Cyclin D3 Immunoreactivity in Gastrointestinal Stromal Tumors Is Independent of Cyclin D3 Gene Amplification and Is Associated with Nuclear p27 Accumulation

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An abnormal expression of cyclin D3, a key regulator of the cell cycle, has been documented in a variety of human malignancies, and the *cyclin D3* gene, mapping to 6p21, may be deregulated in human tumors as a result of the t(6;14)(p21.1;q32.3) translocation or gene amplification. In the current study, we for the first time investigated by immunohistochemistry and fluorescence *in situ* hybridization (FISH) the prevalence of *cyclin D3* abnormalities in gastrointestinal stromal tumors (GISTs), comparing the results with traditional pathological characteristics, p27 immunoreactivity (IR), and Ki-67 labeling index (LI). All the tumors showed nuclear cyclin D3 IR, with a percentage of immunostained neoplastic cells ranging from 10 to 95% (mean, $67.3 \pm 22.9\%$). In 4 (40%) of the 10 cases analyzed by FISH, *cyclin D3* extrasignals were detected. Cohybridization with probes specific for the centromeric region and the long arm of chromosome 6 indicated trisomy in one case, whereas in the remaining three cases the pattern was highly suggestive for the occurrence of an isochromosome 6p. There was no association between the *cyclin D3* gene copy number and IR for the encoded protein. Cyclin D3 IR was positively associated with p27 IR ($P = .004$) but not with Ki-67 LI or tumor malignant potential. On the contrary, p27 IR was inversely associated with Ki-67 LI ($P = .004$) and was more prevalent in tumors of low or intermediate malig-

nant potential, though at a borderline level of statistical significance ($P = .066$) only. These data suggest that cyclin D3 expression in GISTs is independent of gene amplification and that this protein may be involved in the pathogenesis of GISTs by counteracting the inhibitory activities of p27.

KEY WORDS: Cyclin D3, FISH, Gastrointestinal stromal tumors, Immunohistochemistry.

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Cyclin D3 plays a pivotal role in tightly controlling the physiological progression from the G1 to the S phase of the cell cycle. After assembling with the cyclin-dependent kinases (cdk) 4 and 6, cyclin D3 is capable of phosphorylating the retinoblastoma (Rb) gene product, eventually promoting the entry to the S-phase (1). In addition to phosphorylating Rb, cyclin D3 also promotes transition to the S phase by sequestering p27 (2).

Cyclin D3 maps to the 6p21 locus and may be deregulated in a fraction of hematopoietic disorders as a consequence of the t(6;14)(p21.1;q32.3) translocation that juxtaposes the *cyclin D3* gene to the 3' IgH sequences (3, 4). Moreover, amplification of the *cyclin D3* gene has been documented in sporadic cases of malignant gliomas (5) and non-Hodgkin's lymphomas (6, 7). An abnormal expression of cyclin D3 has been documented in a variety of human malignancies (8–10). With regard to soft tissue tumors, immunoreactivity (IR) for cyclin D3 has been found in approximately 20% of leiomyosarcomas (11) and liposarcomas (12).

Gastrointestinal stromal tumors (GISTs) represent the commonest mesenchymal neoplasm of the gastrointestinal tract, accounting for approximately 15% of all soft tissue tumors. Recent data suggest that abnormalities of the genes controlling the cell

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cycle progression may be involved in the pathogenesis and progression of GISTs (13, 14). In this study, we first evaluated by fluorescence *in situ* hybridization (FISH) and immunohistochemistry the prevalence of *cyclin D3* abnormalities in a well-characterized series of GISTs. The results were compared with traditional pathologic characteristics, p27 IR, and Ki-67 labeling index.

MATERIALS AND METHODS

Patients and Samples

Twenty-one consecutive surgically treated primitive GISTs were retrieved from the files of the Division of Pathology and Laboratory Medicine of the European Institute of Oncology. The clinicopathological features of the patients included in the study are shown in Table 1. All the tumors exhibited definite cytoplasmic IR for CD117 (c-kit). There were 13 (61.9%) men and 8 (38.1%) women, with a mean age of 56 years (range, 36–71 y). Sites of involvement were stomach (10 cases, 47.6%), ileum (5, 23.8%), and colon (6, 28.6%). According to the recommendations recently proposed by Fletcher *et al.* (15), the tumors were reclassified as being of low (3 cases, 14.3%), intermediate (4, 19%), and high malignant potential (14, 66.7%).

Immunohistochemistry

Immunoreactivity for cyclin D3, p27, and Ki-67 antigen was evaluated by means of the avidin-biotin peroxidase complex (ABC) method, using the 3,3'-diaminobenzidine tetrahydrochloride chromogen, as described elsewhere (16). Immunostaining

was performed using an automated immunostainer (Autostainer; DAKO, Glostrup, Denmark). For antigen retrieval, the slides were placed in 0.01 M EDTA buffer at pH 8.0 (for cyclin D3 and Ki-67), or 0.1 M citrate buffer at pH 6.0 (for p27), and underwent three 4-minute (for cyclin D3 and Ki-67) or two 5-minute (for p27) cycles at 90° C in a microwave oven operating at 780W. The sections were immunostained with the following monoclonal antibodies: DCS22 (Novocastra, Newcastle upon Tyne, UK, at a working dilution of 1:40) for cyclin D3, Kip-1 for p27 (Transduction Laboratories, Lexington, UK; 1:600), and Mib-1 for Ki-67 (DAKO; 1:200). At least 500 neoplastic cells were evaluated at 400× magnification without knowledge of the genetic and clinicopathological data, and the percentage of neoplastic cells showing definite nuclear staining was recorded. Built-in positive controls were endothelial and ganglion cells for cyclin D3; small lymphocytes, plasma cells, and endothelial cells for p27; and epithelial cells of the gastric and intestinal glands for the three antibodies. Negative-control sections were incubated with nonimmune mouse serum in place of the specific primary antibodies.

FISH Analysis

In the 10 cases analyzed, nuclei were extracted from two 50-μm-thick sections of formalin-fixed and paraffin-embedded samples. Briefly, the sections were dewaxed, rehydrated, and digested in a 0.5% pepsin solution at 37° C. The samples were then filtered through a 30-μm mesh nylon filter. The nuclei were pelleted by centrifugation at 1500 rpm for 10 minutes and resuspended in PBS, then

TABLE 1. Clinicopathologic Characteristics of the Patients Included in the Study Grouped According to the Malignant Potential

Case	Malignant Potential	Age	Gender	Site	Ki-67 LI (%)	Cyclin D3 IR (%)	Cyclin D3 Extrasignals	p27 IR (%)
IEO1	3	53	M	Colon	15	45	Nd	70
IEO2	3	54	F	Ileum	15	90	None	80
IEO3	3	58	F	Stomach	11	80	None	80
IEO4	3	57	M	Stomach	14	85	None	95
IEO6	3	58	F	Ileum	35	60	None	75
IEO8	3	50	M	Colon	21	10	Nd	50
IEO9	3	46	M	Colon	12	95	Nd	70
IEO10	3	66	M	Colon	16	80	None	60
IEO11	3	61	M	Stomach	15	90	Nd	80
IEO12	3	41	F	Stomach	8	70	Nd	90
IEO13	3	36	F	Colon	45	60	Nd	25
IEO15	3	60	M	Colon	38	70	Yes	55
IEO18	3	54	F	Stomach	19	60	Yes	90
IEO20	3	71	M	Ileum	22	30	Nd	50
IEO5	2	50	F	Esophagus	4	25	Nd	40
IEO16	2	67	F	Ileum	9	80	None	90
IEO17	2	70	M	Stomach	8	90	Yes	95
IEO19	2	46	M	Stomach	7	75	Nd	95
IEO7	1	63	M	Stomach	6	65	Nd	95
IEO14	1	55	M	Stomach	4	80	Nd	90
IEO21	1	61	M	Stomach	5	75	Yes	80

M = male; F = female; 1 = low malignant potential; 2 = intermediate malignant potential; 3 = high malignant potential; LI = labeling index; IR = immunoreactivity; Nd = not done; yes = presence of cyclin D3 extrasignals; none = absence of cyclin D3 extrasignals.

processed by a cytospin device at 200 rpm for 8 minutes on positively charged slides and treated with 30% sodium bisulfite (Pretreatment solution; Oncor, Gaithersburg, MD) for 10 minutes, followed by digestion in 250 μ g/mL proteinase K (Oncor) for 10 minutes at 45° C.

FISH experiments were performed with probes labeled with biotin or with the Cy3 fluorochrome (Amersham, Little Chalfont, UK) by nick translation, as described, with minor modifications (17). Briefly, 600 ng of labeled probe was used for each experiment, and hybridization was performed at 37° C in 2 $\times$ SSC, 50% (v/v) formamide, 10% (w/v) dextran sulfate, 7.5 μ g Cot1 DNA (Boehringer Mannheim, Germany), and 3 μ g of sonicated salmon sperm DNA in a volume of 10 μ L. Posthybridization washing was done three times at 60° C in 0.1 $\times$ SSC. Biotin-labeled probes were detected using FITC-conjugated avidin (resulting in a green signal; Vector Laboratories, Burlingame, CA); Cy3-labeled probes were detected directly as a red signal. Nuclei were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) staining. Digital images were obtained using a Leica DMR epifluorescence microscope (Leica Imaging Systems Ltd, Cambridge, UK) equipped with a CCD camera (Cohu Inc., San Diego, CA). FITC-avidin, Cy3, and DAPI fluorescent signals were detected using specific filters. The images were recorded, pseudocolored, and merged using the QFISH software (Leica) and were finally edited using Adobe Photoshop, Version 6.0 (Adobe Systems, Mountain View, CA).

The *cyclin D3* locus was investigated using the PAC clone RP5-973N23 (red signal) (4) in ≥ 200 nuclei for each case. To assess the occurrence of chromosome 6 aneuploidy in interphase nuclei, the cases with cyclin D3 extrasignals were then separately cohybridized with the following pairs of probes: (1) a chromosome 6 Alpha satellite (green signal, Appligene Oncor) and the probe specific for *cyclin D3* locus (red signal); (2) a chromosome 6 Alpha satellite (green signal) and a probe (BAC164C22, red signal) specific for the chromosome region 6q11–12, selected using the NCBI database (www.ncbi.nlm.nih.gov). Figure 1 shows a schematic representation of the locations of the probes used in the study.

Negative controls were uninvolved resection margins from 10 cases of surgically resected gastric adenocarcinoma. At least 200 nuclei were analyzed from these samples, and the percentages of nuclei bearing three or four *cyclin D3* extrasignals were $2.5 \pm 1.46\%$ and $2 \pm 1.52\%$, respectively. Accordingly, the cutoff value was settled at 7% (mean value plus 3 SDs).

RESULTS

Cyclin D3, p27, and Ki-67 Immunoreactivities

The immunohistochemical results in the 21 GISTs analyzed are shown in Table 1.

In normal tissues, nuclear cyclin D3 IR was detectable in a minority of epithelial cells of the gastric and intestinal glands, as well as in endothelial

FIGURE 1. Schematic representation of the location of the probes used for the FISH analysis (left panel) and examples of the FISH results in interphase nuclei from three (IEO15, IEO21, and IEO18) different cases of gastrointestinal stromal tumor (central panel). Based on the results of the hybridizations, a schematic representation of the chromosome 6 aneuploidy is given for each tumor (right panel).

cells and ganglion cells of the Meissner's and Auerbach's plexuses. All the tumors showed nuclear immunoreactivity for cyclin D3, with a prevalence of immunostained neoplastic cells ranging from 10 to 95% (median, 75%; mean, 67.3%; standard deviation, 22.9%; 95% confidence interval, 56.9–77.8%; Fig. 2).

Nuclear p27 IR was detectable in a minority of normal epithelial cells of the gastric and intestinal glands, as well as in small lymphocytes, plasma cells, and endothelial cells. All the GISTs showed nuclear p27 IR, with a percentage of positive neoplastic cells ranging from 25 to 95% (median, 80%; mean, 74 ± 20.2 ; 95% CI, 64.8–83.2%; Fig. 2).

Finally, the Ki-67 LI of the tumors ranged from 4 to 45% (median, 14%; mean, 15.6 ± 11.3 ; 95% CI, 10.5–20.8%; Fig. 2).

Comparison among Cyclin D3, p27, and Ki-67 Immunoreactivities

A statistically significant direct correlation was found between cyclin D3 and p27 IR ($r = .590$, $P = .004$), whereas there was no association between cyclin D3 IR and the Ki-67 LI ($r = -0.216$, $P = .347$). A significant inverse association between p27 IR

and the Ki-67 LI ($r = -0.599$, $P = .004$) was also demonstrated.

Comparison between Immunohistochemical and Pathologic Features

There was no association between cyclin D3 or p27 IR or between Ki-67 LI and age (<56 y *versus* ≥ 56 y; $P = .290$, $.458$, and $.724$, respectively, Mann-Whitney test) and gender ($P = .884$, $.364$, and $.562$, respectively) of the patients.

Also, there was no correlation between cyclin D3 IR and tumor malignant potential ($P = .914$, Mann-Whitney test). Tumors with low or intermediate malignant potential, however, showed an higher prevalence of p27 IR (mean, 83.5 ± 7.5 ; 95% CI, 65.1–100%) than those with high malignant potential (mean, 68.5 ± 19.5 ; 95% CI, 57.2–79.8%), a difference approaching statistical significance ($P = .066$). There was, however, a significant ($P = .003$) difference in Ki-67 LI between the tumors with low or intermediate (mean, 6.2 ± 1.8 ; 95% CI, 4.7–7.7%) and high (mean, 20.4 ± 11.1 ; 95% CI, 14–26.8%) malignant potential.

FIGURE 2. Examples of the immunohistochemical results. All the cases show high levels of nuclear cyclin D3 immunoreactivity (IR). Tumors with high nuclear p27 IR are characterized by a low Ki-67 LI and vice versa (200× original magnification; hematoxylin counterstain).

FISH Analysis

The results of the FISH analysis are shown in Table 1. In 4 (IEO15, IEO17, IEO18, and IEO21) of the 10 cases analyzed (40%), *cyclin D3* gene extrasignals were observed (Fig. 1). The mean percentage of nuclei with *cyclin D3* extrasignals was 50.6% (range, 10.5–90%). In particular, there were two cases (IEO18 and IEO21) harboring three signals of the *cyclin D3* gene and two cases (IEO15 and IEO17) with either three (in 74% and 7% of the cells, respectively) and four signals (in 16% and 3.5% of the cells, respectively). *Cyclin D3* extrasignals were found in two of the three cases of low or intermediate malignant potential and in two of the seven cases of high malignant potential ($P = .500$, Fisher's exact test) analyzed by FISH.

All the cases bearing cyclin D3 extrasignals were cohybridized with an alphoid probe specific for the centromeric region of chromosome 6. Two signals of the centromeric region and three signals of *cyclin D3* were detected in Case IEO21, and three signals of either the centromeric region and *cyclin D3* in Case IEO18 (Fig. 1). The last two cases (IEO15 and IEO17) showed either three signals of the centromeric region and four of *cyclin D3* or two signals of the centromeric region and three signals of *cyclin D3*.

Finally, the cohybridization with probes specific for the 6q11–12 and the chromosome 6 centromeric regions documented one signal of 6q11–12 and two signals of the alphoid probe in Case IEO21 and three signals of both 6q11–12 and alphoid probe in Case IEO18 (Fig. 1). The last two cases (IEO15 and IEO17) had nuclei with either two signals of the 6q11–12 and three signals of the centromeric region, and one signal of the 6q11–12 and two signals of the centromeric region.

In summary, these results indicate the occurrence of trisomy 6 in Case IEO18 and a putative isochromosome involving the short arm of chromosome 6 in Cases IEO15, IEO17, and IEO21 (Fig. 1).

Comparison between Immunohistochemical and FISH Analyses

The mean rate of cyclin D3 IR was $73.7 \pm 12.5\%$ (95% CI, 53.8–93.6%) in the four cases with *cyclin D3* extrasignals and $79.1 \pm 10.2\%$ (95% CI, 68.4–89.8%) in the remaining six cases ($P = .542$, Mann-Whitney test). No association was ascertained either between the results of the FISH analysis and p27 IR ($P = .914$) or Ki-67 LI ($P = .914$).

DISCUSSION

In this study, we analyzed for the first time the prevalence of *cyclin D3* abnormalities in a well-characterized series of GISTs.

We found extrasignals of the *cyclin D3* gene in 40% of the cases analyzed by FISH. The cohybridization with probes specific for the centromeric region and the long arm of chromosome 6 revealed that one case harbored an apparent trisomy of chromosome 6, whereas the remaining three cases showed a pattern highly suggestive for the presence of an isochromosome 6p. The occurrence of an isochromosome 6 has been reported previously in retinoblastomas (18) and non-Hodgkin's lymphomas (19). In the 37 GISTs investigated by traditional cytogenetic approaches, loss of chromosome 14 and/or 22 was the commonest abnormality, whereas the only reported change of chromosome 6 was chromosome loss in approximately 10% of the cases (20–23). Nevertheless, Debiec-Rychter *et al.* (24) reported that almost 10% of the GISTs analyzed by FISH showed additional signals of the centromeric region of chromosome 6. Likewise, El-Rifai *et al.* (25), using comparative genomic hybridization, documented gains or high levels of amplification of the 6p region in 5% of the tumors analyzed. Interestingly, gain of 6p1/p2 has been reported recently to be an independent predictor of poor survival in a series of 151 high-grade soft tissue sarcomas analyzed by chromosomal binding (26). Although we did not find any association between the occurrence of 6p21 extrasignals and tumor malignant potential, the above data suggest verification of the clinical relevance of gain of material from the short arm of chromosome 6 in larger series of GISTs.

The mechanisms by which *cyclin D3* may be deregulated in human tumors have not yet been fully elucidated. The t(6;14) translocation, which juxtaposes the cyclin D3 to the 3' IgH sequences, has been detected in a minority of plasma cell myeloma (3), whereas *cyclin D3* gene amplification has been documented in sporadic cases of malignant glioma (5) and non-Hodgkin's lymphoma (6, 7). Nevertheless, cyclin D3 expression has been reported in a variety of malignancies with a much higher prevalence than gene alterations (8–12). Likewise, we found a high prevalence of cyclin D3 IR in the majority of the cases analyzed, irrespective of the occurrence of *cyclin D3* gene extrasignals. Cyclin D3 IR is significantly higher in GISTs than previously reported in other types of soft tissue tumors. In particular, cyclin D3 IR has been detected in only 14% of liposarcomas (11) and in 21% of leiomyosarcomas (12). Although this discrepancy may be at least in part justified by the different anti-cyclin D3 antibodies used, the data lead to the hypothesis that cyclin D3 deregulation may be involved in the pathogenesis of GISTs. Because cyclin D3 immunoreactivity was not associated with the occurrence of gene extrasignals in the 10 cases analyzed, we also speculate that, similarly to other malignancies (8–12), cyclin D3 deregulation may occur in GISTs as a

result of epigenetic mechanisms. In keeping with recent findings in aggressive B-cell lymphomas (27, 28), we observed a direct correlation between cyclin D3 and p27 IR, also in GISTs. p27 is capable of blocking progression from the G1 to the S phase of the cell cycle by binding cyclin E-cdk2 and cyclin A-cdk2 (29). Counteracting the p27 functions is a common way of escape from the physiological growth control in human tumors, and this may be achieved by promoting p27 ubiquitin proteasome degradation or by sequestering it in cyclins D/cdk or cyclin E/cdk complexes. D-type cyclins and cdk4 competitively bind and down-regulate the activity of p27 and may thereby act on a pathway that reverses cdk2 inhibition and enables G1 progression (30). The present data allow speculation that high levels of cyclin D3 in GISTs could lead to p27 stabilization and functional inactivation, which in turn may contribute to an uncontrolled tumor growth. We did not find any association between cyclin D3 IR and the pathological features analyzed. On the contrary, however, we document a significant inverse association between p27 IR and Ki-67 LI. Moreover, p27 IR decreased in GISTs of high malignant potential, although this trend only approached statistical significance, possibly because of the small series of cases analyzed. In this regard, it should also be underlined that in our series there were only three cases of low malignant potential. Down-regulation of p27 due to ubiquitin-mediated proteasome degradation has been reported to be associated with a worse prognosis in different tumor types, independent of traditional pathologic parameters (31). Taken together, these data suggest that it may be of interest to further investigate the clinical relevance of p27 IR in larger series of GIST patients with adequate follow-up.

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Book Review

Ladanyi M, Gerald WL: *Expression Profiling of Human Tumors, Diagnostic and Research Applications*, 399 pp, Totowa, NJ, Humana Press, 2003 (\$175.00).

The study of the expression of genes has become very important in the diagnosis and therapeutic approach of tumors. The aim of this book is to help understand the technical aspects and applications of gene profiling in human tumors. Given the evolving nature of these issues and the information overload, this book constitutes a welcome introduction and comprehensive review of this important topic.

The book consists of 20 chapters that cover in great detail the technical, clinical, and research aspects of gene expression in human tumors. For this purpose, the editors have assembled a group of researchers, all of them experts in their fields, to write the corresponding chapters.

The first chapters are devoted to the basic concepts of gene profiling, followed by chapters that cover each organ system tumor in great detail, providing in-depth technical and clinical information of each tumor type. The two questions of how and why the gene expression profile of tumors should be studied are discussed in each chapter. The impact that these analyses

have in the management of patients is emphasized throughout the text. The book also includes practical information in two important issues associated with the gene profiling of tumors: tissue arrays and tumor banking/bioinformatics.

The illustrations in the book, many of them in color, are all of high quality, and the references, listed at the end of each chapter, are up to date.

As with any book with multiple co-authors, there are chapters that are more comprehensive than others with minor overlaps of information between them. However, the editors have done a good job in maintaining consistency throughout the entire book.

The target audience of this excellent book includes all of those interested in the study of cancer, ranging from basic researchers to pathologists, surgeons, and medical oncologists. This book should stimulate the medical community, particularly pathologists, to think and look beyond the pure morphologic aspects of tumors.

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