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REVIEW

p53 and CD44 as clinical markers of tumour progression in colorectal carcinogenesis

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Summary

Recent advances in molecular genetics have importantly improved our understanding of the development of colorectal cancer. The present review gives an overview of the clinical value of the tumour-suppressor gene, p53, and the CD44 cell adhesion molecule in colorectal cancer and the pitfalls encountered in the immunohistochemical detection of these proteins. Immunohistochemistry potentially forms a procedure applicable for routine diagnosis and prognostication. Therefore, p53 expression and the independent prognostic importance of CD44v6 expression is given particular emphasis, and other molecular events underlying colorectal carcinogenesis are only mentioned briefly.

Introduction

Colorectal cancer is a common form of cancer in western Europe and the United States, second only to lung cancer and prostate cancer in males and breast cancer in females (Coleman *et al*., 1993; American Cancer Society, 1994). In the United States, about 150 000 new cases are diagnosed every year, representing 12% of invasive cancers and 10% of cancer deaths in men and 13% of invasive cancers and 11% of cancer deaths in women.

Surgery is so far the only effective treatment for colorectal carcinoma, with about 80% of patients considered operable at the time of diagnosis (Gilbert, 1982). However, even after 'curative' resection, 5-year survival is only in the order of 60% (American Cancer Society, 1994). Death is related to recurrent disease, caused by the outgrowth of metastases, which were occult at the time of initial diagnosis and surgery. To reduce morbidity and mortality from minimal residual disease, identification of those patients with a high propensity to develop distant metastases is of great importance, since they might benefit from adjuvant chemotherapy and/or radiotherapy (Mayer *et al*., 1989; Moertel, 1994). Thus, accurate pathological investigation and reporting of colorectal cancer specimens is essential not only for diagnosing the disease, but especially for assessing prognosis and the development of treatment strategies (Hermanek, 1987). In 1932, Cuthbert E. Dukes classified cancers of the rectum based on the extension of the tumour through the bowel wall (Dukes, 1932; Turnbull *et al*., 1967). Tumours limited to the bowel wall were classified as Dukes' stage A, tumours with extension beyond the wall (i.e. beyond the deep muscle) as Dukes' B and, when metastatic spread was present, tumours were classified as stage C tumours. This classification, which can be applied to all cancers of the large bowel, is still considered as the most accurate predictor of prognosis after resection (Turnbull, 1975; Crissman & Barwick, 1993; Fisher *et al*., 1989). However, even within a group of tumours of a specified stage, tumour behaviour and prognosis of the disease is not uniform. For this reason, additional markers that predict tumour behaviour are needed to identify those patients who may benefit from adjuvant therapy (Gastrointestinal Tumor Study Group, 1985; Douglass *et al*., 1986; Laurie *et al*., 1989; NIH Consensus Conference, 1990; Krook *et al*., 1991; Moertel, 1994). Many histopathological parameters

have been evaluated for this purpose, but none of them proved to be an independent and clinically useful predictor of tumour behaviour (Bosman, 1995).

In the last decade, great progress has been made in the identification of the molecular genetic changes underlying colorectal carcinogenesis (Vogelstein *et al.*, 1988; Fearon & Vogelstein, 1990). These genetic alterations may at the same time reflect the biological competence of a tumour, but more insight into their clinical correlates is needed to assess whether they can serve as additional tools to predict prognosis in individual colorectal cancer patients.

Background

The rapid advances in the molecular genetics of colorectal neoplasia are greatly facilitated by the features of the disease itself. Its high incidence in the western world has stimulated investigators and thus provided the necessary specimens for studies. Experimental models of colonic tumorigenesis employing chemical carcinogens in rodents were developed and resulted in animal models with important similarities to human colorectal neoplasia. Moreover, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer (Warthin–Lynch syndrome), inherited syndromes that result in or predispose to colorectal neoplasia, were identified and characterized. These syndromes served as human models of colorectal carcinogenesis enabling the identification of important genetic alterations. Finally, epithelial dysplasia, the precursor lesion of colorectal cancer, was morphologically recognized, and evidence strongly suggested that most colorectal carcinomas arise from pre-existing adenomas, thereby establishing the adenoma–carcinoma sequence (Muto *et al.*, 1975). This series of morphologically identifiable subsequent stages during the adenoma–carcinoma sequence thus provided a unique opportunity for investigating the relationships between molecular genetic alterations, alterations in (nuclear) morphology and tumour biology as the technology of molecular biology emerged in the 1980s.

Molecular genetic alterations during the adenoma–carcinoma sequence

An interplay of activated oncogenes, inactivated tumour-suppressor genes and mismatch repair genes underlies aberrant cell growth leading to colorectal cancer. In addition, recent research has drawn attention to the possible role of cell adhesion molecules in tumour progression, particularly as determinants of metastatic spread. Here, we will briefly discuss the role of these different molecules, but particular emphasis is given to the clinical value of the tumour-suppressor gene p53 and the cell adhesion molecule CD44.

Oncogenes

The members of the *ras* gene family were the first identified oncogenes in 1983, and studies of these genes were subsequently carried out in colorectal neoplasms. Activating *ras* gene mutations involving codon 12, 13, or 61 of K-*ras* and N-*ras* were reported in 1987 to be frequent in advanced adenomas and carcinomas, suggesting an important role in the adenoma–carcinoma sequence (Bos *et al.*, 1987). These mutations resulted in the inability of GTP on the active form of the *ras* gene products to be hydrolysed to GDP, thus providing a continuous stimulus via the signal transduction pathway for cell proliferation (Downward, 1992). Of note, the *ras* gene mutations in human colorectal neoplasms are diverse, providing evidence against a single endogenous or exogenous carcinogen producing activation of these oncogenes, in contrast to chemical carcinogen-induced tumours.

Other oncogenes that may also play a role in the development of colorectal cancer are the c-*myc* gene, which encodes a nuclear phosphoprotein involved in the control of DNA synthesis for cell proliferation, and the c-*src* gene, which encodes a protein tyrosine kinase.

Tumour-suppressor genes

These genes, which generally have inhibitory functions in the cell, were first found in the mid 1980s, the prototype being the retinoblastoma gene. The initial clues for the importance of the suppressor genes in colorectal carcinogenesis came from cytogenetics, and they were expanded by allelic deletion analysis of DNA, which demonstrated non-random loss of chromosomal material of particular chromosomes in a favoured sequence. This suggested that the accumulation of these genetic changes plays a key role in the transition from a benign adenoma into an invasive growing carcinoma (Vogelstein *et al.*, 1988; Fearon & Vogelstein, 1990; Baker *et al.*, 1990). Loss of heterozygosity was most frequently observed in chromosomes 5q, 17p and 18q, suggesting the presence of candidate suppressor genes in particular regions of these chromosomes. Studies directed at the identification of these genes led to the finding of the *p53* gene (named for its 53-kDa molecular weight gene product, and discussed in more detail below) on chromosome 17p (Baker *et al.*, 1989; Rodrigues *et al.*, 1990), the *DCC* gene (deleted in colorectal cancer) on chromosome 18q (Fearon *et al.*, 1990; Hedrick *et al.*, 1992; Cho *et al.*, 1994), and the *MCC* gene (mutated in colorectal cancer) and *APC* gene (involved in adenomatous polyposis coli),

both on chromosome 5q (Kinzler *et al*., 1991; Groden *et al*., 1991; Nishiso *et al*., 1991). The *APC* gene is mutated in the germline of patients with the adenomatous polyposis syndrome, but this gene is also a target for somatic mutations in sporadic colorectal cancer (Nishiso *et al*., 1991). The current concept is that the *APC* gene has a gatekeeper function in the colorectum, i.e. this gene can be considered rate limiting for neoplastic growth and the dysplastic phenotype (Jen *et al*., 1994).

The *p53* gene and protein product

The *p53* gene is located on chromosome 17p and encodes a 53-kDa nuclear phosphoprotein initially thought to be a tumour-specific antigen (Crawford *et al*., 1982). The subsequently found cell-immortalizing and -transforming capabilities of p53 suggested a *p53* function as an oncogene (Jenkins *et al*., 1984; Eliyahu *et al*., 1984; Parada *et al*., 1984). However, the allelic deletions of 17p with accompanying mutations of the *p53* gene (Baker *et al*., 1989; Nigro *et al*., 1989; Levine *et al*., 1991), together with the tumour growth-suppressing capacity of the wild-type *p53* gene (Chen *et al*., 1990), ultimately identified the *p53* gene as a tumour-suppressor gene.

After its identification as a tumour-suppressor gene, research focused on the role of the *p53* gene product in tumorigenesis. The *p53* gene product was shown to function as a transcription factor causing arrest of progression through the cell cycle in G_1 in the presence of DNA damage (Mercer *et al*., 1990; Michalovitz *et al*., 1990; Diller *et al*., 1990; Martinez *et al*., 1991); it thereby allows repair of the DNA or leads to apoptosis via the Bax/BCL-2 pathway when this damage is severe. Wild-type p53 fulfils this function by binding to a specific DNA motif, which represses the initiation of DNA replication (Finlay *et al*., 1989; El-Deiry *et al*., 1990; Bargonetti *et al*., 1991; Kern *et al*., 1991; Funk *et al*., 1992). A variety of somatic mutations of the *p53* gene have been found with 'hot spots' in evolutionarily conserved regions (Hollstein *et al*., 1991; Greenblatt *et al*., 1994). In addition to somatic *p53* mutations, *p53* germline mutations are described as well (Li, 1990; Malkin *et al*., 1990; Srivasta *et al*., 1990). Interestingly, in colorectal carcinomas, mutations of the *p53* gene are usually accompanied by loss of the other allele (Baker *et al*., 1989), abolishing all wild-type p53 production. In contrast to wild-type p53, mutant *p53* gene products have no DNA-binding capability, preventing its tumour growth-suppressing function. The loss of this function as 'guardian of the genome' suggests that *p53* mutations leading to a mutated p53 protein play an important role in the development of colorectal and other solid neoplasms.

Indirect support for this hypothesis came from studies in which allelic loss of chromosome 17p as well as *p53* mutations appeared to be associated with adverse prognosis (Kern *et al*., 1989; Offerhaus *et al*., 1992; Hamelin *et al*., 1994). However, the detection of these genetic alterations at the DNA level involves time-consuming procedures and there is no routine technique. In contrast, immunohistochemical detection of cellular and nuclear proteins currently forms a routine technique in most pathology laboratories. In order to find a feasible alternative for the cumbersome detection of *p53* gene mutations, the immunohistochemical detection of mutant *p53* gene product was evaluated as an alternative to *p53* gene analysis.

Immunohistochemical detection of the p53 protein product

Immunohistochemical detection of the wild-type *p53* gene product is scarcely possible because of the extremely short half-life of the protein. In contrast, the mutant *p53* product is characterized by a conformational change of the protein with resultant prolonged half-life and stability (Reich *et al*., 1983; Finlay *et al*., 1988). This prolonged half-life allows detection of the accumulated mutant p53 protein by immunohistochemistry, suggesting that immunostaining of tissue sections might serve as an important surrogate for *p53* gene analysis (Wynford-Thomas, 1992).

Hence, numerous studies have addressed the prognostic value of p53 protein expression not only in tumours of the colorectum, but in other tumours as well.

In these studies, various immunohistochemical procedures for the detection of the *p53* gene were reported with various monoclonal antibodies, e.g. Bp53-12, 240, 1801, DO7, and polyclonal antibodies, e.g. CM1 and Signet (van den Berg *et al*., 1989; Campo *et al*., 1991; Bodner *et al*., 1992; Kerns *et al*., 1992; Kawasaki *et al*., 1992; Mellon *et al*., 1992; Midgley *et al*., 1992; Pignatelli *et al*., 1992; Porter *et al*., 1992; Thor *et al*., 1992; Vojtesek *et al*., 1992; Bartek *et al*., 1993; Westra *et al*., 1993). Antigen retrieval systems, such as target unmasking fluid, were reported to enhance the immunohistochemical signal (van den Berg *et al*., 1993; Nathanson *et al*., 1994; Mulder *et al*., 1995a). These technical varieties may have contributed to the contradictory results that were initially encountered. In order to reach a more uniform procedure for p53 protein detection, Baas *et al*. (1994) attempted to elucidate and optimize the sensitivity and specificity of the immunohistochemical assay for *p53* mutation by staining a series of paraffin-embedded archival colorectal neoplasms with different commercially available anti-p53 antibodies. These results were

compared with the *p53* mutation status in exons 5–9 and the allelic loss status of chromosome 17p. Substantially different results were observed, depending on the antibody and antigen retrieval system used. Differences in the binding sites for the different p53 antibodies and technical pitfalls like masking of the target epitope in the complex microenvironment of the cell nucleus might explain the different results encountered. No complete concordancy between *p53* gene mutations and p53 protein expression was observed with any of the monoclonal antibodies or procedures. Use of monoclonal antibody DO7 with target unmasking fluid yielded a specificity and sensitivity for *p53* mutations of 90% and 67% respectively. Similar results were found independently by others (Dix *et al*., 1994a). This discrepancy between *p53* gene status and p53 protein expression might come from both technical caveats and alternative biological mechanisms influencing p53 stability. As far as 'false-positive' immunohistochemical results, there are several mechanisms other than a missense point mutation that can result in overexpression of the p53 protein (Wynford-Thomas, 1992; Hall & Lane, 1994). Other genes or proteins may interact with p53, stabilizing the wild-type p53 protein and resulting in positive immunostaining. Genotoxic insults such as radiation can produce immunohistochemical detection of wild-type *p53* product in normal cells (Kuerbitz *et al*., 1992; Fritsche *et al*., 1993; Hall *et al*., 1993). Alteration in the promoter region of the gene, resulting in high expression of wild-type p53, has been suggested in a family with breast cancer syndrome (Barnes *et al*., 1992). Finally, mutation outside the most frequently analysed exons 5–9 or intronic mutation might also explain false positivity. In addition, it is important to realize that the unbridled use of antigen retrieval systems may also lead to false positivity (Baas *et al*., 1996). As far as 'false negativity' is concerned, there are several biological mechanisms that could result in the loss of *p53* activity without accumulation of the p53 protein in the nucleus (Wynford-Thomas, 1992). The underlying genetic lesion may be a point mutation to a STOP codon, thus truncating the protein product and making nuclear accumulation and overexpression impossible. The underlying lesion may not be a missense point mutation but a gross deletion, abolishing all p53 protein product, as described in sarcomas (Oliner *et al*., 1992). Some missense point mutations in the *p53* gene may not stabilize the protein sufficiently for accumulation in the nucleus. Finally, intratumoral heterogeneity could also account for some of the discrepancies between immunohistochemical results and molecular genetic analysis. As a first screening modality for the p53 status, it seems that immunohistochemical detection of the mutated p53 protein forms a useful alternative to *p53* gene mutation analysis, but it has to be kept in mind that, under the current conditions, discrepancies between the results of both techniques do remain. Since mutation of p53 is accompanied by allelic loss in colorectal cancer, analysis of loss of heterozygosity (LOH) of 17p combined with immunohistochemistry of p53 provides a more accurate insight into the possible mutational status of p53.

Expression of p53 during colorectal tumour progression

Mutations of *p53*, often accompanied by loss of the wild-type allele, typically occur when an *in situ* neoplasm becomes an invasive malignancy (Baker *et al*., 1989), suggesting that changes in the *p53* gene seem to be critical for the transformation of a colorectal adenoma into an invasive growing carcinoma. Under the assumption that immunohistochemical detection of the p53 protein forms a relatively reliable alternative for the detection of *p53* mutations, this suggests that p53 protein expression will differ markedly during the subsequent stages of colorectal carcinogenesis. Several studies addressing this subject showed that, in normal colonic mucosa, p53 expression is very rare but that, during the subsequent stages of colorectal tumour progression, p53 expression increases markedly; about 25% of the early adenomas, 33% of the late adenomas and up to 70% of the carcinomas exhibit p53 expression (van den Berg *et al*., 1993; Kaklamanis *et al*., 1993; Mulder *et al*., 1995b). Remarkably, these studies also show that, in adenomas, p53 expression increases with the degree of dysplasia, whereas the several stages of carcinomic growth show no significant difference in p53 expression. These results support the hypothesis that *p53* mutation plays an important role in the transition from adenoma into invasive growing carcinoma.

Expression of p53 and prognosis

On the cellular level, the p53 protein is thought to act as a transcription factor regulating cell growth and inhibiting mutagenic damaged cells from entering the S-phase. Mutagenic damage of the *p53* gene leads to loss of this tumour-suppressive function, facilitating tumorigenic growth. Like *p53* mutation (Hamelin *et al*., 1994; Slebos *et al*., 1996), the increase of p53 protein expression during the subsequent stages of colorectal carcinogenesis makes p53 expression a potential marker of colorectal tumour progression and suggests that it is of prognostic importance as well. In carcinomas arising from other solid organs, such as the breast (Barnes *et al*., 1993), stomach (Martin *et al*., 1992; Starzynska *et al*., 1992), lung (Quinlan *et al*., 1992), ovary (Bosari *et al*., 1993)

and pancreas (DiGuiseppe *et al*., 1994), p53 expression has been correlated with shortened survival, but its clinical value in colorectal tumours is still contradictory. Several authors report p53 expression to be of prognostic importance in univariate analysis (Remvikos *et al*., 1992; Starzynska *et al*., 1992; Bosari *et al*., 1994), but only in two studies did p53 expression remain an independent prognosticator after multivariate analysis in which tumour stage was included (Yamaguchi *et al*., 1992; Auvinen *et al*., 1994). Contradictory results have been described by many others (Scott *et al*., 1991; Sun *et al*., 1992; Bell *et al*., 1993; Nathanson *et al*., 1994). Although in most studies cytoplasmic immunostaining is regarded as non-specific, some authors found this cytoplasmic staining with the anti-p53 polyclonal antibody, CM1, of independent prognostic importance (Sun *et al*., 1992; Bosari *et al*., 1994). As mentioned previously, the large number of studies addressing this subject is characterized by the use of different immunohistochemical techniques and antigen retrieval systems, possibly explaining the unequivocal results encountered. In a large, well-documented study population with long-term follow-up using both p53 antibodies, monoclonal antibody DO7 and polyclonal antibody CM1, in combination with target unmasking fluid, we have also analysed the value of p53 expression retrospectively (Mulder *et al*., 1995a). In this study, p53 expression was objectively quantified with a computerized image analysis system (CAS 200). Slightly higher nuclear p53 expression was observed in metastatic Dukes' C carcinomas compared with non-metastatic Dukes' B carcinomas, but no marked difference from other reports addressing p53 expression in non-metastatic and metastatic tumours was observed (Campo *et al*., 1991; Purdie *et al*., 1991; Scott *et al*., 1991; Remvikos *et al*., 1992; Starzynska *et al*., 1992; Sun *et al*., 1992; Angelis *et al*., 1993; Bell *et al*., 1993; Auvinen *et al*., 1994; Bosari *et al*., 1994; Mulder *et al*., 1995a). Interestingly, in this study, patients with tumours exhibiting intermediate p53 expression had better prognosis than those lacking p53 expression or showing abundant p53 positivity. Although this difference did not reach significance, similar results have been described by others (Dix *et al*., 1994b). This suggests that the extent of p53 expression, rather than p53 stabilization only, might form a reflection of its true biological activity. This might also shed further light on the contradictory results arising from previous follow-up studies in which, in the majority of cases, p53 expression was divided into negative versus positive only. However, we have to conclude that, with current methodology, the clinical significance of p53 expression remains unclear and as yet forms no useful contribution to the prediction of long-term prognosis after resection of colorectal carcinoma.

Mismatch repair genes

Hereditary non-polyposis colorectal cancer syndrome (Warthin–Lynch syndrome) is characterized in pedigrees by autosomal dominant inheritance of colorectal cancer with relatively young age of onset and increased frequency of location in the proximal large bowel compared with sporadic colorectal carcinomas (Warthin, 1913). Recently, four genes that may be responsible for hereditary non-polyposis colorectal cancer in the majority of kindreds have been isolated (*hMSH2*, *hMLH1*, both on chromosome 2p, and *hPMS1* and *hPMS2*, both on chromosome 3p) (Fishel *et al*., 1993; Leach *et al*., 1993; Bronner *et al*., 1994; Papadopoulos *et al*., 1994). These genes are human homologues of the bacterial *mutHLS* complex, which is involved in genetic 'proofreading' (the repair of mismatched basepairs in DNA). Loss of this function is thought to allow basepair mismatches to accumulate, resulting in a replication error phenotype, which is observed in a high percentage of patients with hereditary non-polyposis colorectal cancer and approximately 15% of cases of sporadic colon cancer. Owing to the mutator phenotype, cells enter into a rapidly evolving cascade of genetic changes leading to cancer, and typically this type of colorectal cancer arises in a relatively short period of time.

Cell adhesion molecules

The above outlined cascade of genetic alterations leads to aberrant cell growth characteristics for neoplastic tumours. Metastatic spread is another feature that defines colorectal cancer. Recent research suggests that cell adhesion molecules may be of great importance in this process of metastatic spread. Attention was drawn to their role in this process by the similarity in biological behaviour of activated lymphocytes and metastatic tumour cells. To fulfil their immunosurveillance function, activated lymphocytes continuously migrate through the body. They travel through the blood, extravasate into the lymphoid tissues, then drain into the lymphatics and finally re-enter the blood via the thoracic duct. Like activated lymphocytes, metastatic tumour cells also exhibit such a process of trafficking through the body. In this process, the interaction with endothelial cells and extracellular matrix molecules is thought to be regulated by adhesion molecules. On the basis of these structures, these molecules are divided into separate families, i.e. the integrins, the immunoglobulin superfamily, the selectins, the cadherins and cluster of differentiation 44 (CD44). CD44 is often expressed in the form of various splice variants (Screaton *et al*., 1992, 1993; Tölg *et al*., 1993). Interestingly, in rat carcinoma cell lines, exon v6-containing CD44 splice variants were shown to be

causal in the acquisition of metastatic potential, thus providing additional support for the hypothesis that adhesion molecules are of importance in metastatic spread (Günthert *et al*., 1991; Seiter *et al*., 1993). In the following section, the potential role of the CD44 gene and its protein product in the pathology of colorectal carcinoma will be discussed in more detail.

The CD44 gene and its protein products

Glycoproteins of the CD44 family are encoded by a single gene on chromosome 11p13 that consists of 19 exons (Jalkanen *et al*., 1988; Stamenkovic *et al*., 1989; Aruffo *et al*., 1990; Brown *et al*., 1991; Dougherty *et al*., 1991; Günthert *et al*., 1991; Screaton *et al*., 1992; Heider *et al*., 1993; Lesley *et al*., 1993; Fox *et al*., 1994). All CD44 family members share the N-terminal (extracellular) and C-terminal (transmembrane, cytoplasmic) domains encoded by exons 1–5 and exons 15–19 respectively. However, they differ in the extracellular membrane proximal part as a result of extensive alternative splicing of exons 6–14 (also referred to as exons v2–v10) (Dougherty *et al*., 1991; Günthert *et al*., 1991; Screaton *et al*., 1992; Tölg *et al*., 1993). In this way, more than a thousand CD44 variants (CD44v) can potentially be generated. Further diversity of CD44 results from post-translational modifications with *N*-and *O*-linked sugar chains and with glycosaminoglycan side-chains (Jalkanen *et al*., 1988; Stamenkovic *et al*., 1989; Brown *et al*., 1991; Screaton *et al*., 1992). The expression of CD44 isoforms is tissue specific, for example CD44s, which lacks v2–v10, is the most common form on haematopoietic cells, while larger CD44 splice variants dominate on several normal and on neoplastic epithelia (Jalkanen *et al*., 1988; Stamenkovic *et al*., 1989; Aruffo *et al*., 1990; Brown *et al*., 1991; Dougherty *et al*., 1991; Günthert *et al*., 1991; Screaton *et al*., 1992; Heider *et al*., 1993; Lesley *et al*., 1993; Fox *et al*., 1994) and are also found on activated lymphocytes and malignant lymphomas (Arch *et al*., 1992; Koopman *et al*., 1993; Stauder *et al*., 1995).

CD44 was found to be involved in a number of important biological processes, including haematopoiesis, lymphocyte homing and activation and tumour metastasis. During these processes, CD44 mediates cell binding to extracellular matrix components, most importantly to hyaluronic acid (Jalkanen *et al*., 1986; Haynes *et al*., 1989; Shimizu *et al*., 1989; Koopman *et al*., 1990; Miyake *et al*., 1990; Günthert *et al*., 1991; Faassen *et al*., 1992; Sy *et al*., 1992; Lesley *et al*., 1993; Cooper & Dougherty, 1995). The binding site for hyaluronic acid is located on the N-terminal part of CD44. Although all isoforms bear this binding site, hyaluronic acid binding was shown to be modulated by both alternative splicing and post-translational modification (Bennet *et al*., 1995; Katoh *et al*., 1995; van der Voort *et al*., 1995). Besides hyaluronic acid, CD44 also binds fibronectin, serglycin, invariant chain and osteopontin (Lesley *et al*., 1993; Toyama-Sorimachi *et al*., 1995; Weber *et al*., 1996).

Initial evidence for a role of CD44 in cancer comes from studies of human non-Hodgkin's lymphomas. In these tumours, the expression of CD44 was associated with tumour dissemination and unfavourable prognosis (Pals *et al*., 1989; Horst *et al*., 1990; Jalkanen *et al*., 1990, 1991). Subsequently, experimental studies were reported in which the CD44 standard form was found to promote tumour growth and metastasis through interaction with hyaluronate (Sy *et al*., 1992; Lesley *et al*., 1993), and specific CD44 splice variants were shown to confer metastatic potential to a non-metastatic rat adenocarcinoma cell line (Günthert *et al*., 1991). These findings prompted studies of CD44 expression in a number of human malignancies, including non-Hodgkin's lymphomas, colorectal cancer, breast cancer, cervical and bladder cancer. In most of these studies, increased levels of CD44 and/or different patterns of splice variants were found in tumours in comparison with their normal counterparts (Matsumara & Tarin, 1992; Heider *et al*., 1993; Herrlich *et al*., 1993; Lesley *et al*., 1993; Koopman *et al*., 1993; Tanabe *et al*., 1993; Wielenga *et al*., 1993; Dall *et al*., 1994; Finn *et al*., 1994; Kim *et al*., 1994; Mulder *et al*., 1994; Kauffmann *et al*., 1995; Cooper & Dougherty, 1995; Orzechowski *et al*., 1995; Rodriguez *et al*., 1995; Stauder *et al*., 1995; Imazeki *et al*., 1996).

In the following paragraph, a detailed description of the expression of CD44 splice variants during the subsequent stages of the adenoma–carcinoma sequence of colorectal cancer is given.

Expression of CD44 isoforms during colorectal tumour progression

Different techniques have been used by various groups to analyse CD44 expression. In general, CD44 expression has been examined either at the mRNA level or at the protein level. For the assessment of mRNA levels and CD44 exon composition, reversed transcriptase–polymerase chain reaction (RT–PCR) and, in a few studies, mRNA *in situ* hybridization and Northern blot analysis were used. For CD44 protein detection, immunohistochemistry was performed on frozen or paraformaldehyde-fixed tissue sections, with a variety of monoclonal and polyclonal antibodies that differ in exon specificity and affinity. Furthermore, in some studies, Western blot analysis was used.

In studies that (only) used RT–PCR to assess

CD44 expression at the mRNA level, more and higher molecular weight CD44 isoforms were consistently found in tumours than in the normal mucosa. Along the adenoma–carcinoma sequence, the most prominent up-regulation was found at the transition from normal to adenoma (Matsumara *et al*., 1992; Heider *et al*. 1993; Tanabe *et al*., 1993; Finn *et al*., 1994; Rodriguez *et al*., 1995; Yoshida *et al*., 1995; Higashikawa *et al*., 1996; Imazeki *et al*., 1996). With the exception of the study by Rodriguez *et al*. (1995), no correlation was found with this technique between CD44 expression and tumour parameters, including tumour grade and Dukes' stage. This lack of correlation might be explained by the fact that RT–PCR, although highly sensitive, is not quantitative.

At the protein level, the normal colorectal mucosa expresses small amounts of CD44, as shown by immunohistochemistry. The cells of the villi are negative, whereas the crypt cells show weak and focal expression of CD44v and moderate expression with monoclonal antibodies that recognize the standard part of CD44 (Abbasi *et al*., 1993; Heider *et al*., 1993, 1995; Fox *et al*., 1994; P. A. Jackson *et al*., 1995; Gorham *et al*., 1996; Gotley *et al*., 1996). At later stages of the adenoma–carcinoma sequence, an increased expression of CD44v4 and especially CD44v6 is found (Wielenga *et al*., 1993; Mulder *et al*., 1994).

In the majority of colorectal tumours, the expression of CD44 is enhanced in comparison with normal mucosa, although there is inter- and intratumour heterogeneity. Along the adenoma–carcinoma sequence, the major up-regulation of CD44 occurs at the transition from normal mucosa to adenoma (Abbasi *et al*., 1993; Wielenga *et al*., 1993; Fox *et al*., 1994; Kim *et al*., 1994; Mulder *et al*., 1994; P. A. Jackson *et al*., 1995; Gorham *et al*., 1996; Gotley *et al*., 1996; Yamaguchi *et al*., 1996). Kim *et al*. (1994) reported that this up-regulation of CD44 in colorectal tumours occurs before K-*ras* and p53 mutations. These immunohistochemical findings reflect regulation of CD44 expression at transcriptional level as was shown by RNA *in situ* hybridization (Orzechowski *et al*., 1995; Gorham *et al*., 1996).

The results of studies addressing the relationship between CD44 expression at the protein level and tumour parameters, such as tumour grade and Dukes' stage, have not been uniform. We have described CD44 variant expression on colorectal tumours as being correlated with Dukes' stage (Wielenga *et al*., 1993). This finding was recently confirmed by others (Yamaguchi *et al*., 1996). However, a study by Gotley *et al*. (1996) did not find this correlation. This discrepancy might reflect technical differences, e.g. the use of different monoclonal antibodies that differ in their specificity and affinity.

CD44 as a prognostic marker in colorectal carcinomas

As a consequence of studies in rats showing that CD44v6 can confer metastatic potential to rat carcinoma cell lines (Günthert *et al*., 1991), studies addressing the prognostic and biological significance of CD44 variant expression in human cancer have largely been focused on CD44v6. As described above, CD44 variants that contain exon v6 were indeed found to be up-regulated in colorectal cancer, and their expression was found to correlate with tumour stage (Wielenga *et al*., 1993; Finn *et al*., 1994; Fox *et al*., 1994; Mulder *et al*., 1994; Orzechowski *et al*., 1995; Rodriguez *et al*., 1995; Yoshida *et al*., 1995; Gorham *et al*., 1996; Gotley *et al*., 1996; Higashikawa *et al*., 1996; Imazeki *et al*., 1996). As for prognosis, it was shown that expression of CD44v6 correlates with poor survival and is an independent prognosticator in patients who underwent radical surgery (Dukes' B and C). Hence, it identifies individuals with a high propensity to develop metastases. These patients might benefit from adjuvant therapy (Mulder *et al*., 1994; V. J. M. Wielenga *et al*., unpublished results). In addition to CD44v6, expression of other variant exons also correlates with unfavourable prognosis: Yamaguchi *et al*. (1996) reported that CD44v8–10 was an independent unfavourable prognosticator, and we have found that CD44v3 expression is also related to unfavourable disease outcome (V. J. M. Wielenga *et al*., unpublished results). This overexpression of CD44v3 is of potential biological interest, since isoforms containing v3 can be modified by heparan sulphate side-chains, which can bind heparin-binding growth factors (D. Jackson *et al*., 1995; Bennet *et al*., 1995). By presenting these growth factors to their high-affinity receptors, CD44v3 might promote the growth and motility of tumour cells (Ruoslahti & Yamaguchi, 1991; Yayon *et al*., 1991; Schlessinger *et al*., 1995).

Conclusion

This article was written in an attempt to give a brief overview of the clinical significance of two important molecular events underlying colorectal tumour progression: mutation and expression of the *p53* tumour-suppressor gene and overexpression of the cell adhesion molecule CD44. It seems that, despite its up-regulation during the various stages of colorectal tumour progression, p53 expression cannot prove its initially suggested function as an independent marker for prognosis after resection of colorectal carcinoma.

In contrast, CD44 splice variants are up-regulated during colorectal tumorigenesis. In follow-up studies, it was shown that CD44 is a marker for prognosis that is independent of other known

prognosticators. This suggests that, in the future, assessment of CD44 expression may guide the clinician in delineating a subset of patients with biologically unfavourable tumours that may profit from post-operative adjuvant therapy.

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