Expression of Cell Cycle Control Proteins in Primary Colorectal Tumors Does Not Always Predict Expression in Lymph Node Metastases[1]

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ABSTRACT

Analysis of tumor markers focuses on expression in primary tumors with the assumption that this is representative of metastatic tumor, against which treatment is targeted. Few studies have compared the expression of such markers in primary and secondary tumors. In this study, several key genes involved in cell cycle regulation were investigated in colorectal tumors and corresponding lymph node metastases. The cell cycle regulators p53, cyclin D1, p21, p27, retinoblastoma protein (Rb), and proliferating cell nuclear antigen (PCNA) were examined in a series of 42 paired samples of primary colorectal and secondary lymph node tumors by immunohistochemistry. Expression of p53, p27, and Rb was similar in virtually all paired samples (p53, 38 of 42; p27, 39 of 42; Rb, 40 of 42), indicating that the pattern of these proteins in colorectal tumors may be used to predict that in lymph node tumors. It also suggests a lack of direct involvement in the metastatic process. A lower concordance for p21 and cyclin D1 staining was observed between primary and secondary tumors (p21, 19 of 42; cyclin D1, 22 of 42). p21 expression was more often observed in primary colorectal cancers, whereas cyclin D1 expression was more frequently seen in lymph node metastases, in keeping with the contrasting roles of these proteins as a cell cycle inhibitor (p21) and activator (cyclin D1). The PCNA-labeling index was found to vary considerably in a number of cases, thus limiting the ability to predict expression of this protein in lymph node metastases from the primary tumor. In addition, PCNA-labeling indices between paired samples were neither consistently higher nor lower, suggesting that the proliferative capacity of tumor cells is not directly related to their ability to metastasize.

INTRODUCTION

Colorectal cancer is a leading cause of cancer death in the Western world, with ~125,000 mortalities due to this disease each year in Europe (1). Despite surgical and therapeutic advances, the mortality rate has remained relatively constant for the past 40 years (2). The natural history of colorectal cancer has been widely studied, and a model was established by Fearon and Vogelstein (3) proposing a pathway of genetic mutations arising in the sporadic disease. This model suggests that not only is an accumulation of mutations required for progression from dysplasia to carcinoma, but also that there may be an ordering of events. It is now recognized that alternative genetic pathways from those initially proposed are also in existence (4), and more may yet be implicated.

Aberrations occurring in cancer frequently involve components of the cell cycle machinery (5), and it has been proposed that this may be a prerequisite for tumorigenesis. The checkpoint occurring late in G_1 is known as the restriction point, beyond which the cell no longer requires growth factors to enter the S phase and is committed to complete the division cycle. Cell cycle checkpoints are carefully controlled by complex interactions involving cyclins, CDKs,[3] their activators, and inhibitors. The major driving force of the phase transition from the G_1 to S phase is provided by complexes of D-type cyclins and CDKs 4 and 6, the activity of which is stringently regulated (6). Inhibition of CDK/cyclin D complexes is effected by the CIP (p21, p27 and p57) and INK (p15, p16, p18 and p19) protein families (7). Active CDK/cyclin D complexes act to hyperphosphorylate Rb, which in turn derepresses E2F-responsive promoters allowing transcription of genes essential for progression through the S phase (5). Overexpression of components of the G_1-S checkpoint also appears to influence sensitivity to some chemotherapeutic agents (8, 9).

In addition to cyclins, CDKs, and their activators/inhibitors and substrates, the G_1-S phase checkpoint is also influenced by p53. In response to cellular signals, p53 is activated and, through its action as a transcription factor, switches on the transcription

Received 7/8/99; revised 11/15/99; accepted 11/26/99.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported by a University of Aberdeen Development Trust Colorectal Cancer Initiative Grant and by the Aberdeen Colorectal Initiative (Steering Committee: Jim Cassidy, Howard L. McLeod, Graeme T. Murray, Neva Haites, Julian Little, and William T. Melvin).
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[3] The abbreviations used are: CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; PCNA, proliferating cell nuclear antigen; PCNA-LI, PCNA-labeling index.

Table 1 Monoclonal antibodies employed in immunohistochemical analysis

Protein	Clone	Supplier	Dilution	Positive control tissue
p53	DO-7	Dako A/S (Glostrup, Denmark)	1/200	Colorectal tumor
Cyclin D1	P2D11F11	Novocastra (Peterborough, United Kingdom)	1/10	Tonsil
p21	EA10	Oncogene Research Products (Cambridge, MA)	1/40	Breast tumor
p27	1B4	Novocastra	1/40	Tonsil
Rb	Rb1	Dako A/S	1/50	Tonsil
PCNA	PC10	Dako A/S	1/50	Normal colon

of a number of genes that can regulate the cell cycle, in particular the CDK inhibitor p21 (10).

PCNA is an auxiliary factor essential for DNA polymerases δ activity, which is required for both replication and repair of DNA (11). In addition, PCNA can exist in a quaternary complex with CDK/cyclin/p21 (12). PCNA is expressed in cycling cells and is frequently used as a measure of the proliferative activity of tissues (13).

Although several studies have implicated factors involved in the cell cycle in the development of colorectal cancer, few have examined their expression in metastatic deposits. Because therapy is targeted toward metastatic disease, the assumption is made that expression of tumor markers in secondary lesions reflects the situation observed in the primary tumor. To investigate this supposition, the present study has evaluated the expression profile of key G_1-S transition proteins (p53, cyclin D1, p21, p27, Rb, and PCNA) in primary colorectal tumors and their corresponding lymph node metastases to establish any patterns of expression in this disease and its progression.

MATERIALS AND METHODS

Tumor Samples. Forty-two samples of primary colorectal cancer and their corresponding lymph node metastases were randomly selected from the 1996–1998 archive in the Pathology Department at the University of Aberdeen, United Kingdom. There were 26 male and 16 female patients, with an age range of 44–88 years (median, 64.5 years). Forty of the tumors were Dukes' stage C, 2 were Dukes' stage D, 38 were moderately differentiated tumors, and 4 poorly differentiated tumors. Fourteen of the primary tumors were situated in the proximal colon, 13 were in the distal colon, and 15 were in the rectum. Tissue samples had been fixed in 10% neutral buffered formalin for 24 h before being routinely processed to paraffin wax.

Immunohistochemistry. Expression of PCNA, p53, cyclin D1, p21, p27, and Rb proteins was investigated in primary colorectal tumors and their corresponding lymph node metastases by immunohistochemistry using an avidin-biotin-peroxidase development system (14). Briefly, 5-μm paraffin-embedded sections prepared on 3-aminopropyltriethoxysilane-coated slides were dewaxed and rehydrated, and endogenous peroxidase activity was blocked by incubation with H_2O_2/methanol. Following antigen retrieval, which was achieved by microwaving in 10 mM citrate buffer (pH 6.0)— or in 1 mM EDTA (pH 8.0), for cyclin D1 sections—for 20 min (no antigen retrieval was carried out for PCNA), endogenous biotin was blocked using a biotin blocking kit (Vector Laboratories Ltd., Peterborough, United Kingdom). Slides were then sequentially incubated with primary antibody (for dilutions and sources of primary antibodies, see Table 1), biotinylated rabbit antimouse immunoglobulin (Dako Ltd.), and streptavidin/biotin/horseradish peroxidase complex (sABComplex, Dako Ltd.). Sites of bound peroxidase were visualized using diaminobenzidine (Sigma Chemical Co. Ltd., Poole, Dorset, United Kingdom) and enhanced by incubation with $CuSO_4$ in saline before sections were counterstained with Mayer's hematoxylin and mounted. Control slides included in each experiment consisted of tissue previously shown to express the factor of interest as positive controls (see Table 1), whereas primary antibody was replaced by Tris-buffered saline in the case of negative controls.

Scoring Systems. Slides were examined by two independent investigators with no prior knowledge of clinical information, and in the case of discrepancies, the case was reviewed and a consensus score was agreed upon. Immunostaining for p53, cyclin D1, p21, p27, and Rb was graded according to the percentage of positive tumor cells [<5% (negative), 5–25% (weak positive), >25% (strong positive)]. PCNA-LI was calculated as the percentage of positive cells following the evaluation of >500 cells in greater than or equal to three high-power fields.

Statistical Analysis. Associations between expression of cell cycle proteins in paired primary and secondary tumor samples were examined with the kappa (κ) test (15), and PCNA-LIs were compared using the Wilcoxon test. The relationship of differences in cyclin D1 and p21 expression between primary and secondary tumors was investigated using the χ^2 test. All statistical analyses were carried out using SPSS for Windows 95 version 7.0 (SPSS UK Ltd., Woking, Surrey, United Kingdom), and the significance level was set at $P < 0.05$.

RESULTS

Immunohistochemical analysis of p53, cyclin D1, p21, p27, Rb, and PCNA was carried out on 42 paired samples of primary colorectal cancer and lymph node metastases. Levels of expression of each protein in these tumors are detailed in Table 2. Although the frequency of expression of several factors (*i.e.*, p53, p27, Rb, PCNA) was similar in primary colorectal and secondary lymph node tumors, immunoreactivity patterns were not always concordant between both members of a paired sample. The kappa test, which is a measure of agreement between paired samples, was used to assess the relationship between expression in primary and secondary tumors for each protein, and the results are detailed in Table 3. Guidelines are available to judge the strength of agreement between samples (15), and approximate significance can be calculated. Using 5% positive expression as a cutoff point, p53 showed "good" agreement in colorectal and lymph node tumors (38 of 42 paired samples with identical scores; $\kappa = 0.77$; $P < 0.001$), as did Rb (40 of 42 cases

Table 2 Frequency of expression of examined proteins in paired colorectal and lymph node tumors ($n = 42$)

Cell cycle factor	<5%		5–25%		>25%	
	n	(%)	*n*	(%)	*n*	(%)
p53						
Colorectum	14	(33.3)	1	(2.4)	27	(64.3)
Lymph node	10	(23.8)	3	(7.1)	29	(69.1)
Cyclin D1						
Colorectum	21	(50.0)	10	(23.8)	11	(26.2)
Lymph node	11	(26.2)	15	(35.7)	16	(38.1)
p21						
Colorectum	20	(47.6)	17	(40.5)	5	(11.9)
Lymph node	26	(61.9)	10	(23.8)	6	(14.3)
p27						
Colorectum	3	(7.1)	9	(21.4)	30	(71.4)
Lymph node	2	(4.8)	11	(26.2)	29	(69.1)
Rb						
Colorectum	3	(7.1)	8	(19.0)	31	(73.8)
Lymph node	3	(7.1)	4	(9.5)	35	(83.3)
PCNA						
Colorectum	3	(7.1)	7	(16.7)	32	(76.2)
Lymph node	3	(7.1)	10	(23.8)	29	(69.1)

Table 3 Comparison of cell cycle protein expression between paired colorectal and lymph node tumors using a positive expression level of 5% as a cutoff point

Expression in lymph node tumor	Expression in colorectal tumor			Agreement		Kappa (κ)	Approximate significance (*P*)
	<5%	>5%	Total	*n*	%		
p53							
<5%	10	0	10				
>5%	4	28	32				
Total	14	28	42	38/42	(90.5)	0.77	<0.001
cyclin D1							
<5%	6	5	11				
>5%	15	16	31				
Total	21	21	42	22/42	(52.4)	0.05	0.73
p21							
<5%	12	14	26				
>5%	8	8	16				
Total	20	22	42	20/42	(47.6)	−0.04	0.81
p27							
<5%	1	1	2				
>5%	2	38	40				
Total	3	39	42	39/42	(92.9)	0.36	0.016
Rb							
<5%	2	1	3				
>5%	1	38	39				
Total	3	39	42	40/42	(95.2)	0.64	<0.001
PCNA							
<5%	0	3	3				
>5%	3	36	39				
Total	3	39	42	36/42	(85.7)	−0.08	0.62

with matching staining; $\kappa = 0.64$; $P < 0.001$), whereas p27 demonstrated "fair" agreement (39 of 42 paired samples in agreement; $\kappa = 0.36$; $P = 0.016$). A "poor" agreement between primary and secondary tumors was observed with all other examined proteins (*i.e.*, cyclin D1, p21, and PCNA; $\kappa < 0.20$; $P > 0.05$). Cyclin D1 was overexpressed at a higher frequency in lymph node tumors, whereas p21 expression occurred more often in primary colorectal lesions (Table 3); however, the change in expression of these proteins following metastasis was not correlated (χ^2; $P = 0.82$). In the case of PCNA, a concordance of 85.7% was observed using PCNA-LI of 5% as a cutoff point. However, expression was neither consistently higher nor lower in lymph node tumors compared to their paired colorectal tumor when examined using actual PCNA-LI values, and no consistent relationship was found in PCNA-LI (Wilcoxon test, $P = 0.58$). When the data were reanalyzed using <5%, 5–25%, and >25% expression levels, the relationship between primary and lymph node tumor remained similar to the initial analysis

for p53 ($\kappa = 0.800$; $P < 0.001$), cyclin D1 ($\kappa = -0.009$; $P = 0.928$), p21($\kappa = -0.046$; $P = 0.682$), and PCNA ($\kappa = 0.057$; $P = 0.643$). p27 and Rb, however, demonstrated a lower degree of agreement ($\kappa = 0.202$; $P = 0.116$ and $\kappa = 0.078$; $P = 0.497$, respectively) than found using a 5% positive expression cutoff point.

DISCUSSION

This study has examined the expression of various proteins involved in the cell cycle, in particular the G_1-S phase checkpoint, in a series of 42 primary colorectal tumors and their corresponding lymph node metastases. This is one of the largest sample sizes used to compare the expression of a comprehensive range of cell cycle regulators in primary and secondary colorectal tumors. Using immunohistochemistry, we have established the expression of each protein in both tumor types and investigated the level of agreement in immunostaining results from paired cases. To compare complex staining patterns between primary and secondary tumors, a scoring system was used based on the percentage of positively staining tumor cells in the examined section.

Alterations of the tumor suppressor gene p53 are frequent in a variety of tumor types and have been related to prognosis of colorectal cancer in a number of reports (reviewed in Ref. 16). p53 overexpression was found in 66.7% of primary tumors and exhibited >90% agreement for patterns of immunoreactivity in primary and secondary tumors. There have been several previous reports describing p53 in colorectal tumors and their metastases. Kimura *et al.* (17) used flow cytometry to measure p53 expression, and whereas ~40% of primary and secondary tumors were found to overexpress p53, only 9 of 25 (36%) cases gave similar results in paired colorectal and lymph node samples. A higher rate of agreement between colorectal and lymph node tumors was demonstrated using either immunohistochemistry (67.2% for nuclear staining and 77.6% for cytoplasmic staining; $n = 58$; Ref. 18), PCR-single strand conformational polymorphism analysis (87.5%; $n = 40$; Ref. 19), or both (100%, $n = 8$; Ref. 20). One further study on p53 in primary and secondary tumors found an identical mutation in the single paired sample of colorectal and lymph node tumor they examined using direct sequencing of PCR products (21). Taken together, these results suggest that acquisition of p53 mutations in colorectal cancer generally occurs before cell dissemination from the primary tumor and also that p53 mutation or overexpression of wild-type p53 is not a prerequisite for metastatic growth because primary tumors with nonmutated or immunohistochemically undetectable p53 can spread to distant sites. However, if p53 is proven to be of value as a prognostic marker, there is a high likelihood of predicting p53 expression in secondary deposits from that observed in the primary lesion.

The Rb tumor suppressor is a target substrate for CDK/cyclin complexes, central to control of the cell cycle at the G_1-S phase checkpoint. Although inactivation of the *Rb* gene has been demonstrated in a variety of tumor types, this does not appear to be the case in colorectal cancer (22–24). In agreement with this, we found that >90% of primary and secondary colorectal tumors expressed Rb protein. In addition, staining patterns were similar in 40 of 42 cases (95.2%; $P < 0.001$). This suggests that loss of Rb function is not important for either colorectal tumorigenesis or metastasis. This is the first report on Rb protein expression in paired samples of primary and secondary colorectal tumors, and as with p53, knowledge of expression in the primary tumor could be used to assess the likely situation in the secondary tumor.

A further putative tumor suppressor gene involved in control of the cell cycle at the G_1-S transition is p27. Previous reports have shown a lack of p27 expression to correlate with poor prognosis in colorectal cancer (25, 26). Further, Loda and coworkers (25) suggested that this deficiency of p27 protein may be due to increased proteasome-mediated protein degradation rather than altered gene expression. This increased protein turnover may account for the loss of p27 tumor suppressor function in tumors rather than mutation or loss of the *p27* gene, which has only been described infrequently (27). We found that >90% of primary colorectal tumors expressed p27, which is in agreement with previous studies (25, 26), whereas Thomas *et al.* (28) recorded a lower frequency of p27 expression in this tumor type. In addition, 39 of 42 (92.9%) cases we studied showed similar patterns of p27 immunoreactivity in primary and secondary tumors. Although there have been no previous studies on p27 expression in colorectal lymph node metastases, Thomas *et al.* (28) found liver metastases present at the time of resection of colorectal tumors that expressed p27 at similar levels as the primary tumor. Interestingly, liver metastases that occurred >6 months after resection were found to exhibit much lower levels of p27 protein than their corresponding primary tumor (28), and it was suggested that this loss of p27 may facilitate the metastatic process.

Cyclin D1, one of the major regulators of the G_1-S phase checkpoint, is known to be overexpressed in a variety of tumor types, including colon (24, 29, 30). The reported frequency of overexpression of cyclin D1 protein in colorectal tumors, as ascertained by immunohistochemical analysis, ranges from ~10–45%; however, variations in scoring systems used makes direct comparisons between individual studies difficult. We detected cyclin D1 overexpression (>5% of cells demonstrating positive immunostaining) in 26% of primary colorectal tumors and 38% of secondary lymph node tumors. In a previous study, concordant staining patterns were found in 9 of 9 (100%) cases of primary and metastatic colorectal cancer (29); however, the site of the secondary lesion was not detailed. In contrast, only 22 of 42 (52.4%) of our samples demonstrated equivalent immunoreactivity in primary and secondary paired tumors. Moreover, in the majority of discordant samples, we found cyclin D1 to be overexpressed in the lymph node tumor at a higher frequency than the primary lesion (see Table 3), which may suggest a role for this protein in the metastatic process. Increased cyclin D1 protein may drive the cell cycle forward, providing a growth advantage.

The tumor suppressor gene p21, which can be induced by p53-dependent and -independent mechanisms, is a potent inhibitor of CDK activity. Localization is altered early in neoplastic transformation (31), and a decrease in the frequency of p21 expression has been noted accompanying adenoma development and progression to carcinoma (32). We found that 52.4% of primary colorectal carcinomas expressed p21, which is in agreement with the frequency described by Doglioni *et al.* (33).

Although frequencies of p21 expression ranging from ~30–90% have been reported in colorectal cancer (32, 34), this may be due to differences in antibodies used or discrepancies in scoring systems used to assess the presence or absence of p21 immunostaining. In addition, we found that the frequency of p21 expression was decreased in secondary lymph node tumors (38%) compared with primary colorectal lesions (52%), which is in keeping with the proposed progressive loss of p21 during neoplastic transformation (32). Loss of p21 protein could result in an inability to halt the cell cycle in the absence of growth stimulatory signals or in the presence of growth inhibitory signals, leading to unrestrained and untimely proliferation. Although loss of p21 may play a role in metastatic colorectal tumor growth, it is of little value as a predictor of expression because less than half of the cases we examined showed concordant immunostaining in primary and secondary tumors ($P = 0.81$).

Cell proliferation was investigated in this series of tumors by assessing expression levels of PCNA. PCNA has been associated with improved survival in advanced colorectal cancer (35); however, two further studies examining all stages of disease did not find such a correlation (36, 37). In addition, Sun and coworkers (37) examined PCNA expression in 56 cases of primary colorectal and secondary lymph node tumors and found similar immunostaining patterns in 42 of 56 (75%) cases using a 25% PCNA-LI as a cutoff point, which is in very close agreement to our findings of concordance in 73.8% of cases using the same cutoff point. However, using actual PCNA-LI values, we found no relationship between expression of PCNA in primary and secondary tumors ($P = 0.58$). A similar lack of association has previously been shown by Mayer and coworkers (38) in a sample of 18 paired colorectal and lymph node tumors. This suggests that not only is PCNA an unsuitable marker for predicting expression in secondary tumors, but also that the proliferative capacity of tumor cells may not be directly related to their ability to metastasize.

Poor prediction of the expression of certain cell cycle proteins in lymph node metastasis from that observed in primary tumor may have a number of explanations. To metastasize, the tumor may require the presence of mutations or alterations in gene regulation, which are not initially present in the primary tumor. Expression of the G_1-S proteins may be important in this process or may be incidental, with other proteins mediating the process of metastasis. In addition, factors such as differential growth rates between primary and secondary tumors may also affect the expression of proteins involved in cell cycle regulation. Regardless of the mechanism behind differential protein expression between the primary and metastatic tumor, this has important implications for prognostic prediction. All present studies of protein prognostic markers rely on primary tumor assessment. It is therefore not surprising that inconsistent predictive ability of certain markers is found for approaches analyzing primary tumor because it does not necessarily reflect the situation in metastatic disease.

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Clin Cancer Res 2000;6:1113-1118.