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Expression of CD44v6 is Associated with Cellular Dysplasia in Colorectal Epithelial Cells

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There is increasing evidence that the expression of variants of the glycoprotein CD44 is related to the invasive and metastatic potential of tumour cells. By *in situ* hybridisation, we analysed the cellular expression of human homologues of a rat metastasis-associated CD44 variant v6 in invasive and non-invasive colorectal neoplasia and normal colonic mucosa. No specific hybridisation signals could be detected in epithelial cells of the normal crypt ($n = 10$). In contrast, we found moderate epithelial hybridisation signals in adenomatous polyps of mild dysplasia ($n = 6$). Adenoma cells of moderate or severe dysplasia ($n = 7$) showed increased hybridisation signals compared to mildly dysplastic adenomas ($P \leq 0.01$). We could not demonstrate significant differences in CD44v6 transcript levels between cells of dysplastic adenoma and primary adenocarcinoma ($n = 11$) ($P \geq 0.05$). Furthermore, we were not able to demonstrate a significant difference between primary and metastatic tumours ($n = 7$) ($P \geq 0.05$). However, there was a significant difference between metastatic carcinoma and adenomas with advanced dysplasia ($P \leq 0.01$). Our data demonstrate that significant transcriptional expression of CD44v6 is not confined to invasive tumour cells, but is already detectable in cells of adenomatous polyps showing mild dysplasia. The results of this study show a close relationship between cellular dysplasia and steady state levels of CD44 variant v6 transcripts in colorectal neoplasms.

Key words: CD44 variants, colorectal carcinoma, *in situ* hybridisation, malignant transformation
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INTRODUCTION

COLORECTAL CARCINOMA is the second most frequent cause of cancer mortality in Western countries. The prognosis of the disease is largely dependent on the tumour stage at the time of surgery and is worse when development of metastasis has occurred. The molecular events leading to the expression of the metastatic phenotype of tumour cells and particularly the functional role of cell adhesion molecules are under intensive study. In this context, expression of variants of the glycoprotein CD44 is of special interest. CD44 was originally described as an integral lymphocyte homing receptor involved in endothelial transmigration and binding to lymph node stroma [1, 2]. Various CD44 isoforms of higher molecular weight have been cloned which contain additional extracellular domains inserted at a unique site of the molecule and are expressed on epithelial tumour cells [3–6]. The genomic structure of the *CD44* gene revealed at least 12 alternatively spliced exons spanning approximately 50 kilobases of DNA [7]. The significance of variant CD44 expression for metastatic capacity of tumour cells was emphasised by Günthert and associates [8]. In transfection studies, they demonstrated that overexpression of a CD44 protein variant, expressing two additional domains encoded by alternatively spliced CD44 exons v4 to v7, conferred metastatic potential to rat pancreas carcinoma cells. Further studies linked the expression of CD44 with development and spread of cancer. In solid tumours, elevated levels of CD44 have been associated with high grade malignant gliomas [9], and tumour spread and poor prognosis of large cell lymphomas [10]. Overexpression of defined variable CD44 exons has been demonstrated in Northern blots or reverse transcriptase-polymerase chain reaction (RT-PCR) followed by Southern blot hybridisation (PCR) of RNA from pulmonary adenocarcinoma, oesophageal carcinoma, gastric carcinoma, mesothelioma and melanoma, hybridised with cDNA probes of this gene [11]. Recently, expression of a human homologue of the rat metastasis-associated CD44 variant v6 was detected by immunohistochemical staining and PCR analysis in

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human colorectal adenomas and carcinomas [11, 12]. Further studies indicate that expression of variants containing exon v6 is largely restricted to advanced stages of tumour development [13]. However, controversial results have been reported. Immunohistological studies using antibodies against standard CD44 revealed a strong correlation between the proliferative status of neoplastic and normal epithelial cells and CD44 expression [14]. Furthermore, results of an immunohistological study demonstrated a frequent expression of CD44 prior to *K-ras* and p53 alterations in colorectal adenomas [15]. Recently, Mulder and associates demonstrated that expression of CD44v6 coded proteins and p53 increased with the degree of dysplasia in adenomas [16]. The question arises whether the association of CD44 variant v6 in colorectal neoplasia is linked to proliferative status or other aspects of tumour development. To address this question, we used *in situ* hybridisation to determine the precise cellular localisation and steady state levels of variant CD44 transcripts homologous to the rat metastasis-associated CD44 exon v6 in cells of human colorectal neoplasia. *In situ* hybridisation analysis of tissue sections allows the correlation between cellular morphology and gene specific RNA expression and, therefore may be useful to investigate the distribution of CD44v mRNA in tissues.

MATERIALS AND METHODS

Tissues

Tumour specimens were obtained from the Department of Surgery at the Benjamin Franklin Hospital, Germany, and submitted for histological examination to the Institute of Pathology, Germany. Biopsies of adenomas were obtained from the endoscopy services of the Department of Medicine at Benjamin Franklin Hospital. Samples of colonic mucosa were removed from macroscopically normal areas. Clinical data of the tissue samples are summarised in Table 1. Samples were frozen in liquid nitrogen within 45 min after resection and stored under liquid nitrogen until needed. Cryostat sections (4 μm) were mounted on APS-treated glass slides, air-dried on a hot plate at 80°C for 3 min and fixed in 4% (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS; pH 7.4) for 20 min. After two washes in PBS and dehydration in graded ethanols sections were air-dried and stored at −80°C. Dysplasia of adenomas were classified as mild or advanced, differentiation of carcinomas were classified as high, moderate and low [17].

Table 1. Clinical and histological data of the tumour samples analysed for CD44v6 RNA expression by in situ *hybridisation*

No.	Site	Histology	Grade	TNM
Adenomatous polyps				
1	colon	tu-vi	G1*	
2	colon	tu-vi	G1*	
3	rectum	tu	G1	
4	colon	tu-vi	G1*	
5	colon	tu-vi	G1	
6	colon	tu-vi	G1	
7	colon	vi	G1	
8	colon	tu-vi	G2	
9	sigmoid	tu-vi	G2	
10	colon	tu-vi	G2	
11	sigmoid	vi	G2	
12	sigmoid	tu-vi	G2	
13	colon	tu-vi	G3	
Primary adenocarcinoma				
1	rectum	Adeno	G2	T3N0
2	colon	Adeno	G2	T3N0
3	sigmoid	Adeno	G2	T3N0
4	sigmoid	Adeno	G2	T3N0
5	rectum	Adeno	G2	T3N0
6	coecum	Adeno	G2	T4N0
7	colon	Adeno	G2	T3N0
8	colon	Adeno	G2	T3N0
9	colon	Adeno	G2	T3N0
10	coecum	Adeno	G3	T3N1
11	rectum	Adeno	G3	T4N1
Metastatic adenocarcinoma				
1	lung	Adeno	G2	
2	liver	Adeno	G2	
3	liver	Adeno	G2	
4	liver	Adeno	G2	
5	lung	Adeno	G2	
6	liver	Adeno	G2	
7	lymph node	Adeno	G3	

* Presence of moderately dysplastic foci in adenomas characterised by predominantly mild dysplasia, tu = tubulous, vi = villous.

Detection of CD44v6

The presence of CD44v6 splice variants was analysed by immunohistochemistry using an indirect immunoperoxidase technique. Sections were fixed in acetone and preincubated with rabbit anti-human serum (DAKO A206, Hamburg, Germany) for 30 minutes to reduce non-specific staining. This was followed by incubation with 0.2% hydrogen peroxidase for 15 min to inhibit endogenous peroxidases. The primary antibody (anti-CD44var(v6) (BMS116) (Serva, Heidelberg, Germany)) was then added at a 1:100 dilution in PBS and incubated for 60 min at room temperature. The second layer antibody used was biotinylated rabbit anti-mouse immunoglobulin (DAKO, Z109), diluted at 1:200 and incubated for 30 min. Slides were then incubated with streptavidin–biotin complex (DAKO, K391), diluted 1:100 for 30 min, and then 3,3′-diaminobenzidine tetrahydrochloride for 15 min to develop the peroxidase. This was followed by thorough washing and counterstaining with haematoxylin and eosin.

Hybridisation probes

Using specific primers, a template (nucleotide positions 941 to 1108 of the alternatively spliced CD44 exons v5 and v6 according to the published human cDNA sequence) [7, 11] have been made by PCR. Identity of the amplification product was confirmed by DNA sequence analysis (data not shown). The amplified fragment consisting of a 170 bp cDNA sequence was cloned into the expression vector pCR™3 (Invitrogen, San Diego, California, U.S.A.). The vector contains T7 and SP6 polymerases as promoters, for generation of radiolabelled RNA probes by run-off transcription. After linearisation with HindIII or EcoRI restriction endonucleases, RNA probes complementary (antisense) or anticomplementary (sense, negative control) to the cellular RNA were synthesised using T7 or T3 RNA polymerase (Gibco BRL, Berlin, Germany), respectively. The specific activity routinely obtained by incorporation of [^{35}S]uridine 5′-[a-thio]triphosphate (3000 Ci/mmol; New England Nuclear, Dreieich, Germany) was 1.2–1.4 × 10^9 cpm/μg.

Hybridisation and autoradiography

For *in situ* hybridisation, posthybridisation washing including removal of non-specifically bound probe by RNase A digestion and autoradiography were performed essentially as previously described [18]. Briefly, tissue sections were treated with 0.2 N HCl for 20 min, digested with 0.125 mg/ml pronase (Boehringer Mannheim, Mannheim, Germany) for 10 min, rinsed in 0.1 M glycine/PBS and fixed for 20 min in 4% paraformaldehyde/PBS. After washing in PBS, sections were acetylated in a freshly prepared solution of acetic anhydride (diluted 1:400 in 0.1 M triethanolamine, pH 8.0) for 10 min, again washed in PBS, dehydrated in graded ethanols, and air-dried. Hybridisation was performed in a humid chamber for 18 h at 50°C using 2×10^5 cpm of ^{35}S-labelled RNA probe in 25 µl hybridisation mixture (50% formamide, 10% dextran sulphate, 10 mM dithiothreitol, 0.1 M Tris–HCl, pH 7.5, 0.1 M $NaPO_4$, 0.3 M NaCl, 50 mM EDTA, 1 × Denhardt's [0.002% Ficoll 400, 0.002% polyvinylpyrrolidone, 0.002% bovine serum albumin], 0.5 mg/ml yeast tRNA). Slides were washed for 5 h in hybridisation buffer without Denhardt's and yeast tRNA and subjected to RNase A digestion (20 µg/ml in 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl) for 30 min at 37°C. After stringency washing in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate) and 0.1 × SSC at room temperature, slides were dehydrated in graded ethanols containing 0.3 M ammonium acetate and air-dried prior to immersion into Amersham LM-1 photographic emulsion (Amersham, Braunschweig, Germany) containing 0.3 M ammonium acetate. After air-drying, slides were stored in light-tight boxes and exposed at 4°C for 35 days. Slides were developed in a Kodak D19 developer (Kodak, Hemel Hampstead, U.K.) for 3 min, rinsed in 1% acetic acid, and fixed in Kodak fixer for 3 min. After washing, slides were counterstained with haematoxylin and eosin, again dehydrated in graded ethanols, xylene treated and mounted in corbit balsam for microscopy.

To quantify the expression of CD44v6 transcripts revealed by *in situ* hybridisation experiments, all slides were examined using a Zeiss microscope at ×200 magnification. For this approach, tissue samples were selected according to two main criteria: (a) excellent morphology showing preserved tissue architecture and well-defined cellular (i.e. nuclear) borders, (b) even and low non-specific hybridisation background. For each tissue sample, we analysed four different areas that were reasonably representative of the tissue sample. The grains were counted in 20 to 50 cells per area and the median of grains per cell was calculated for each sample. Results of tissues in each group (normal colonic mucosa, adenomas with epithelial cells showing mild dysplasia, adenomas with epithelial cells showing advanced dysplasia, primary adenocarcinomas, metastatic adenocarcinomas) were expressed as median values. Slides were counted by two independent observers and interobserver results varied by less than 10%.

Statistical analyses

Statistical analyses as a multiple group comparison, e.g. analysis of variance, using the Wilcoxon-rank test indicated significant differences between the groups. Therefore, a Mann–Whitney test, a non-parametric statistical test, was performed to determine if the difference in the expression of CD44v6 transcripts determined by *in situ* hybridisation between normal colonic mucosa, adenomas and colon cancer lesions were significant. Differences between the groups were considered to be significant at the $P \leq 0.05$ level.

RESULTS

Expression of CD44v6 splice variant

Immunostaining of CD44v6 was not detected in epithelial or mesenchymal cells in normal colonic mucosa. CD44v6 expression was evident in epithelial cells with mild dysplasia and in the majority of epithelial cells with advanced dysplasia in adenomas. Since CD44v6 expression in epithelial cells with mild dysplasia was very rare (<5% were positive), the percentage of positive cells within adenomas increased with malignant transformation. A moderate expression of proteins coded by exon v6 was observed in adenomas containing epithelial cells with advanced dysplasia in 30% (Figure 1A). However, in most carcinomas (8/11) and metastases (7/7), the majority of tumour cells were positive for CD44v6 (Figure 1B). Sections of only three carcinomas showed focally moderate and weak expression.

Expression of CD44v6 RNA in normal colonic mucosa

To elucidate further the role of the CD44 splice variant v6 in colorectal tumorigenesis, we analysed expression of CD44v6 specific transcripts in human colorectal tissues during the malignant transformation process using *in situ* hybridisation. These experiments demonstrated differential expression of CD44v6 transcripts in epithelial cells of normal colonic mucosa, adenomas, primary carcinomas and metastases. In normal colonic mucosa, only a faint CD44v6 transcript-specific signal localised to the base of the crypts could be detected. However, *in situ* hybridisations using the sense probe yielded sections with a similar pattern. An overexpression of CD44v6 was observed at an early stage of colorectal tumour expression, i.e. in epithelial cells with mild dysplasia of adenomas. At the more advanced stages of colorectal carcinogenesis, i.e. in adenomas with advanced dysplasia or invasive carcinomas, the level of CD44v6 transcripts increased. In addition, a strong, but heterogenous expression of CD44v6 transcripts could be detected in metastases. Neither mesenchymal nor lymphatic cells showed any specific CD44v6 expression.

Quantification of CD44v6 transcripts

Ten samples of normal colonic mucosa were analysed for CD44v6 transcript expression. In epithelial cells of the normal colonic mucosa, the intensity of the hybridisation signal was equal to that of the background signal, thus we were not able to detect specific CD44v6 transcriptional expression, either in superficial (Figure 2A) or epithelial cells located at the basal proliferative zone of colonic crypts (Figure 2B). Quantification of cellular signals revealed 0–3 grains per cell (median 0.5) (Figure 3). The median cellular hybridisation signal detected in samples of normal and neoplastic tissue was approximately 0.2 grains per cell (minimum: 0, maximum: 2.1) in control hybridisations using a corresponding RNA sense probe (Figure 2F). As mentioned above, the amount of CD44v6 transcripts in colorectal neoplasia differed from that in normal epithelium. The range of hybridisation signal intensities observed in adenomas showing mild dysplasia ($n = 6$) (Figure 2C) was between 1.7 to 3.1 (median 2.3) (Figure 3). Compared to epithelial cells of normal mucosa, the difference was statistically significant ($P \leq 0.01$). Careful histological examination identified some foci of moderate dysplasia in three of the adenomas graded as mildly dysplastic. These foci displayed overtly increased median cellular transcript levels when compared to the surrounding cells showing mild nuclear atypia. Because only one or two foci could be identified in each section, semiquantitative analysis of the hybridisation signal was not performed. However, in aden-

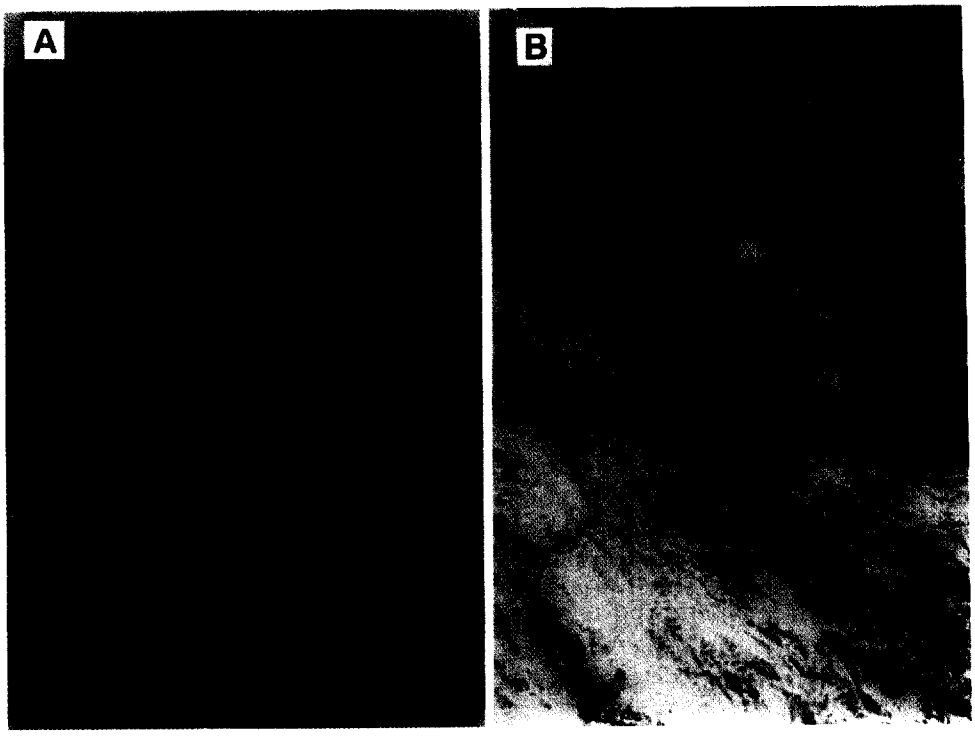

Figure 1. Expression of CD44v6 coded proteins on epithelial cells of an adenoma with moderated dysplasia (A) and a colorectal carcinoma (B). Cryostat tissue sections were subjected to immunohistochemistry using the monoclonal antibody anti-CD44var(v6) (BMS116) as described in Materials and Methods. Staining with antibodies to CD44v6 was either weak or undetectable in epithelial cells with low grade dysplasia, whereas cells with epithelial cells with advanced dysplasia (arrows) expressed CD44v6 at moderate levels. Counterstained with haematoxylin and eosin. Magnification ×200.

omatous polyps of advanced dysplasia (n = 7) (Figure 2D) we found average cellular hybridisation signals ranging from 2.8 to 9.1 grains per cell (median 6.2) (Figure 3). The difference in signal intensity compared to mildly dysplastic adenomas was significant ($P \leq 0.01$). Quantification of CD44v6 transcripts in primary adenocarcinomas (n = 11) revealed signal intensities ranging from 4.2 to 13.3 grains per cell (median 8.3) (Figure 3). The difference in hybridisation signal intensity when compared to adenomatous polyps of advanced dysplasia was not significant ($P \geq 0.05$). Therefore, we did not detect a difference at the cellular level of CD44v6 RNA between adenomas showing advanced dysplasia and primary adenocarcinoma. Seven cases of metastastic colorectal tumours were analysed, most localised in the liver (Table 1). In metastatic tumours, we found a hybridisation signal range from 7.1 to 18.3 grains per cell (median 11.2) (Figure 3). Compared to primary adenocarcinomas, the difference was not significant. However, the difference between metastatic tumours and adenomatous polyps showing advanced dysplasia was statistically significant ($P \leq 0.01$).

In summary, our data demonstrate significant upregulation of the cellular CD44v6 RNA level in early adenomas of the colon. During malignant transformation, we found increasing cellular CD44v6 RNA levels (Figure 3). However, neither the difference between primary carcinomas and dysplastic adenomas nor the difference between primary and metastatic tumours proved to be significant at the 0.05 level.

DISCUSSION

The interaction between basement membrane components and cell adhesion receptors in malignant cells may influence the aggressiveness of local infiltrative growth and metastasis of colonic cancer cells. Expression of CD44 molecules has recently been linked to tumour dissemination in several systems including colorectal carcinomas [4, 8, 12]. It has been hypothesised that expression of CD44 variants generated by alternative splicing, particularly those containing v6, is strongly related to colorectal tumour metastasis [13]. However, immunohistological studies have revealed that this variant isoform is also expressed in non-infiltrative adenomatous polyps [12, 16]. Masking of the CD44 v6 epitope may lead to an underestimation of prevalence of this protein in immunohistological studies, and differences in the intensity of protein expression are difficult to estimate in immunohistological studies. Another major advantage of the *in situ* hybridisation technique compared to an immunohistochemical approach is that the latter technique cannot distinguish between cells that have captured antigens from those that have the capacity to synthesise the gene product. Therefore, we analysed the steady state levels of CD44 transcripts expressing exon v6 in epithelial cells of colorectal neoplasia during benign to malignant tumour transformation using *in situ* hybridisation. Our results clearly demonstrated that variant CD44 transcripts were expressed in epithelial cells of non-invasive adenomas as well as carcinomas and metastases. In adenomatous polyps, a close relationship between the cellular steady state levels of CD44 transcripts expressing variant exon v6 and the dysplastic degree of cells was observed. Low but distinct variant CD44 transcript levels were detected in adenoma cells showing mild dysplasia, but the transcript level was significantly enhanced in cells displaying moderate or severe dysplasia. A similar correlation between expression of CD44 v6 coded proteins and the degree of dysplasia has recently been described by Mulder and colleagues [16]. In contrast to recently published data by Wielenga and associates [13], we did not observe a significant increase in CD44 v6 mRNA levels during transformation from non-invasive

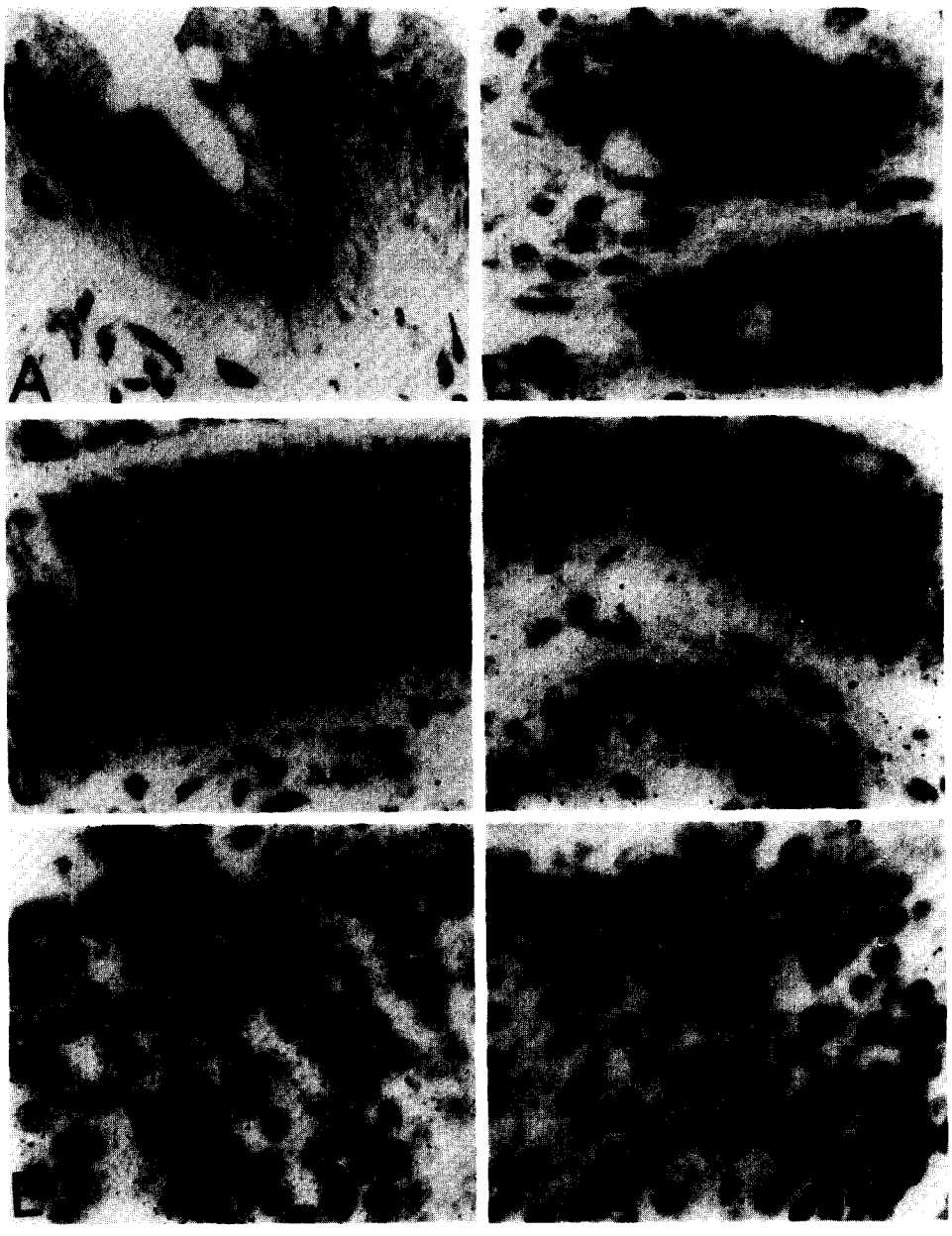

Figure 2. RNA transcripts containing variant exon 6 in cells of colorectal tumours and normal colonic mucosa. Cryostat tissue sections were subjected to *in situ* hybridization using ^{35}S-UTP-labelled antisense RNA transcripts specific for alternatively spliced CD44 exon v6 as described in Materials and Methods. (A)–(E) antisense probe, (F) sense probe. (A) Normal colonic mucosa, surface epithelium. In epithelial and underlying mesenchymal cells of the lamina propria, specific hybridisation signals were not detectable. (B) Normal colonic mucosa, basal crypt. Compared to sense controls, specific hybridisation signals were not detectable. (C) Adenomatous polyp, mild dysplasia. Adenoma cells are moderately positive. (D) Adenomatous polyp, moderate dysplasia: intermediate hybridisation signal intensity clearly increased compared to (C). (E) Colonic adenocarcinoma, histological grading G3. Strong hybridisation signal. (F) Colonic adenocarcinoma (serial section of the adenocarcinoma seen in (E)), control hybridisation. No specific hybridisation signal was visible. Exposure time 35 days after posthybridisation washing, counterstained with haematoxylin and eosin. Magnification ×400.

adenomas to infiltrative carcinomas. Semiquantitative analysis of our *in situ* hybridisation results indicated that the steady state concentration of transcript levels found in adenoma cells of moderate and severe dysplasia was largely similar compared with the average level detected in cells of invasive tumours, although maximum levels of expression was seen in single tissue samples of the carcinoma and metastasis group. In addition, the number of strongly positive cells (more than 5 grains per cell) in invasive neoplasia was not notably different from those in adenomatous polyps with severe dysplasia (see Figures 2D and E). With respect to our semiquantitative assessment and limitations of the method, we could not find any overall significant differences between dysplastic adenoma and carcinoma. Furthermore, within the carcinomas, no correlation of CD44 v6 expression with TNM classification or biological grading was obvious. However, we cannot exclude that an increased translation activity in carcinomas or a decreased degradation of proteins results in increased CD44 variant protein level in carcinomas compared with adenomas. In this context, it is important to note that the group of S. Pals describes expression of exon v6-containing CD44 proteins as a predictive prognostic parameter of patients with colorectal cancer [19]. In this study, immunohis-

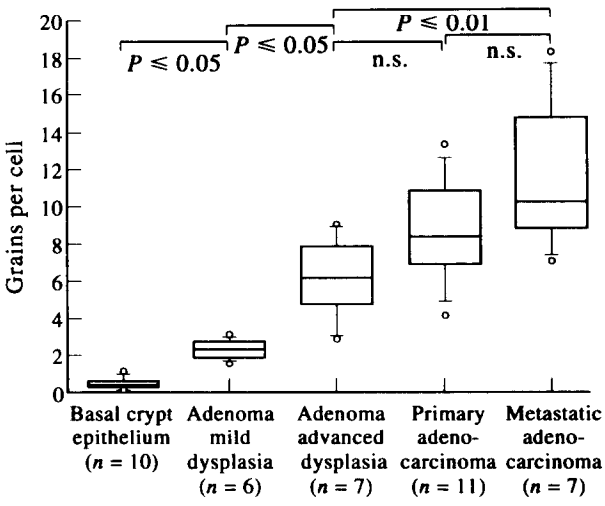

Figure 3. Quantification of CD44v6-specific grains in epithelial cells of normal colonic mucosa and colorectal neoplasms. Grains were counted in 20 to 50 cells per area and the median of grains per cell was calculated for each sample as described in Materials and Methods. For each tissue group, data were summarised using the Boxplot technique. Shown is the median, 25 and 75% percentile. Open circles represent values out of the interquartile range.

tological analysis of 68 primary colorectal carcinomas revealed that expression of CD44v6 coded proteins was highly variable, ranging from negative to strongly positive. They demonstrated that expression of CD44v6 was correlated with adverse prognosis. However, immunohistochemical analysis of adenocarcinomas in our study revealed no CD44v6 negative sections and only three carcinomas with focal moderate expression. All other carcinomas and metastases studied showed strongly positive staining of epithelial cells.

The observation that variant CD44 expression is not a characteristic of invasive or metastatic tumour cells is also suggested by the detection of CD44 exon v6 expression in normal human tissues such as keratinocytes, pancreatic duct epithelium and bronchial epithelium. In rats, transient expression of variant CD44 exon v6 was detected after antigenic stimulation of lymphocytes and macrophages and in newborn animals [20]. Depending on the tissue or cell line investigated, controversial observations concerning the role of *CD44* gene products in the process of malignant transformation have been reported. In contrast to colorectal carcinomas, downregulation of physiologically expressed CD44 exon v6 was recently reported for squamous cell carcinomas [21].

Carcinoma progression is a complex multistep process involving multiple modifications of cell-surface components, intracellular alterations and genetic changes. Our study revealed increased CD44 v6 expression in dysplastic cells of non-invasive adenomas indicating that other factors besides CD44v expression may contribute to the capacity for infiltrative growth. For example, Fearon and associates [22] have described a gene that is absent or altered in 70% of colorectal carcinomas and codes for a protein with great homology to the members of the N-CAM family. It was postulated that loss of this cell–cell adhesion molecule may result in disturbed cell–cell interaction which promotes the infiltrative phenotype. Additional cumulative changes in the expression of cell surface receptors, e.g. loss of integrins and increased expression of the 32/67 kD laminin receptor, may induce changes to the adhesive properties of tumour cells, finally resulting in the invasive and metastatic phenotype [23–26]. Another factor promoting infiltrative tumour growth is the secretion of proteolytic enzymes, including plasminogen activators [27], lysosomal hydrolases [28], and collagenases [29]. With progression from a benign epithelial growth to an infiltrative carcinoma, a decrease of basement membrane material in many types of tumours including colorectal carcinomas has been observed [30]. Immunohistological studies indicate that the percentage of collagenase-positive epithelial cells is significantly higher in colorectal carcinomas than in epithelial cells of adenomas or normal colonic mucosa [30].

In summary, this study demonstrates that expression of CD44v6 transcripts is related to the degree of dysplasia in colorectal neoplasms. Our data may serve as a basis for further studies aiming at the identification of regulatory factors (e.g. hormones, lymphokines, cytokines) that modulate the expression of the CD44 variants in colonic epithelial cells. This might open up new concepts for influencing local tumour growth and metastasis of colorectal carcinomas.

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