Expression of mutant p53 protein and CD44 variant proteins in colorectal tumorigenesis

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Abstract

Colorectal tumorigenesis evolves through a series of molecular genetic changes, providing putative markers for tumour progression. This study investigated the relation between expression of the tumour suppressor gene p53 and splice variants v5 and v6 of the cell adhesion molecule CD44 by immunohistochemistry on tissue samples of early adenomas (n=12), late adenomas (n=12), Dukes's A and B carcinomas (n=21), and Dukes's C and D carcinomas (n=22) and compared these results with expression of these proteins in normal colonic mucosa (n=17). A statistically significant trend of increasing expression was seen for both p53 ($p<0.005$) and CD44 variant exon v6 ($p<0.0005$) in subsequent stages of this tumour progression model. High expression of CD44 v5 was seen in most colorectal neoplasms (83%–96%), independent of stage. A statistically significant correlation was present between p53 expression and expression of variant v6 of CD44 ($p<0.01$). Both p53 expression and CD44 v6 expression in adenomas increased with the degree of dysplasia ($p<0.05$). The results of this study show that mutant p53 protein and variant v6 of the CD44 glycoprotein are markers of tumour progression in colorectal cancer.

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Colorectal cancer is common in the Western world and represents the second leading cause of cancer related death.¹ The molecular genetics of colorectal cancer are the best understood of any solid neoplasm. This is because colorectal cancer evolves through a series of morphologically recognisable stages known as the adenoma-carcinoma sequence.² Morphologic markers of tumour progression in the adenoma-carcinoma sequence are increase in size of the adenomas, degree of dysplasia, and architectural distortion with extension of the villous component.^{3,4} During tumour progression there is an accumulation of genetic changes in a preferential order in which specific oncogenes and tumour suppressor genes take part.^{5–10}

One of the most common genetic changes encountered in colorectal cancer concerns chromosome 17p where the p53 tumour suppressor gene is located. Mutations of p53, often accompanied by loss of the wild type

allele, typically occur when an in situ neoplasm becomes an invasive malignancy.⁶ Thus changes in p53 seem critical for the transformation of an adenoma into an invasive colorectal carcinoma.⁸ Previous studies have shown that allelic loss of 17p, indicative of p53 mutation, is associated with a higher likelihood of distant metastasis, poor prognosis, and DNA aneuploidy.¹¹ The mutant p53 gene product has a prolonged half life and is therefore detectable by immunohistochemistry. Immunohistochemistry seems to be a valid test for p53 mutation in colorectal cancer.¹²

Another molecule that is expressed in the colon and may influence prognosis is CD44.^{13–15} CD44 is a cell adhesion molecule often expressed in the form of various splice variants, some of which have a role in tumour metastasis.¹⁶ CD44 variant glycoproteins with sequences encoded by exon v6 confer full metastatic potential to rat carcinoma and sarcoma cell lines, which can be blocked by anti-v6 monoclonal antibodies.^{16,17} In colorectal carcinomas CD44 proteins, homologous to those with metastatic potential in the animal, are overexpressed during tumour progression.^{13,15}

Molecular markers like mutant p53 and CD44 variant proteins could be of clinical value in colorectal cancer. In particular, such markers may help to predict the biological behaviour of tumours and the risk of recurrence or metastasis after apparently radical surgery; this would delineate a subset of patients who would potentially benefit from adjuvant chemotherapy and thus improve prognosis in colorectal cancer.¹⁸

This study investigates the relation between p53 and CD44 during colorectal tumorigenesis and the potential role of these two molecules as prognostic markers in colorectal cancer.

Methods

TUMOUR SAMPLES

The study comprised 84 fresh frozen tissue samples collected at the Department of Pathology, Academic Medical Center, University of Amsterdam, The Netherlands. Seventeen samples consisted of normal colon mucosa, 24 samples were adenomas, and 43 samples were colorectal carcinomas.

Adenomas were subdivided into early adenomas (diameter <1 cm, n=12) and late adenomas (diameter >1 cm, n=12), and the degree of dysplasia was graded as low (n=13),

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Figure 1: (A) Colorectal carcinoma exhibiting p53 protein expression. Expression of p53 protein is restricted to the nucleus; (B) colorectal carcinoma exhibiting CD44 variant exon v6 expression. Expression of glycoprotein CD44 variants is confined to the membrane of the cell.

moderate (n=6), and severe (n=5) according to the criteria of the US national polyp study.⁴ Eleven of the polyps were tubular adenomas, 11 were tubulovillous adenomas, and two were villous adenomas.

Stage of the carcinomas was determined according to the original Dukes's classification¹⁹ in Dukes's A (n=4, disease confined to the bowel wall), Dukes's B (n=17, extension through the muscularis propria into the pericolic fat without metastasis), and Dukes's C/D (n=22, tumours with regional or distant metastasis, or both).

Nineteen tumours came from the proximal colon (caecum through flexura lienalis) and 42 tumours were resected from the left colorectum (colon descendens through rectum). Six adenomas came from familial adenomatous polyposis patients; their exact location was unknown. Normal mucosa came from various locations of the colon.

MATERIALS

Polyclonal rabbit IgG antibody CM1 was obtained from Novacastra Laboratories (Newcastle upon Tyne, UK). The monoclonal antibodies (mAbs) VFF4, VFF7, and VFF8²⁰ were obtained from Bender Co (Vienna, Austria). Normal goat serum, bovine serum albumin, human AB-serum, biotinylated rabbit antimouse IgG, biotinylated swine antirabbit IgG, and avidin-biotinylated horseradish-peroxidase complex (ABC-HRP) were from Dakopatts (Glostrup, Denmark). 3-Amino-9-ethylcarbazole (AEC) was obtained from Sigma (St Louis, USA).

DETECTION OF p53 PROTEIN

Nuclear overexpression of p53 protein was analysed by immunohistochemistry as described previously,^{21 22} using the polyclonal rabbit IgG antibody CM1, diluted 1:1000 in phosphate buffered saline.

DETECTION OF CD44 VARIANTS

Presence of CD44 splice variants was analysed by immunohistochemistry as described previously,¹³ using the monoclonal antibodies (mAbs) VFF8 against exon v5 and VFF4 and VFF7 against exon v6 of the human CD44. A detailed description of these antibodies and their specificity has been published elsewhere.²⁰ Antibodies were diluted 1:1000 in PBS-BSA.

STATISTICAL ANALYSIS

Statistical analysis was performed with a STATA software package (Computing Resource Center, Santa Monica, CA). Expression of the various antibodies was quantitatively ranked as follows: positive staining in less than 1% of the tumour cells was considered 'no expression', positivity in 1% to 30% of the cells 'moderate expression', and positivity in more than 30% of the cells 'high expression'. Subdivision in these three categories was based on a previous study, in which the immunohistochemistry was quantified using computerised image analysis.¹² For this study the immunohistochemistry was assessed by two experienced observers, who were blinded for other variables. When immunohistochemistry results of both antibodies against v6 were not identical, the antibody with the highest expression was used for analysis.

Comparison between groups and trends across groups were tested for significance with the χ^2 test.

Results

Immunohistochemical staining was performed with an antibody against p53 and mAbs against CD44 variant exon sequences v5 and v6 on all 84 specimens and controls. Expression of the p53 protein occurred typically in the nucleus, whereas the membrane glycoprotein CD44 variants were expressed on the cell membrane (Fig 1). Figures 2 and 3 summarise the results of the immunohistochemical procedures.

EXPRESSION OF p53 PROTEIN

Figure 2 shows that expression of stabilised p53 protein significantly increased during the various stages of tumour progression. Normal colonic mucosa did not show increased expression of p53 protein; of the early adenomas 25% were positive, 33% of the late adenomas, 71% of the Dukes's A and B and 54% of the Dukes's C and D carcinomas were positive (χ^2 test for trend, $p<0.005$, $df=4$).

In the adenomas, expression of p53 protein increased significantly with the grade of dysplasia (χ^2 test for trend, $p=0.05$, $df=2$) (Fig 3). Low grade adenomas showed 8% positivity, whereas intermediate grade and high grade adenomas showed 50% and 60% positivity respectively.

p53 Expression was not related to tumour site, either in adenomas or in carcinomas. No correlation was seen between architecture of the adenomas and p53 overexpression.

Figure 2: Expression of mutant p53 protein and CD44 variant proteins v5 and v6 in colorectal tumour progression. Black bars show high expression: >30% of the tumour cells positive. White bars show moderate expression: 1–30% of the tumour cells positive.

EXPRESSION OF CD44 SPLICE VARIANT EXON v5

Expression of variant v5 was detected in 12% of the normal mucosa samples. In early and late adenomas 83% of the specimens stained with antibodies to exon v5 sequence. In carcinomas, 95% of the Dukes's A and B tumours and 96% of the Dukes's C and D tumours showed v5 positivity (Fig 2). Thus, expression of v5 was seen in almost all colorectal neoplasms independent of stage. Exon v5 expression was not correlated to tumour site. In the adenomas no correlation was found between v5 expression and grade of dysplasia or architecture.

EXPRESSION OF CD44 SPLICE VARIANT EXON v6

The two different antibodies against exon v6 showed almost identical results by immunohistochemistry ($p < 0.005$). Immunostaining of exon v6 was not seen in normal colonic mucosa; in early and late adenomas CD44 v6 was found in 17% and 25% respectively; v6 staining was present in 67% of the Dukes's A and B carcinomas and in 82% of the Dukes's C and D carcinomas (χ^2 test for trend, $p < 0.0005$, $df = 4$) (Fig 2). The percentage of positive cells within a tumour also increased during tumour progression – that is, in adenomas only moderate expression (1–30% of the tumour cells positive) of v6 was found, whereas high expression (>30% of the tumour cells positive) of v6 was present in 14% of the Dukes's A and B carcinomas and in 36% of the Dukes's C and D carcinomas. Exon v6 expression was not related to tumour site, either in adenomas or in carcinomas.

In the adenomas a significant correlation was found between v6 expression and the grade of dysplasia (χ^2 test for trend, $p < 0.05$, $df = 2$). Low grade dysplastic adenomas were v6 negative, whereas in adenomas with moderate and severe dysplasia positive staining was seen in 33% and 40% respectively (Fig 3).

COEXPRESSION OF p53 AND CD44 VARIANT PROTEINS

The increase in expression of mutant p53 protein during tumour progression was significantly correlated with expression of CD44 variant v6 ($p < 0.01$) – that is, most tumours expressed both mutant p53 and CD44 simultaneously. No specific pattern in expression of the different antibodies was seen. In particular, expression of CD44 variants was not more noticeable at the infiltrating edge of tumours.

Discussion

The prognosis after resection of colorectal carcinoma depends on the presence or absence of residual tumour cells in the patient. In patients with seemingly curative resection, occult metastatic disease often accounts for tumour related mortality. Currently, the prediction of outcome is based mainly on the stage of colorectal carcinoma at the time of resection. Although several clinically useful staging classifications are available,²³ a more specific recognition of colorectal carcinomas with biological competence or propensity to metastasise would provide a rationale to adjust different therapeutic approaches, for example, potentially effective adjuvant chemotherapy and radiotherapy, to individual patients and improve the outcome.¹⁸

The results of this study show that expression of the tumour suppressor gene p53 and splice variant v6 of the adhesion molecule CD44 are potentially useful biomarkers to predict tumour behaviour of colorectal carcinomas. The increase of p53 expression during tumour progression suggests that a more aggressive tumour biology is accompanied by changes in the p53 tumour suppressor gene. Other studies also found that increased p53 expression delineates a subset of biologically unfavourable neoplasms.^{24–26} The precise prognostic value of p53 expression remains nevertheless to be elucidated and so far follow

Figure 3: Expression of mutant p53 protein and CD44 variant protein v6 in various grades of dysplasia of colorectal adenomas. Black bars show high expression: >30% of the tumour cells positive. White bars show moderate expression: 1–30% of the tumour cells positive.

up studies have given equivocal results.²⁷⁻²⁹ Interestingly, one recently published study reported poorer prognosis in colorectal tumours that had positivity for p53 in the cytoplasm,³⁰ but in our study no specific cytoplasmic staining of p53 was seen. Future longitudinal studies should not only look at p53 expression, but at the same time consider underlying mutations and allelic loss of the p53 tumour suppressor gene.¹²⁻³¹ Such studies would establish the mutual relation between changes at the DNA level and protein expression and the independent prognostic value of each of these parameters. The finding of an increase in p53 expression in increasingly dysplastic adenomas in this study as well as others,²⁴⁻²⁵ supports the concept of p53 expression as an indicator of tumour aggressiveness.

Expression of the cell adhesion molecule CD44 has been recently described on colonic epithelium¹³ and a differential expression of splice variants seems to occur during neoplastic transformation.¹⁵ This study suggests that exon v5 expression occurs early during colorectal tumour progression whereas v6 expression coincides with changes in the p53 tumour suppressor gene. Interestingly expression of exon v6 of CD44 is accompanied by metastatic potential in rat carcinoma and sarcoma cell lines.¹⁶ Also, in longitudinal studies of other organ systems CD44 expression influences prognosis³²⁻³⁵ and follow up studies for colorectal carcinomas are underway. The simultaneous occurrence of changes in the p53 tumour suppressor gene and expression of exon v6 of CD44 is intriguing. It is conceivable that autonomous cell turnover and migration are the main prerequisites for malignant – that is, invasive growth. It could be speculated that acquisition of the CD44 v6 phenotype only leads to significant growth advantage when ultimate control by p53 is lost resulting from its mutated status.³⁶⁻³⁸ This would be in agreement with the postulated role of p53 as a guardian of the genome,³⁹ and other recent work that shows how inactive p53 may lead to unregulated tumour growth.⁴⁰

In summary, this study shows that expression of the p53 gene product and CD44 variant exon v6 are correlated with tumour progression and therefore may be important markers for delineating subsets of tumours with more aggressive biology. Follow up studies, with special emphasis on 'good' Dukes's C and 'bad' Dukes's B tumours, will be needed to elucidate the potential prognostic value of expression of p53 protein and CD44 variants. Such studies should also look at the mutation status of the p53 gene and allelic loss of chromosome 17p.

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