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ARTICLE

Overexpression of Cyclin D1 mRNA in Colorectal Carcinomas and Relationship to Clinicopathological Features: An *In Situ Hybridization* Analysis*

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Increased expression of a key cell cycle regulator, cyclin D1, may have relevance to carcinogenesis and clinicopathological characteristics of some cancers. This study represents the first application of *in situ hybridization*, ISH, to detect cyclin D1 mRNA in tissue sections from colorectal carcinomas. This approach was selected because of its unique potential to clarify whether increased expression of cyclin D1 mRNA correlates with clinical and pathological parameters. The ISH of a non-radioactive oligonucleotide probe (Biogenex) was immunocytochemically detected in paraffin embedded sections from biopsy or resection specimens. Tumors ranged from well to poorly differentiated, and from stages A, B, C, and D. Ten year survival data were available on the majority of patients. Intensity of tumor and background (smooth muscle) signals were independently scored from 0 to 3. Overexpressed cyclin D1 mRNA was seen in 86% of cases compared to background. This frequency is similar to that reported for pancreatic carcinoma. The average signal intensity score in tumor foci was 1.9 with a background score of 0.05 ($p < 001$). All cases showed specific staining judged by the cytoplasmic localization and a tumor signal:background ratio > 1. Expression did not differentiate cancers based on grade, stage or survival ($p > 1$), but did differentiate carcinoma and severe dysplasia from mild dysplasia. We conclude that ISH of cyclin D1 mRNA is an effective and relatively specific means of detecting activity of this gene in colonic neoplasms. The high frequency of overexpression implies that gene activity by itself is not likely to predict a tumor's biological or clinical behavior. On the other hand, these data suggest that increased cyclin D1 gene activity may be an early event in colorectal carcinogenesis. They also are consistent with findings showing cyclin D1 is inducible by a variety of oncogene products. (Pathology Oncology Research Vol 6, No 1, 65–70, 2000)

Keywords: cyclin D1, in situ hybridization, mRNA, colon cancer, colorectal carcinoma, survival, grade, stage

Introduction

Abnormal regulation of proliferation is characteristic of malignant cells, and generally reflects alterations of cell cycle events and their molecular participants.[14] During this cycle, a cell becomes committed to DNA replication in the initial phase, referred to as G_1. Cyclin D1 regulates progression through a key G_1 checkpoint in the transition into the next phase (S), in which DNA is actually synthesized.[5]

Because of the functional importance of cyclin D1 in cell cycle progression, it is reasonable to expect that deregulation of its expression would play a role in oncogenesis.[19] D-type cyclins also could contribute to malignant transformation by effecting phosphorylation of the Rb tumor suppressor protein.[9] The inducibility of cyclin D1 by oncogenes such as *myc*[7] and *ras*[10], and growth factors[21] could represent a further possible locus of action in the process of carcinogenesis. These theoretical considerations are in keeping with the occurrence of cyclin D1 amplification and/or overexpression in many different types of cancers, including carcinomas of breast,[4,12] colon,[2,3,24] pancreas,[11] esophagus,[15,22,26] prostate,[16] liver[25] and lymphomas.[7,18,19]

Based on the foregoing, expression of cyclin D1 also should have relevance to tumor behavior or clinical para-

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meters. However, currently there is no clear consensus on this issue. A single study of non-small cell lung carcinoma, showed that overexpression was associated with a poorly differentiated histology.[6] Other studies have not shown a relationship between cyclin D1 and clinicopathological characteristics[2,3,11,22,24] except survival.[3,11,22] Moreover, the correlation with decreased survival is not straight forward either, since it only has been consistently noted when the protein is immuno-cytochemically demonstrated.[3,11,22] In contrast, in a Northern blot analysis cyclin D1 mRNA did not appear to be correlated.[11] It is unclear whether this discrepancy reflects the technical differences between Northern blots and immunocytochemistry or the intrinsic biological properties of cyclin D1 mRNA vs. protein in these cancer cells.

The present study was undertaken to help clarify this situation. We investigated cyclin D1 gene activity in terms of mRNA within tissue sections from *colorectal carcinomas* using *in situ hybridization.* The ISH observations provided a basis to re-examine whether overexpression correlated with a tumor's clinical and pathological parameters including survival. Colon cancer is similar to other malignancies, in terms of the rate of cyclin D1 expression,[2,3,24] but the relationship to survival has not been reported previously. With an eye to potential applications in pathological practice, the *in situ hybridization* procedure utilized a non-radioactive probe to cyclin D1 mRNA that was detected immunocytochemically.

Materials and Methods

Patient Material

Material was examined from 23 patients ranging from 50-88 years of age. Clinical information is summarized in *Table 1*. Randomly selected biopsies (5) or resection specimens (18) were received in the Pathology Department of Rabin Medical Center, Golda Campus during either 1989 (n=16) or 1998/99 (n=7). The 22 carcinomas were either endophytic or exophytic masses from all regions of the colon, and included examples of Duke's stages A, B, C, and D. Histologically they ranged from well to poorly differentiated. Ten year followup or date of death was available on all 16 patients from 1989. One example of a benign tubular adenoma was also examined, as explained in results.

Table 1. **Patient information and cyclin D1 expression for colorectal carcinomas**

Patient	*Age*	*Sex*	*Site*	*Grade*	*Stage (Duke's)*	*PO Survival–Followup (yr.)*	*Cyclin D1 Score*[a]	
							Tumor	*SM*[b]
1	84	F	Rectum	Mod	B	Alive – 1	2.5	0
2	77	F	Rectum	Well	D	Alive – 1	2.0	0
3	72	F	Right Colon	Mod	B	Alive – 1	1.0	0
4	68	M	Rectum	Mod	C	Alive – 1	2.5	0
5	76	M	Right Colon	Mod	C	Alive – 1	0	0
6	79	M	Right Colon	Mod	D	Alive – 1	3.0	0
7	78	M	–	Mod	A – adenoma	Dead – 1	3.0	0
8	73	M	–	Well	D	Dead – 2/12	0	0
9	52	M	Rectum	Well	(Bx[b])	Alive – 10	2.5	0
10	76	F	Rectum	Poorly	C	Dead – 3	0	0
11	69	M	Left Colon	Mod	B	Alive – 10	1.0	0
12	57	F	Rectum	Mod	C	Alive – 10	1.0	0
13	82	M	Right Colon	Mod	B	Dead – 2	3.0	1.0
14	78	M	Left Colon	Mod	(Bx)	Dead – 1	3.0	0
15	77	M	Rectum	Mod	A	Dead – 3	2.5	0
16	72	M	–	Mod	(Bx)	Dead – 7	2.5	0
17	69	F	Rectum	Mod	(Bx)	Alive – 10	2.5	0
18	67	F	Right Colon	Poorly	B	Alive – 10	1.5	0
19	72	M	Left Colon	Mod	(Bx)	Dead – 7	1.5	0
20	65	F	Rectum	Well	C	Alive – 10	2.0	0
21	69	M	Right Colon	Mod	C	Dead – 2	2.5	0
22	–	F	Rectum	Mod	B	Alive – 10	2.5	0
23	40	F	–	Dyspl[b] – No CRC	Villous adenoma	Alive – 10	Mild Dyspl 0 / 0 Severe Dyspl 2 / 0	

a – Cyclin D1 score: signal intensity in tumor cells and adjacent smooth muscle: 0 to +3 (negative, light, medium, high level of signal). If two different scores are present in different areas of the section for a single tissue type an average value is noted.
b – Abbreviations: PO – postoperative; SM – smooth muscle; Bx -biopsy; Dyspl – dysplasia.

All specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 μ, and placed on positively charged slides without further pre-treatment of the glass surface. Following in situ hybridization, sections were lightly stained with Mayer's hematoxylin.

In Situ Hybridization

An in situ hybridization kit (DPO30-SS) and probe produced by Biogenex (San Ramon, Ca) was used according to the supplied protocol, with the incorporation of all recommendations to minimize background signal. The probe was 20-35 base pairs long, but the sequence is proprietary information of Biogenex. Four separate runs were undertaken which included 6 pairs of duplicate sections and the controls described below. The procedure is based on the use of a non-radioactive, fluorescein tagged, mRNA probe for cyclin D1(HK854-2K) that hybridizes to the cyclin D1 mRNA in formalin fixed, paraffin-embedded tissue sections. The fluorescein tag is subsequently detected immunocytochemically by a biotin/streptavidin system utilizing alkaline phosphatase with aminoethyl carbazole (AEC), as a substrate. When high stringency conditions were maximized a high signal to noise ratio was achieved. Briefly, the in situ hybridization (ISH) procedure is as follows. Sections are initially, deparaffinized, subjected to microwave irradiation, and washed with RNase blocking solution. The slides were then sequentially, dehydrated, incubated in the prehybridization solution containing salmon sperm, hybridized with the supplied nucleotide probe solution placed over each section, coverslipped, and heated for 8-10 min. at 95°C. The slides were then place in a humidity chamber at 37°C for 3 hr. After a number of washes in SSC (sodium chloride/sodium citrate), and SSC with formamide, a protein blocking agent in PBS (phosphate buffered saline) was added to each section, followed by additional PBS washes. The final phase of the procedure was the immunocytochemical labeling of the fluorescein, using an anti-fluorescein antibody (IgG) secondarily linked to a biotin-conjugated anti-IgG which binds to a strepavidin-enzyme-substrate complex. A fluoresceinated poly (A) oligonuclotide probe (Biogenex) served as a positive control, and the omission of any probe as a negative control. Smooth muscle from blood vessels and muscularis propria were used in all cases as an intrinsic negative control, and basal crypts of adjacent colonic mucosa as an intrinsic positive control.

Microscopic Evaluation

The signal intensity for tumor and smooth muscle were independently assessed. The evaluation entailed scoring these elements semi-quantitatively from 0 to 3 (negative, light, moderate and heavy staining). Tumor foci were examined in which > 50% of cells were immunoreactive for the fluorescein labeled probe, and the smooth muscle samples (vascular and mural) were found in or adjacent to the evaluated tumor foci. Reactivity in lymphocytes, colonic crypts, and the inflammatory/desmoplastic response surrounding the infiltrative epithelium were qualitatively assessed.

Statistical Analysis

The non-parametric Mann-Whitney U-test was used to evaluate differences in scores between: (1) tumor signal and background signal, and (2) between survival groups.

Results

Tumor expression

Overexpression of cyclin D1 mRNA in tumor cells was found in 86% (19/22) of carcinomas. The neoplastic epithelium in positive cases contained fine orangophilic grains distributed at varying densities predominantly in the cytoplasm of the cells (*Figure 1*). This is the expected localization of mRNA[1], and parallels a previous report on ISH detection of cyclin D1 expression in esophageal carcinoma.[22] Table 1 shows the semi-quantitative scores for tumor and background signal intensity of all cases. Most malignant cells in a focus were reactive at more or less the same level, although there was variability from focus to focus in some cases of approximately 1 score unit. The average signal score for the tumors was 1.9 (median 2.5). Duplicate sections were run in 6 cases with approximately comparable results in all instances. A duplicate run of one negative case was also negative.

One case (*Table 1*, #7) of an early carcinoma originating in a villous adenoma showed reaction product limited to

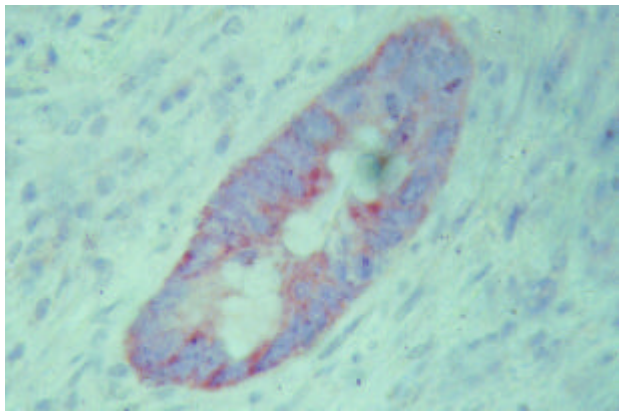

Figure 1. Expression of cyclin D1 demonstrated with in situ hybridization in colonic carcinoma. Cells from a malignant glandular formation show a strong signal, consisting of orange grains generally restricted to the cytoplasm of the cell.. In contrast, the surrounding muscularis propria that is being invaded, remains unstained. Nuclei are lightly stained blue with Mayer's hematoxylin. x580.

the malignant epithelium. As a comparison, we examined a second adenoma without evidence of carcinoma. In the latter example, reactivity was restricted to foci with thickened, highly atypical epithelium, characteristic of severe dysplasia; mildly and moderately dysplastic cells – even in the same glandular formation – were not reactive.

Clinicopathological correlations

Expression did not differentiate cancers based on grade, stage or survival. Nearly all tumors were reactive regardless of clinicopathological status (*Table 1*). Negative cases had no other common feature and were found among both well and poorly differentiated specimens.

Since variation in intensity of signal did occur between tumors, we further analyzed the survival data in relation to signal intensity. The patients were divided into four groups based on their length of survival: ≤1 yr. (n=3), <5 yr (4=7), >1 yr. (n=13), >5 yr. (n=9). The mean scores for each group were: 2.0, 2.0, 1.9, 1.9, respectively. The scores are not significantly different ($p > 0.1$).

Specificity

In 18 samples, sections contained normal colonic structures adjacent to foci of tumor. This enabled direct comparison of tumor signal with that of tissues in the immediate vicinity of the tumor focus. Smooth muscle (vascular and mural) was considered a prototype of a non-proliferative tissue without any expectation of cyclin D1 expression under physiological conditions of the bowel. It was therefore used as a basis to quantify the background signal.

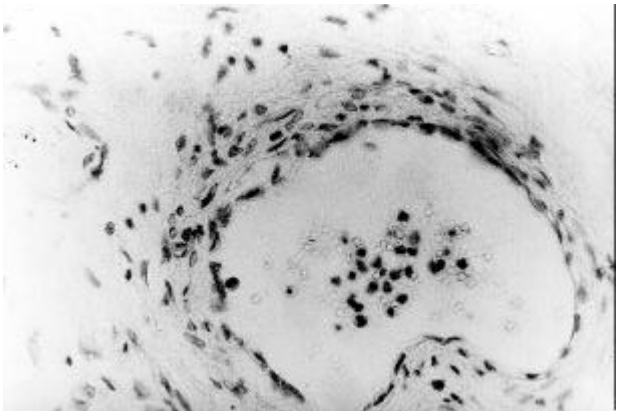

Figure 2. Intrinsic negative control for in situ hybridization with cyclin D1 probe. Vascular smooth muscle (surrounding lumen), and contiguous fibroadipose tissue (upper right) fail to show any signal. This blood vessel is adjacent to a tumor focus with a strong signal, similar to Figure 1. The photograph was made with the substage condenser slightly closed to enhance contrast of unstained vascular smooth muscle. Only nuclei are apparent that are lightly stained with Mayer's hematoxylin. x300.

No smooth muscle staining was noted in 22/23 cases (*Table 1, Figure 2*). The one exception, with a strong tumor signal, exhibited light cytoplasmic staining of muscle. The average signal in smooth muscle for all cases was 0.05 (median 0), in contrast to 1.9 (median 2.5) for tumor. The difference is highly significant ($p < 0.001$). Additionally, all cases exhibited specific staining judged by the predominantly cytoplasmic localization of signal in the vast majority of cells. In general, mRNA is expected to predominantly accumulate in the cell cytoplasm.[1]

Duplicate sections were hybridized with a poly A nucleotide positive control probe, in place of the specific probe for cyclin D1. These slides showed strong reactivity of almost all cells present including smooth muscle and fat. Reaction product in these cells was located both within the nucleus and the cytoplasm. Omission of a probe resulted in a completely negative section.

Using the specific probe, lymphoid aggregates adjacent to tumor often contained scattered cells with light reactivity. Adjacent colonic crypts contained a few lightly reactive cells basally, with an expanded topological distribution (upwards) in the crypt paralleling that found with Ki67 in the same setting.[17] Serosal fat in resection specimens was always negative. The desmoplastic response showed scattered lightly stained positive inflammatory cells and fibroblasts in many cases. These cells also are immunoreactive for cyclin D1 protein.[4,11] In no instance did these cells have the high frequency and intensity seen in the poly A control. It is also relevant that the areas of mild and moderate dysplasia in the two polypoid adenomas examined, failed to show reactivity. We conclude, that from a technical point of view the Biogenex ISH kit and probe is an effective and a relatively specific means of detecting activity of the cyclin D1 gene in tissue sections. A similar conclusion has been reached in a previous application of this kit to detect viral DNA.[20] Although some of the material had been in paraffin for 10 yrs., there was no discernable impact on the outcome.

Discussion

This study represents the first application of *in situ hybridization*, ISH, to detect cyclin D1 mRNA in tissue sections from colorectal carcinomas. This approach was selected because of its unique potential to clarify whether increased expression of cyclin D1 mRNA correlates with clinical and pathological parameters.

Most of the tumors evidenced moderate to high signal levels in the face of very low background. This indicates that increased cyclin D1 gene activity is a common feature of this type of carcinoma, in agreement with previous findings.[2,3,24] In particular, we found increased expression of cyclin D1 mRNA in 86% of our cases. These results are comparable to those in studies on other malignancies using

Northern blotting of amplified RNA.[11,18] The latter work showed 82% of pancreatic carcinomas[11] and 80% of mantle cell lymphomas[18] overexpressed cyclin D1 mRNA. In contrast, another study using Northern blot analysis on colonic carcinoma only found a 50% frequency of cyclin D1 overexpression.[24] The basis for this discrepancy may be that the later workers normalized their findings with respect to activity in mucosa bordering the tumors. The data from all other studies represent the frequency of non-normalized expression in each tumor. A normalization procedure was not adopted here for two reasons. First, the ISH procedure is not quantitative in the manner of a Northern blot. Second, in a previous study we found increased proliferative activity in mucosa at the edge of colorectal carcinoma,[17] so that increased signal in these border crypts would be expected. This effect should reduce the reported relative frequency of tumors overexpressing cyclin D1. Indeed, if we subtract the cases in the current series with a score of 1.5 or less (*Table 1*) – which probably would be eliminated as a positive case in a normalization procedure – our relative frequency of "highly" expressing tumors would have been 64%. Nonetheless, because of the intrinsic technical variability and non-quantitative nature of the ISH it seems unwise to base our analysis of prognostic relevance of gene activity on a normalized value.

As a consequence of the high frequency of increased cyclin D gene activity, overexpression was detected in tumors of all phenotypes and behavior. This implies that overexpression, per se, is not likely to be predictive of biological or clinical behavior. Previous evaluations of cyclin D1 in colonic carcinomas,[2,24] have reached the same conclusion. We have additionally evaluated the relationship of survival to the relative intensity of signal. In three groups of patients, differing in longevity, no statistically significant difference in cyclin D1 expression was found.

The basis for this high frequency is also not entirely resolved. Genetic alterations in tumor cells, such as translocations, and amplifications, only account for 16-42% of cases.[11,12,15,18,22] Consequently, cyclin D1 overexpression may be partially due to functional, non-genomic mechanisms that deregulate gene activity.[13] For instance, the role of cyclin D1 in proliferation is subject to influences by the products of several oncogenes including *ras*,[10] *myc*[7], and growth factors.[21] It is also inducible by cyclin-dependent kinase inhibitors[8] which involve cyclin D1 in the regulation of apoptosis and the arrest of cell cycle progression.[23] Together, this work suggests that cyclin D1 is involved in many different facets of cell function, which could account for the high frequency of expression precluding clinical correlations. Additionally these polymodal effects of cyclin D1 may be an independent reason why increased cyclin D1 is not linearly related to the biological characteristics of these cancers.

On the other hand, these data do not abrogate the reasonable relationship of cyclin D1 with oncogenesis at several levels (see above). The finding of overexpression in colorectal carcinomas of all phenotypes and stages may be particularly relevant. It would support the possibility that cyclin D1 even contributes to early stages of neoplastic progression, as suggested previously.[2]

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