CD44v2	2/36 (6)	4/15 (27)⋆	10/62 (16)
CD44v5	1/36 (3)	5/15 (33)⋆	18/62 (29)‡
CD44v6	2/36 (6)	7/15 (47)⋆	26/62 (42)‡

⋆$P \leq 0.05$; †$P \leq 0.01$; ‡$P \leq 0.001$ compared with normal mucosa.

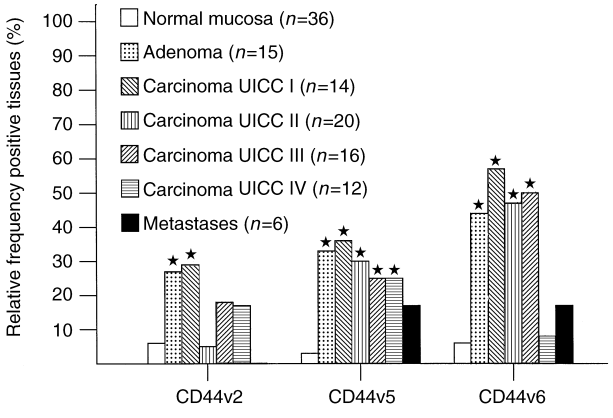

Figure 2. Relative frequencies for CD44v2, CD44v5, and CD44v6 in normal colonic mucosa, colorectal adenomas, carcinomas and metastatic tissue in relation to stage of the tumour (UICC classification: I=T1-2N0M0; II=T3-4N0M0; III=TxN1-3M0; IV=TxNxM1). ⋆$P \leq 0.05$ compared with normal mucosa.

and v6 was observed in 10 cases. CD44v2 or CD44v5 occurred in three adenomas and 11 carcinomas in combination with CD44v6. Expression of CD44v2 and/or CD44v5 without coexpression of CD44v6 was rare (one UICCIII and two UICC IV tumours of 83 neoplastic tissues). In contrast, CD44v6 expression together with common isoforms but no other variants was frequent (one adenoma and nine carcinomas of stage UICC I–III of 83 neoplastic tissues). Only three of 12 metastatic primary tumours (UICC IV) showed variant expression; one had only CD44v5, one had only CD44v2 and v5, the third one expressed CD44v2, v5 and v6. In one of six metastases, expression of CD44s, CD44e, CD44v5 and v6 was observed. The other metastatic samples were CD44 negative, but GAPDH positive.

DISCUSSION

Certain splicing variants of the cell adhesion molecule CD44 confer metastatic potential to malignantly transformed cells in animal models. Overexpression of CD44v6 has been demonstrated in colorectal neoplasia by immunohistochemistry, RT–PCR and *in situ* hybridisation [4–10]. Inconsistent data have been published about CD44v6 expression in colorectal tumours depending on the expansion of the tumour. Since data obtained by immunohistochemical staining might be biased by methodological problems, we applied a transcriptional approach by RT–PCR and subsequent exon-specific probing to examine tissues of colorectal carcinomas, adenomas and healthy colonic mucosa for their expression patterns of CD44 splicing variants. Our most important finding was that CD44v2 and CD44v6 expression depend on stage of the tumour. In normal colonic mucosa, both variants were seen in only a few cases. CD44v2 was observed in 27% of colorectal adenomas and in 29% of colorectal carcinomas of UICC stage I (T1-2N0M0). Therefore, this variant seems to be associated with early colorectal neoplasia. CD44v6 was expressed in 45% of colorectal adenomas and in 50% of carcinomas without distant metastases. In metastatic colorectal cancer, however, a decreased expression of CD44v6 was observed both in primary as well as metastatic tissues. These data correspond with immunohistochemical findings by Finke and coworkers [16] who demonstrated a decrease of CD44v6 expression in primary colorectal tumours with distant metastases and also in metastatic tissue. Sugino and colleagues [17] reported similar observations in invasive bladder carcinomas, with reduced CD44 expression frequencies in deeply invasive carcinomas, indicating that this phenomenon can also be found in other types of carcinoma.

Frequencies of CD44s were high in all study groups, reflecting ubiquitous expression of the standard form of CD44 in epithelial and mesenchymal cells. The epithelial form of CD44 was expressed with high frequencies in normal colonic mucosa and neoplastic tissues. Although frequencies were high in all groups, semiquantitative analysis of bands on ethidium bromide stained gels indicated that a quantitative enhancement of CD44e expression occurred in benign and malignant colorectal tumours compared with normal colonic mucosa. This is in accordance with recent data of Imazeki and coworkers [6] who observed enhancement of the densitometrically determined ratio of the epithelial to the standard CD44 isoform in neoplastic tissue compared with normal mucosa. These authors found expression of CD44s in all, and of CD44e in most, of the investigated colorectal tumour specimens. They did not mention if they found CD44 negative tumours and if they conducted a quality check. So we assume that the number of CD44s and CD44e positive tumours might have been overestimated in this study.

Despite intensive research in recent years, very little is known about differences in functional properties between the different CD44 splicing variants. Since a changing pattern of CD44 expression has been reported during lymphocyte activation [18, 19], it has been proposed that CD44 might work as a homing receptor during migration of lymphocytes [20]. Since metastasising cells and lymphocytes share some properties, it has been suggested that certain splicing variants of CD44 play a crucial role in migration processes. Nevertheless, consistent data on the molecular mechanisms of CD44 variant interaction with their ligands are still lacking [21]. Since several studies agreed on enhanced expression of CD44v6 in non-invasive colorectal adenomas, the initial hypothesis of CD44v6 as a molecule conferring metastatic potential to malignant transformed cells cannot be upheld. CD44 variants might have functions in the regulation of cell growth and migration that have not yet been clarified.

Since overexpression of CD44v2 and CD44v6 can already be observed with our method in early alterations of the adenoma–carcinoma sequence, we suggest that these variants might be a promising marker for screening patients with precancerous lesions. This seems to be especially interesting in patients with ulcerative colitis. Rosenberg and associates [22] recently reported an enhanced expression of CD44v3 and CD44v6 in inflammatory and dysplastic lesions in patients with ulcerative colitis. Thus, detection of CD44 splicing variants might facilitate early identification of patients with ulcerative colitis at special risk for the development of colorectal malignancies.

Our study clearly shows that CD44 variant expression in colorectal neoplasia depends on the stage of the tumour. A shift is visible from complex CD44 variant expression patterns, most of them containing CD44v6, in adenomas and non-metastatic carcinomas, to less complex patterns with a reduced frequency of CD44v6 in metastatic tumours.

This suggests the existence of regulatory mechanisms that influence the expression patterns of CD44 variants in benign and malignant colorectal tumours. Only a few studies have been conducted concerning regulations of alternative mRNA splicing of CD44. Mackay and colleagues [23] have shown that cytokines interferon-γ and (TNF-α) tumour can influence the expression of variant CD44 isoforms not only in T-lymphocytes, but also in epithelial cell lines, probably due to an unidentified signal transduction mechanism. Cell fusion experiments with generation of transient heterokaryons indicate the existence of trans-activating factors playing a role in the regulation of CD44 mRNA splicing [24]. Recent data [25] indicate that members of the SR-protein family, a group of essential and alternative splicing regulators, might influence CD44 expression patterns.

In view of the results presented, further studies need to be conducted to clarify the regulatory mechanisms for CD44 mRNA splicing in colorectal tumours.

1. Stauder R, Günthert U. CD44 isoforms. Impact on lymphocyte activation and differentiation. *Immunologist* 1995, **3**, 78–83.
2. Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* 1992, **89**, 12160–12164.

3. Günthert U, Hoffmann M, Rudy W, *et al.* A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991, **65**, 13–24.
4. Heider KH, Hofmann M, Hors W, *et al.* A human homologue of the rat metastasis-associated variant of CD44 is expressed in colorectal carcinomas and adenomatous polyps. *J Cell Biol* 1993, **120**, 227–233.
5. Wielenga VM, Heider KH, Offerhaus JA, *et al.* Expression of CD44 variant proteins in human colorectal cancer is related to tumour progression. *Cancer Res* 1993, **53**, 4754–4759.
6. Imazeki F, Yokuzuka O, Yamaguchi T, Ohto M, Isono K, Omata I. Expression of variant CD44-messenger RNA in colorectal adenocarcinomas and adenomatous polyps in humans. *Gastroenterology* 1996, **110**, 362–368.
7. Higashikawa K, Yokozaki H, Ue T, *et al.* Evaluation of CD44 transcription variants in human digestive tract carcinomas and normal tissues. *Int J Cancer* 1996, **66**, 11–17.
8. Rodriguez C, Monges G, Rouanet P, Dutrillaux B, Lefrancois D, Theillet C. CD44 expression patterns in breast and colon tumours: a PCR-based study of splice variants. *Int J Cancer* 1995, **64**, 347–354.
9. Orzechowski HD, Beckenbach C, Herbst H, Stölzel U, Riecken EO, Stallmach A. Expression of human homologues of a metastasis-associated CD44 variant is associated with cellular dysplasia in colorectal epithelial cells. *Eur J Cancer* 1995, **31A**, 2073–2079.
10. Gorham H, Sugino T, Woodman AC, Tarin D. Cellular distribution of CD44 gene transcripts in colorectal carcinomas and normal colonic mucosa. *J Clin Pathol* 1996, **46**, 482–488.
11. Mulder JWR, Kruyt PM, Sewnath M, *et al.* Colorectal cancer prognosis and expression of exon-v6-containing CD44 proteins. *Lancet* 1994, **344**, 1470–1471.
12. Matsumura Y, Hanbury D, Smith J, Tarin D. Non-invasive detection of malignancy by identification of unusual CD44 gene activity in exfoliated cancer cells. *Br Med J* 1994, **308**, 619–624.
13. Beahrs OH. Colorectal cancer staging as a prognostic feature. *Cancer* 1992, **50**, 2615.
14. Dukas K, Sarfati P, Vaysse N, Pradayrol L. Quantitation of changes in the expression of multiple genes by simultaneous polymerase chain reaction. *Anal Biochem* 1993, **215**, 66–72.
15. Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. *J Biol Chem* 1988, **263**, 15335–15341.
16. Finke LH, Terpe HJ, Zörb Q, Haensch W, Schlag PM. Colorectal cancer prognosis and expression of exon-v6-containing CD44-proteins. *Lancet* 1995, **345**, 583.
17. Sugino T, Gorham H, Yoshida K, *et al.* Progressive loss of CD44 gene expression in invasive bladder cancer. *Am J Pathol* 1996, **149**, 873–882.
18. Arch R, Wirth K, Hofmann M, *et al.* Participation in normal immune responses of a metastasis inducing splice variant of CD44. *Science* 1992, **257**, 682–685.
19. Koopman G, Heider KH, Horst E, *et al.* Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med* 1993, **177**, 897–904.
20. Jalkanen S, Jalkanen M, Bargatze IR, Tammi M, Butcher EC. Biochemical properties of glycoproteins involved in lymphocyte recognition of high endothelial venules in man. *J Immunol* 1988, **141**, 1615–1623.
21. Sherman L, Sleeman J, Dall P, *et al.* The CD44 proteins in embryonic development and in cancer. *Current Top Microbiol Immunol* 1996, **213**, 249–269.
22. Rosenberg WMC, Prince C, Kaklamanis L, *et al.* Increased expression of CD44v6 and CD44v3 in ulcerative colitis but not colonic Crohn's disease. *Lancet* 1995, **345**, 1205–1209.
23. Mackay CR, Terpe HJ, Stauder R, Marston WL, Stark H, Günthert U. Expression and modulation of CD44 variant isoforms in humans. *J Cell Biol* 1994, **124**, 71–82.
24. König H, Moll J, Ponta H, Herrlich P. Trans-acting factors regulate the expression of CD44 splice variants. *EMBO J* 1996, **15**, 4030–4039.
25. Screaton GR, Cáceres JF, Mayenda, A *et al.* Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J* 1995, **14**, 4336–4349.

Acknowledgements—This work was supported by a grant Sta 295/2-2 of the Deutsche Forschungsgemeinschaft. For technical assistance, the authors are indebted to Ms Reinhild Goebel.