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Original Paper

No Evidence for Cancer-related CD44 Splice Variants in Primary and Metastatic Colorectal Cancer

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The expression of alternatively spliced CD44 adhesion molecules has been implicated in the pathogenesis and metastasis of colorectal cancer. Using a new set of primers for exon-specific reverse transcription–polymerase chain reaction (RT–PCR) we delineated the exact exon composition of CD44 mRNAs in normal colorectal mucosa, including isolated colonic crypts, in colorectal carcinomas and in their hepatic metastases. In addition, the surface expression of CD44 isoforms was analysed by immunohistochemistry. We identified by RT–PCR eight variant transcripts expressed in colorectal carcinomas and their metastases, but also constitutively in normal colorectal epithelia. In the normal colorectal epithelium, the surface expression of CD44 standard and variant molecules was restricted to proliferating cells at the bottom of the crypts. Despite expression of these transcripts in colorectal cancers and their metastases, monoclonal antibodies specific for standard or variant epitopes encoded by exons v5 and v6 stained only a few neoplastic lesions. These data point to a differentiation-specific CD44 expression and splicing pattern in proliferating colorectal epithelia. However, they do not support a cancer- or metastasis-specific CD44 splicing pattern. Instead, cell surface availability of CD44 epitopes was reduced rather than increased in primary tumours and particularly in liver metastases. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: exon-specific RT–PCR, immunohistochemistry, adhesion molecules, alternative splicing, metastasis
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INTRODUCTION

THE GENE encoding the CD44 adhesion molecule is composed of 20 exons. Up to nine of these exons v2–v10, are expressed in various alternatively spliced isoforms in specific normal and neoplastic epithelia. The CD44 standard molecule does not encompass these variant exons and is rather ubiquitously expressed in haematopoietic, mesodermal and epithelial cells. The CD44 standard isoform confers important functions for cellular adhesion to the extracellular matrix and for lymphocyte homing, whereas the role of CD44 splice variants is still poorly understood [1]. The expression of CD44 molecules encompassing variant exons v4–v7 induced metastatic growth of a rodent pancreatic carcinoma cell line [2, 3], and various studies have suggested that the expression of a complex pattern of alternatively spliced variant CD44 molecules also contributes to metastasis of human neoplasms, including colorectal carcinomas [4–16]. However, immunohistochemical studies have suggested that in epithelia of the upper gastrointestinal tract, as well as in cryptic cells of the colon, CD44 variant isoforms encompassing v7 and v8–v10 sequences are expressed [7, 17–19]. In other studies, expression of v3 and v6 epitopes on normal colon epithelia could not be consistently detected [12, 13, 20, 21]. While in a variety of tumours derived from tissues constitutively expressing CD44 variant isoforms malignant transformation has been found to be associated with reduced expression of CD44, divergent results have been reported on colorectal primary tumours and metastases derived thereof [10–19]. To clarify, whether putative 'cancer-specific' CD44 splice variants

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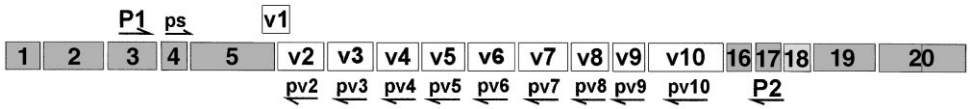

Figure 1. Schematic presentation of CD44 coding region, variant exons and localisation of primers utilised for standard (P1 and P2) and exon-specific polymerase chain reaction (ps and pv2–pv10).

are indeed selectively expressed in colorectal carcinomas, we analysed the expression of CD44 variants in the normal colorectal epithelium and neoplastic or metastatic lesions derived thereof by immunohistochemistry and delineated the exon composition of CD44 transcripts using a recently described exon-specific reverse transcription–polymerase chain reaction (RT–PCR) protocol [22, 23].

MATERIALS AND METHODS

Biopsy samples obtained during surgical resection were immediately snap frozen in liquid nitrogen and stored at − 70°C until further processing. Cryostat sections approximately 5 μm thick were mounted on gelatine covered slides, fixed in methanol and acetone, air dried and stored at − 20°C until used for immunohistochemistry. Total RNA was extracted from tissue sections or cell lines using a commercial micro-isolation system (GlassMAX RNA micro-isolation system, Life Technologies, Karlsruhe, Germany) as recommended by the supplier. As described previously [22, 23], approximately 1 μg of total cellular RNA was reverse transcribed and amplified using primers, ps and pv2–pv10, for exon-specific PCR with the following sequences (Figure 1): ps, AGT CAC AGA CCT GCC CAA TGC CTT T; pv2, GTT TCT TGC CTC TTG GTT GCT GTC TCA; pv3, GGT GTC TGT CTC TTT CAT CTT CAT TTT CTT CAT TT; pv4, GTC CAG TCC TGG TTC TGT TTT GTG TGG; pv5, GGG TGT GCT TCT GGG TTC CAG TTT C; pv6, TCT GTT GCC AAA CCA CTG TTC CTT CTG; pv7, TCC TGC TTG ATG ACC TCG TCC CAT; pv8, CCT GTC CTG TCC AAA TCT TCC ACC A; pv9, GTC TTT ATC TTC TTC CAA GCC TTC ATG TGA TG; pv10, CAA CAG TAA CTG CAG TAA CTC CAA AGG ACC C. The primers P1 and P2 were used to amplify the CD44 standard isoform [5].

The PCR reaction consisted of 35 cycles (93°C for 30 sec, 65°C for 30 sec, 72°C for 2 min), 1.0 U Taq DNA polymerase and reaction conditions as recommended by the supplier (Life Technologies). After amplification, the reaction mixture was incubated at 72°C for 10 min to allow final extension of the reaction products. Amplified fragments were then electrophoresed in 1% agarose gels and blotted onto nylon filters (Genescreen, NEN Research Products, Boston, Massachusetts, U.S.A.). Subsequently, blots were hybridised with ^{32}P-labelled exon specific primers pv2–pv10 (data not shown).

Exon-specific PCR was performed on biopsy specimens of 30 primary colorectal cancers and adjacent normal colon mucosa (sampled at least 20 cm from the primary tumour), eight hepatic metastases, nine colon cancer cell lines and eight samples of isolated crypts from normal colonic mucosa. The isolation of crypts was performed essentially as described by Whitehead and colleagues [24]. Briefly, small, pieces of colonic mucosa were incubated immediately upon surgical resection in phosphate buffered saline (PBS) containing 1 mM ethylene diamine tetra-acetic acid (EDTA), 1 mM ethylene glycol-aminoethyl-tetra-acetic acid (EGTA) and 0.5 mM dithiothreitol for 1 h on ice and for another 15 min at 37°C. After a short washing step in PBS, whole crypts were liberated from the mucosa by vigorously shaking the tube containing the tissue pieces. Histological examination of the snap-frozen crypts revealed minimal contamination of the isolated crypts with intra-epithelial lymphocytes.

Immunohistochemistry was performed on 5 μm thick cryostat-derived tissue sections using monoclonal antibodies SFF-2, VFF-8 and VFF-7 directed against CD44 standard and variant exons v5 and v6, respectively.

RESULTS

CD44 transcripts were amplified from RNA preparations derived from biopsy samples of normal colorectal mucosa using primers (PIP and P2) located in the standard region upstream and downstream of the variant exons. Consistent with previous reports [5, 9, 15] we obtained only amplimeres corresponding to the CD44 standard transcript, but not to any variant CD44 mRNA (Figure 2a). In contrast, 30 biopsies derived from colorectal carcinomas and eight hepatic metastases clearly revealed a complex pattern of expression of alternatively spliced CD44 transcript lines (data not shown). A similar pattern of CD44 transcripts was also identified in nine colon carcinoma cell.

Immunohistochemical staining of biopsies of colonic mucosa subjected to RT–PCR analysis demonstrated that the CD44 standard isoform is abundantly expressed in stromal tissues (Figure 3a), whereas the expression of this isoform within the colorectal epithelium is restricted to proliferating cells at the bottom of colonic crypts. Staining for epitopes encoded by the variant exons v5 and v6 indicated that stromal tissues do not express the respective CD44 variants, whereas proliferating epithelial cells at the bottom of the colonic crypts clearly stained positive (Figure 3b). These results indicate that CD44 variants are expressed in a very small fraction of cells in biopsies of the normal colorectal

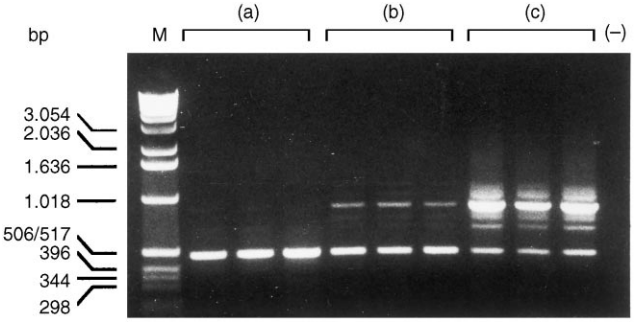

Figure 2. Reverse transcription–polymerase chain reaction amplification of differentially spliced CD44 transcripts in a biopsy sample of normal colonic mucosa with primers P1 and P2; (a) using 1 μg RNA of whole colonic mucosa; (b) using 2 μg RNA of whole colonic mucosa; and (c) using 1 μg RNA of enriched colonic crypts; bp, base pairs; M, size marker; (−), negative control without RNA.

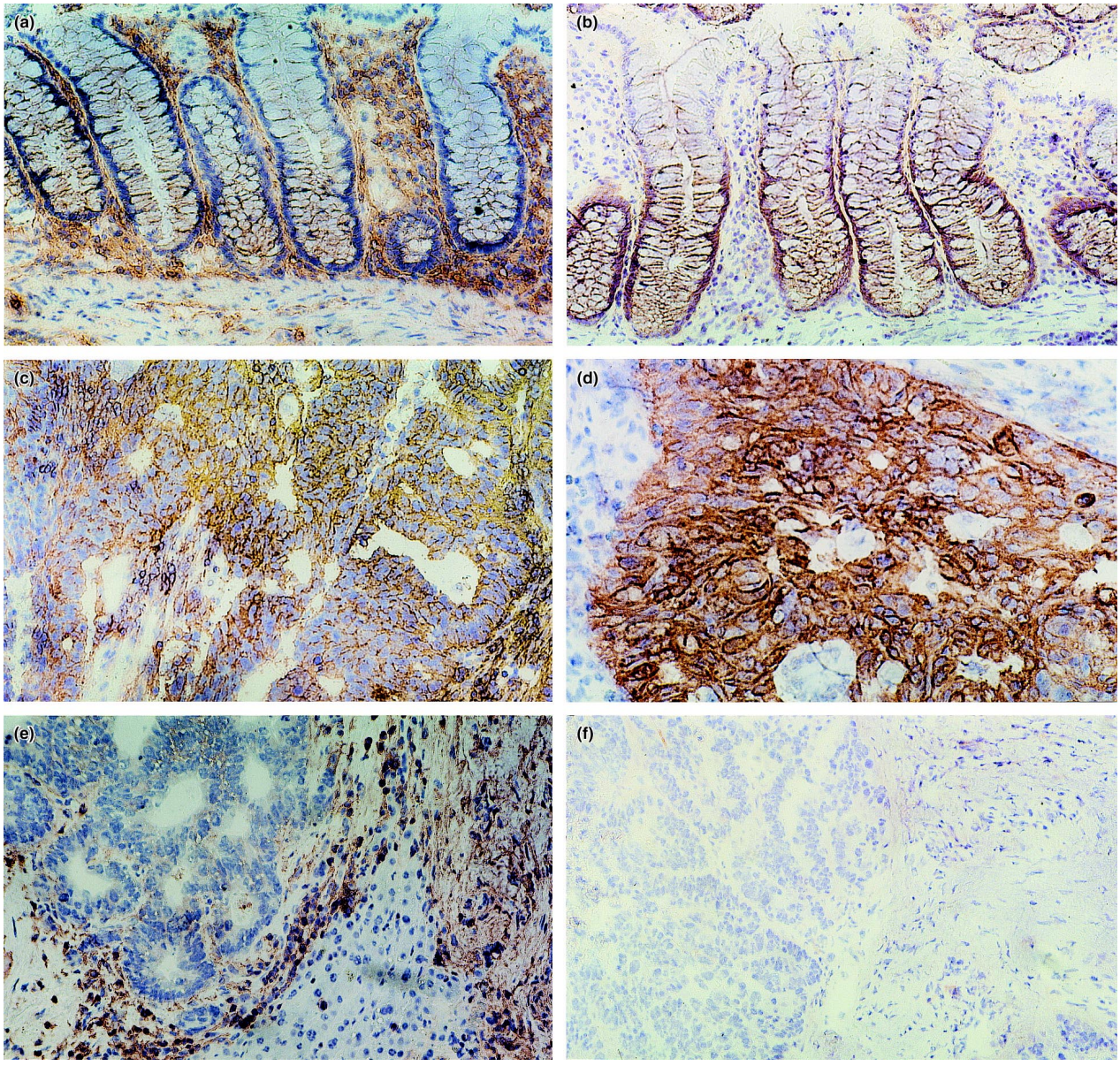

Figure 3. Immunohistochemical staining of normal colorectal epithelium (a, b), colorectal carcinomas (c, d) and hepatic metastasis (e, f) with antibodies specific for standard CD44 (a, c and e) and variant exon v6 (b, d and f). Magnification: (a) ×500; (b) ×500; (c) ×400; (d) ×600; (e) ×500; (f) ×450.

mucosa, whereas the CD44 standard form is abundantly expressed in most cells of normal colorectal tissues. In 30 biopsies obtained from colorectal carcinomas or their hepatic metastases, a positive staining for the CD44 standard form was observed in all mesenchymal cells. Again no staining with antibodies specific for variant exons v5 and v6 was achieved in these cells. A different staining intensity of tumour cells was achieved in only 16 biopsies independent of tumour stage (Figure 3c and e). However, despite the expression of the respective transcripts, only 17 of 30 lesions were stained with the v6 specific antibody VFF-7, and only six of them stained with the v5 specific antibody VFF-8 (Figure 3d and f, Table 1). No correlation to tumour stage could be detected.

Since immunohistochemistry revealed that only very few epithelial cells express variant CD44 molecules in the normal colorectal mucosa, we repeated the RT–PCR analysis using the primer pair P1 and P2, but subjected twice as much RNA to the reaction. Under these conditions, a faint larger PCR fragment of approximately 1 kb was detected, suggesting that in the normal colonic mucosa variant CD44 transcripts are indeed expressed (Figure 2b). Therefore, we enriched the epithelial cells from the biopsy samples as previously described [24] and subjected RNA extracted from the epithelial cell fraction to the RT–PCR analysis. Now a complex pattern of variant CD44 amplimeres was amplified (Figure 2c), suggesting that within normal colorectal epithelial cells variant CD44 mRNAs are abundantly expressed. Since most cells within biopsies of normal colorectal mucosa selectively express the CD44 standard molecule (Figure 3a) and only few epithelial cells at the bottom of the crypts express CD44 splice variants (Figure 3b), the variant CD44 transcripts can easily be missed in RT–PCR reactions using RNA preparations derived from biopsies, unless the epithelial cells are specifically enriched.

To compare the exon composition of the CD44 transcripts expressed in the normal colorectal epithelium and colorectal cancers and their metastases, respectively, we amplifed CD44 transcripts using a recently described exon-specific RT–PCR protocol [22, 23]. In all mRNA preparations either derived from normal epithelium (Figure 4b), primary colorectal cancers or metastasis (Figure 4a) we identified a similar pattern of alternatively spliced CD44 transcripts. Interestingly, a similar expression pattern was detected in nine colon carcinoma cell lines (Figure 4c).

DISCUSSION

Our data indicate that normal proliferating colon epithelial cells express a specific pattern of eight alternatively spliced CD44 transcripts which are retained during neoplastic and metastatic progression (Figure 5), but they exclude cancer- or metastasis-related alternative splicing of CD44 transcripts. However, immunohistochemical analysis of 14 primary colorectal cancers and hepatic metastases revealed no staining with antibodies directed against CD44 standard and variant transcripts whereas RT–PCR analysis of the same biopsies revealed high levels of CD44 standard and variant transcripts. This could indicate that CD44 epitopes in these metastases are masked, for example, by altered glycosylation of the CD44 molecules. The reduced availability of functional CD44 epitopes rather than *de novo* expression of CD44 variants might be important for metastatic progression of colorectal cancers. This assumption awaits further experimental support, particularly by defining the biological function of distinct CD44 isoforms. Nevertheless, other tumours derived from tissues expressing CD44 variant isoforms show a similar tendency of reduced CD44 surface expression on metastatic lesions [22–28]. However, the exon-specific PCR of enriched colonic crypts confirms and extends the recent report on the CD44 expression pattern in colorectal mucosa [19].

Table 1. *Expression of standard and variant CD44 molecules in surgical specimens of primary colorectal cancers and hepatic metastases**

Biopsy	TNM	SFF-2 (s)	VFF-8 (v5)	VFF-7 (v6)
1	T3N0G2M0	++	++	+++
2	T3N0G2M0	++	+	++
3	T2N1G2M0	+++	++	+++
4	T4N3G2M1	++	+	++
5	T3NOG2M0	–	–	–
6	T3N2G2M0	+	–	–
7	T3N3G2M0	+++	–	–
8	T3N2G2M0	–	–	+
9	T4N3G3M0	++	++	+++
10	T4N0G2M0	+	–	+
11	T2N2G2M1	–	–	–
12	T2N0G2M0	–	–	–
13	T3N2G3M0	–	–	–
14	T1N0G2M0	++	–	–
15	T2N0G2M0	+	–	++
16	T3N0G1M0	–	–	++
17	T3N0G2M0	+	–	++
18	T2N0G2M0	–	–	–
19	T3N1G2M0	–	–	+++
20	T3N2G3M0	–	–	++
21	T3N1G2M0	–	–	–
22	T3N1G2M0	–	–	–
23	T3N1G3M0	–	–	–
24(pTu)	nd	–	–	–
(LM)	–	–	–	–
25(pTu)	T2N0G2M1	++	–	+
(LM)	–	+	–	+
26(pTu)	T4N×G3M1	+	–	++
(LM)	–	–	–	–
27(pTu)	T3N3G2M1	+	–	+
(LM)	–	+	–	+
28(pTu)	T3N1G2M0	+	–	+
(LM)	–	+	–	+
29(pTu)	T3N2G2M1	+	+	+
(LM)	–	–	–	–
30(pTu)	T3N2G2M1	–	–	–
(LM)	–	–	–	–

*Immunohistochemistry with monoclonal antibodies directed against standard and variant CD44 epitopes, +++, intense staining; ++, distinct staining; +, weak staining; –, no staining; pTu, primary tumour; LM, liver metastasis; TNM, primary tumour, lymph node, and metastasis stages at the time of the primary resection; nd, not determined.

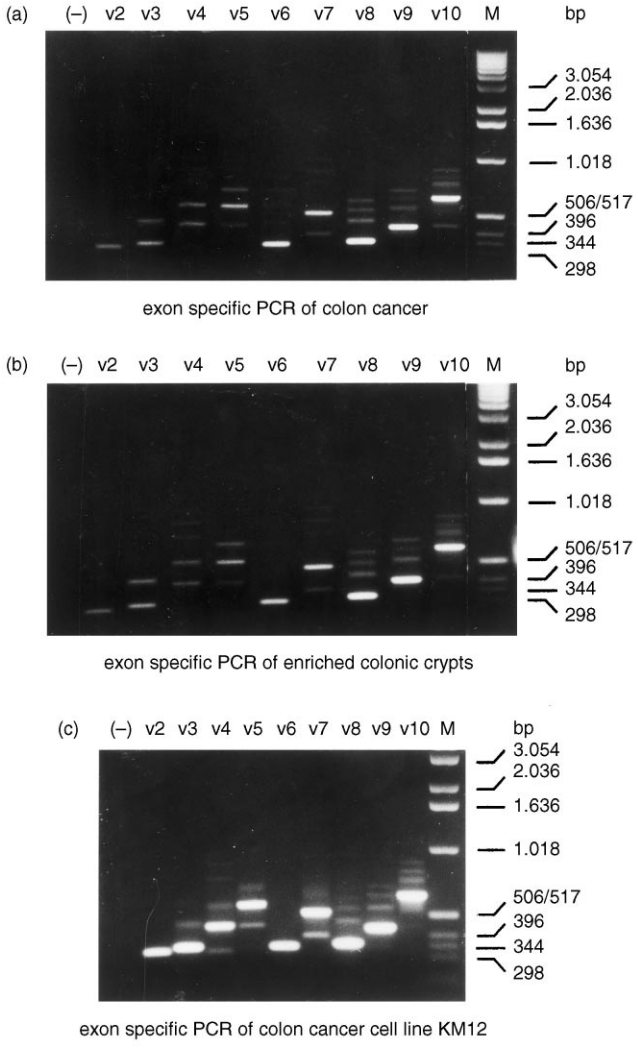

Figure 4. Exon-specific reverse transcription–polymerase chain reaction analysis of CD44 splice variants (a) in a biopsy sample of colorectal cancer, (b) in enriched colonic crypts and (c) in the colorectal cancer cell line, KM12. bp, base pairs; M, size marker, (–), negative control without RNA.

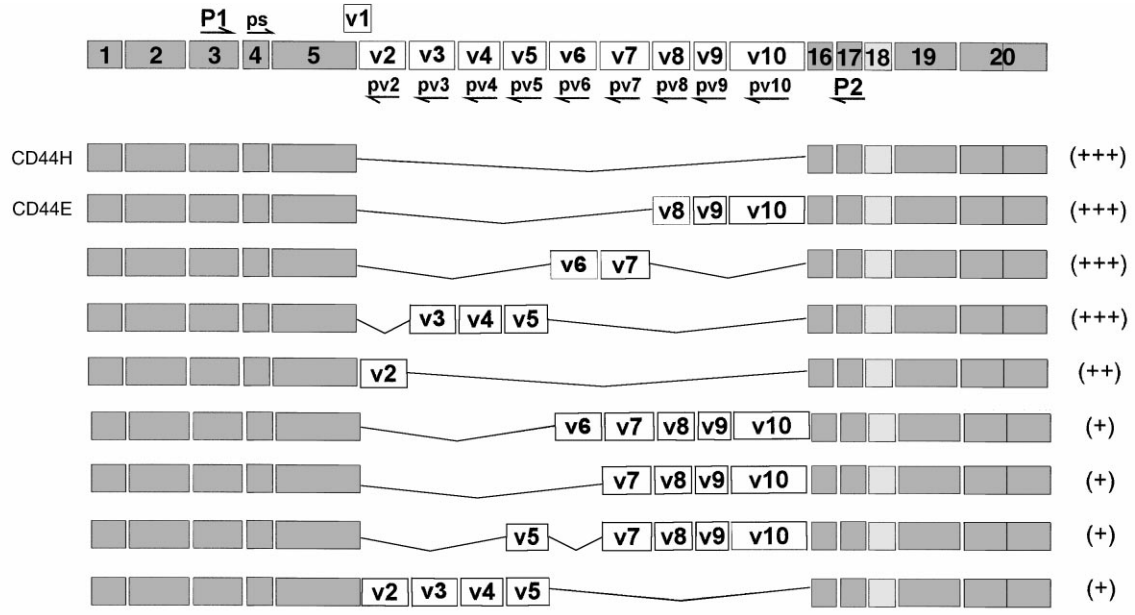

Figure 5. Schematic presentation of the common CD44 splice transcripts detected in colorectal cancer biopsies, in the KM12 cell line and in enriched colorectal epithelium. CD44H, haematopoietic form; CD44E, epithelial form. The relative abundance of transcripts is indicated; (+++), abundant transcripts; (++), distinct transcripts; (+), rare transcripts.

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