

Expression of CD44 Variant Proteins in Human Colorectal Cancer Is Related to Tumor Progression¹

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Abstract

Specific CD44 variant glycoproteins are overexpressed at particular stages of colorectal tumor progression. Some variants of the CD44 glycoprotein without exon v6 sequences appear at the earliest stage of tumorigenesis, *i.e.*, in early adenomas. Expression of variants containing exon v6 sequences is largely restricted to the advanced stages of tumor development and in addition is more prevalent and intense in metastatic (Dukes C/D) than in nonmetastatic (Dukes A/B) carcinomas. The observation that CD44 variants containing a protein domain of CD44 that confers full metastatic potential to rat carcinoma and sarcoma cell lines is increasingly expressed during colorectal tumor progression indicates that this domain may have an important role in tumor progression and metastasis in humans. Information on v6 expression, which can be obtained by routine immunohistochemistry, may prove of important prognostic value, particularly in carcinomas (Dukes A and B) that have not yet given rise to detectable metastases.

Introduction

During tumor progression, a subset of cells acquires metastatic properties, presumably through a series of genetic alterations. As a result, cells detach from the primary tumor, penetrate the basement membrane into the connective tissue, and invade adjacent structures including lymph and blood vessels. The tumor cells are subsequently transported to sites of metastatic outgrowth via lymph and/or blood. Loss of adhesive functions and gain of new adhesive functions are thought to play a crucial role in this metastatic cascade (1). Since tumor metastasis is the principle cause of death for cancer patients, there is consensus that a search for tools that allow effective assessment of the metastatic potential of tumors is a prime goal for cancer research. Recently, we have shown that CD44 variant glycoproteins containing sequences encoded by exon v6 confer full metastatic potential to rat carcinoma and sarcoma cell lines. Coinjection of variant-specific mAb³ with the metastasizing cells led to retardation or even complete block of metastatic spread *in vivo* (2). Moreover, overexpression of specific CD44 variants in nonmetastasizing tumor cell lines led to metastatic behavior (3). Several CD44 variants, including homologues of those that confer a metastatic phenotype to rat carcinomas, have recently been found to be overexpressed in human tumors including colorectal carcinoma (4-7). In the present study, we have used the unique stepwise progression model (8) of colorectal

tumorigenesis to explore the relation between the expression of CD44 splice variants and tumor progression.

Materials and Methods

Tumor Samples. Normal and pathological tissues were selected from the files of the Department of Pathology, Academic Medical Center, University of Amsterdam, the Netherlands. A total of 70 normal and pathological colon specimens was examined. Colorectal carcinomas ($n = 38$) were staged according to the original Dukes' classification (9) in Dukes A ($n = 8$), disease limited to the bowel wall; Dukes B ($n = 12$), extension through the deep muscle without metastases; and Dukes C/D ($n = 18$), tumors with regional and distant metastases, respectively. Adenomas were subdivided into early adenomas (diameter, <1 cm) ($n = 11$) and late adenomas (diameter, >1 cm) ($n = 11$) and were graded as low or high grade dysplasia using standard criteria.

Detection of CD44 Variants. Frozen tissue sections were tested for expression of CD44 and CD44 splice variants by immunohistochemistry as described previously (4, 5) using a polyclonal serum and a panel of monoclonal antibodies raised against a bacterially expressed fusion protein encoded by exons v3-v10 (4, 5) of the human HPKII-type CD44. v3-v10 are variant exons corresponding to exons 8-15 of the human genomic CD44 (10). The structure of CD44 and the location of the epitopes that are recognized by the polyclonal anti-CD44v serum and by the mAbs VFF4, VFF7, VFF8, VFF11, VFF14, VFF16, and VFF17 are shown in Fig. 1. A detailed description of most of these antibodies and their specificity has been published elsewhere (4, 5). Tumors were designated "positive" when they were estimated to show staining in more than 10% of the tumour cells. Staining in less than 10% of the tumor cells was designated "focal."

Southern blot analysis of reverse transcription-PCR amplification products from samples of normal colonic mucosa and colorectal adenocarcinomas was performed as described previously (4, 5). For complementary DNA synthesis a primer homologue to positions 900 to 922 of the 3'-standard portion of CD44 was used (11). The PCR primers were specific for CD44 exons adjacent to the variant exon sequences (5'-oligo, positions 513-540; 3'-oligo, positions 849-874) (11).

Results and Discussion

Immunohistochemical studies demonstrated differential expression of CD44 epitopes on normal colorectal mucosa, adenomatous polyps, and colorectal carcinomas (Fig. 2). In normal colon epithelium, expression of CD44 proteins as determined by the pan-CD44 mAb NKI-P1 directed against an epitope on the NH₂-terminal constant part of CD44 was limited; staining was weak and localized to the base of the crypts. A similar expression pattern (Fig. 2, *) was observed for epitopes encoded by v7 and v8-10 (Fig. 2). Expression of other variant CD44 exons (v3-v6) was not detectable in normal colon epithelium (Fig. 2).

The pattern of CD44 expression in colorectal tumors differs dramatically from that in normal epithelium. Pan-CD44 mAb shows an overall increase of CD44 on the surface of tumor cells. Similarly, epitopes encoded by exons v7-v10 were also present and increased.

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³ The abbreviations used are: mAb, monoclonal antibody; PCR, polymerase chain reaction.

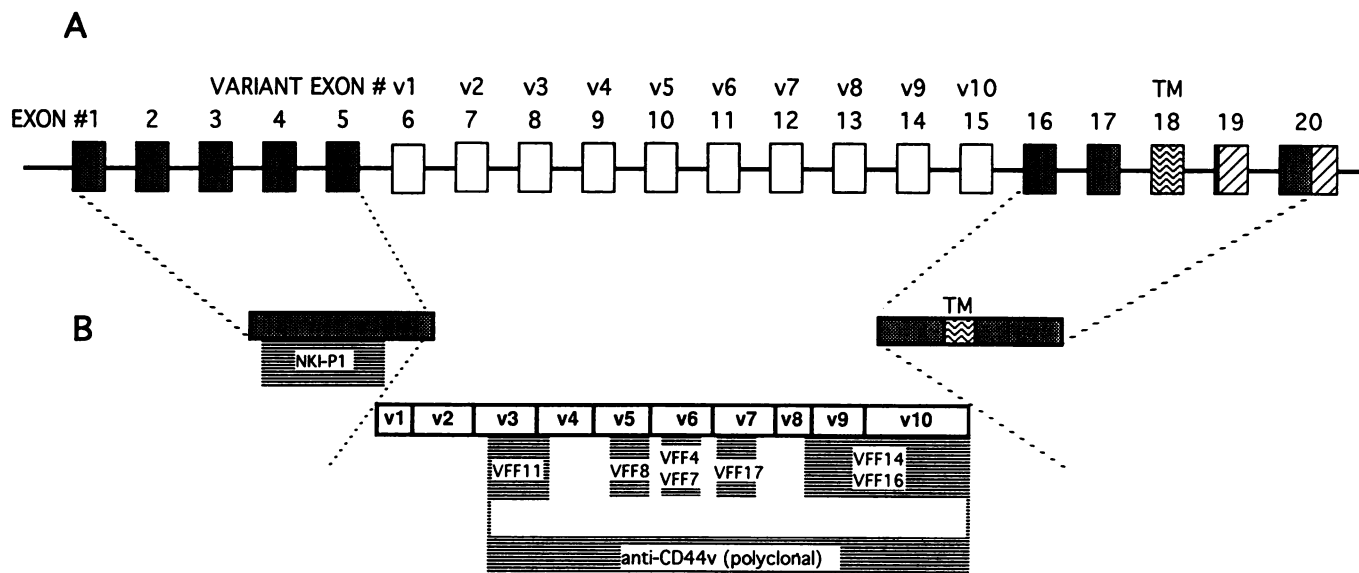
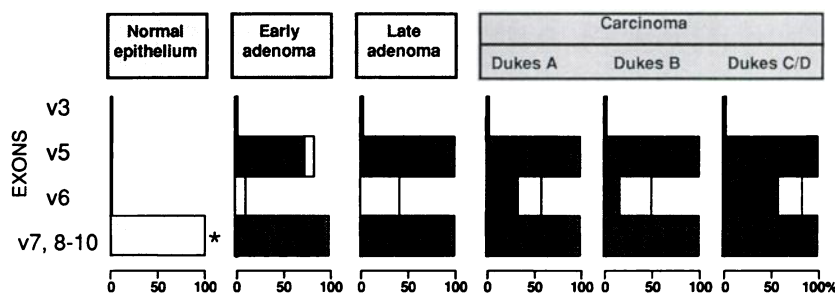


Fig. 1. A, schematic representation of the CD44 gene. □, exons that are spliced in the "standard" type of CD44. TM, transmembrane region. B, schematic representation of the CD44 protein with location of the epitopes which are recognized by the monoclonal antibodies NKI-P1, VFF4, VFF7, VFF8, VFF11, VFF14, VFF16, and VFF17 and the polyclonal antiserum CD44v. Anti-variant antibodies were raised against a bacterially expressed fusion protein encoded by pGEX CD44v HPKII (v3-v10). □, standard CD44; v1-v10, domains encoded by variant exons.

Fig. 2. Expression of CD44 variant proteins in colorectal tumor progression. Dark gray columns, percentage of positive tumors. Light gray columns, specimens with focal staining only. *, weak staining at base of crypts.



Moreover, new epitopes not found on normal epithelium were detected on the tumor cells. Overexpression of CD44 and appearance of a new epitope was already observed at a very early stage of colorectal tumor progression, *i.e.*, in early adenomas. In these adenomas almost all cells expressed an exon v5 epitope. From the fact that not all v7-v10 positive cells carried also the v5 epitope and from PCR data (Fig. 3), we conclude that there is progressive change of CD44 splice variant production and that, compared to normal colon mucosa, the expression pattern of CD44 variants in tumor cells is much more complex (Fig. 3). At the more advanced stages of colorectal tumor progression, *i.e.*, in advanced polyps and invasive carcinomas, the level of expression of v5 containing CD44 isoforms increased. Interestingly, tumor progression was strongly related to overexpression of CD44 isoforms carrying epitope(s) of exon v6, which in the rat has been shown to be involved in metastasis formation (Fig. 2). Expression of this exon was detectable in none of the normal colon specimens but in 9, 45, and 68% of the early and advanced polyps and invasive carcinomas, respectively (χ^2 trend 1 d.f. = 13.1; $P = 0.0003$). Moreover, in carcinomas, the expression of exon v6 was correlated to Dukes stage. The percentage of positive cases in the nonmetastatic Dukes A and B tumors was 55%, whereas in the metastatic Dukes C/D group 83% of the cases were positive (χ^2 1 d.f. = 4.4; $P = 0.03$). Importantly, the overexpression of v6 containing CD44 isoforms during tumor progression was reflected not only by increasing percentages of positive cases but also by increasing numbers of positive cells within the tumors as well as by higher average expression levels apparent from the more intense staining (not

shown). Focal expression of v6 in adenomas was correlated to another parameter of tumor progression, *i.e.*, to the histological tumor grade. Expression was detectable in 1 of 17 low-grade but in 4 of 5 high-grade adenomas.

Our present finding that expression of CD44 splice variants, particularly those containing v6, in colorectal cancer is strongly related to tumor progression supports the concept that these CD44 variants play a role in human colorectal tumor metastasis similar to that in the rat model (2, 3). Interestingly, CD44 variants containing v6 are also up-regulated on activated lymphocytes (4, 12) and are involved in the normal immune response (12). Activated lymphocytes and metastasizing tumor cells share many properties, *e.g.*, invasive behavior, migration involving reversible adhesive contacts, accumulation and expansion in draining lymphoid tissue, release into the circulation, and extravasation. CD44 splice variants appear to play a decisive role in one (or several) of these steps (13).

Multistep carcinogenesis, exemplified in the colorectal adenoma-carcinoma sequence, is believed to involve mutation and clonal selection (8). From normal mucosa through various adenoma stages to invasive and metastatic carcinoma, morphological phenotypes have been associated with progressive accumulation of genetic changes involving oncogenes and tumor suppressor genes like *ras*, *APC*, *DCC*, and *p53*, which appear to cause growth advantage. Our finding of progressive overexpression of CD44 variants at the successive stages of colorectal tumor progression suggests that expression of these variants also confers a selection advantage to the tumor cells. Altered adhesion mediated via the CD44 variants and growth signals associ-

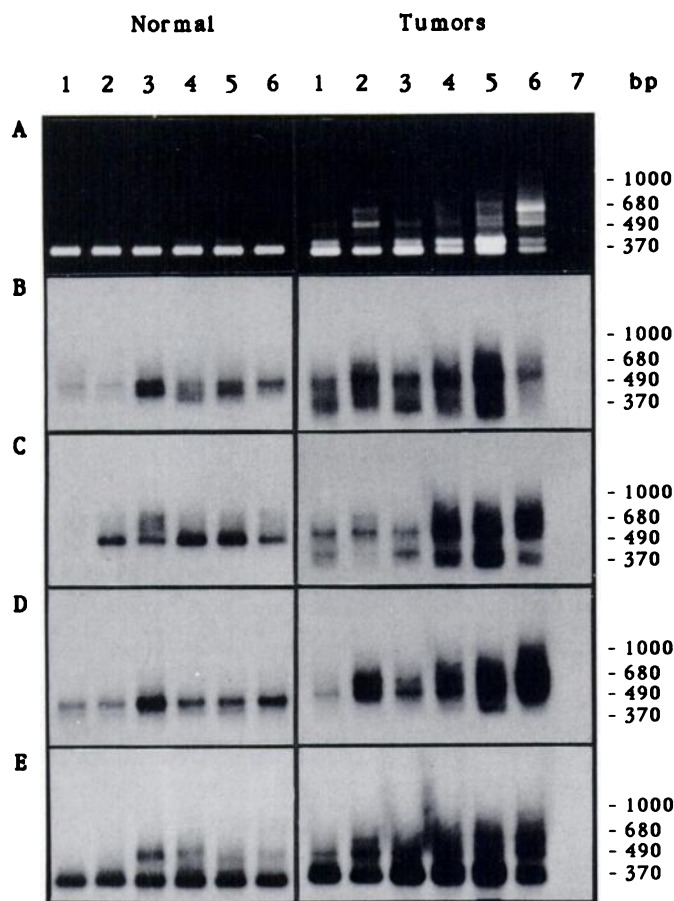


Fig. 3. Southern blot analysis of reverse transcription-PCR amplification products from specimens of normal colonic mucosa and colorectal adenocarcinomas. In A, the PCR products obtained with CD44 specific primers 5' and 3' of the variant part were resolved on 1.2% agarose, stained with ethidium bromide, and visualized under UV light. The bright 350-base pair (bp) band present in both normal and tumor samples corresponds to the expected standard CD44 amplification product. Compared with normal colon mucosa, colorectal carcinomas grossly overexpression several larger splice variants. In B-E, after transfer to a Hybond N+ membrane, the same filters was hybridized consecutively to (B) exon v5, (C) exon v6 (D), exon v9, and (E) standard CD44 specific probes. The results show that, compared with normal colon mucosa, the expression pattern of CD44 variants in colorectal carcinomas is much more complex. The relative strong bands obtained with normal tissue in B-D result from long exposure times needed to allow adequate qualitative analysis of the variants. Hence, it should be stressed that a quantitative comparison between expression of variants in normal tissues and tumors in B-D is not possible. Lane 7, negative control.

ated with such adhesion might create this advantage, leading to expansion of v6 positive cells that than metastasize. We envisage that information on v6 expression, which can be obtained by routine immunohistochemistry, may prove of important prognostic value, particularly in invasive carcinomas (Dukes A and B) that have not yet given rise to detectable metastases.

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