Increased Cyclin D1 Expression Is Associated with Features of Malignancy and Disease Recurrence in Ovarian Tumors

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ABSTRACT

Alterations in the expression of cyclin D1 have been reported frequently in several human cancers, but their significance in the multistep model of carcinogenesis has been scantly described. To define the pattern of cyclin D1 expression in the development of ovarian cancer and clinical outcome, 55 cases of benign ovarian tumors, 12 borderline cases, and 37 ovarian carcinomas (32 primary and 5 recurrent carcinomas) were studied. Analyses were carried out on fresh tumor specimens by Western blotting and reverse transcription-PCR and provided significant superimposable results ($P = 0.00001$). Cyclin D1 abundance was classed according to the densitometric values as undetectable, detectable, well detectable, and highly detectable. A significant increase ($P < 0.000001$) in median cyclin D1 values was observed from benign (0.038; range, 0.001–0.705) to borderline (0.226; range, 0.001–0.623) to malignant (0.347; range, 0.027–2.330) to recurrent (0.887; range, 0.309–2.2260) tumors. In addition, higher median cyclin D1 values were reported in serous carcinomas ($P = 0.058$) and advanced-stage diseases ($P = 0.003$). Survival analyses carried out in the 32 primary carcinomas showed no significant difference in overall survival between detectable *versus* well/highly detectable cyclin D1 neoplasms. Conversely, a significant relationship between cyclin D1 expression and progression-free survival was found ($P = 0.031$). These results may elucidate the function of altered cyclin D1 expression in ovarian tumorigenesis and provide a basis for additional studies on its prognostic role.

INTRODUCTION

Ovarian cancer is the leading cause of death among gynecological diseases in Western countries. A significant factor contributing to the high mortality rate is that most patients are diagnosed with advanced stages of disease, and, despite the introduction of aggressive chemotherapy regimens, the long-term prognosis for these patients remains poor (1, 2). Ovarian epithelial neoplasms are divided into three histological and biological subtypes: (*a*) benign; (*b*) low malignant potential (borderline); and (*c*) malignant.

Very little is known about the exact sequence of cellular and molecular events contributing to the development of ovarian malignancy; indeed, benign and borderline tumors probably occur from the same precursor, but the demonstration of the carcinoma as a part of a disease continuum or a separate disease entity is far from unequivocal (3, 4). In fact, some molecular changes, such as telomerase expression and DNA methylation, are associated with both borderline tumors and carcinoma but not with adenoma (5, 6), whereas others, such as p53 abnormalities, are specific for malignancy (7).

Alterations of the mechanisms controlling cell cycle progression play a relevant role in the pathogenesis of different human neoplasias, and among the molecules involved in cell cycle regulation, cyclin D1 abnormalities may contribute to such malignant transformation (8). Cyclin D1 is a G_1-specific protein essential for progression through the G_1 phase to S phase of the cell cycle in eukaryotes. The cyclin D1 locus has been mapped to chromosome 11 band q13, and amplifications of this region, as well as the cyclin D1 gene as a component of such an amplicon, have been observed in a variety of human carcinomas (9). The indication that the cyclin D1 gene is somehow centrally relevant to cancer, functioning as an oncogene, is supported by several experimental observations (10, 11). Altered expression of cyclin D1 may result from rearrangement [isolated as PRAD-1 in parathyroid adenomas (12)], translocation [isolated as bcl-1 in B-lymphocytic malignancies (13)], and amplification and/or overexpression in head and neck, breast, and squamous cell carcinomas, non-small cell lung cancer, and colon and urinary bladder cancer (14). In addition, overexpression of cyclin D1 has been reported in ovarian cancer as well (15–17).

To gain more insight about the significance of abnormal cyclin D1 content in the controversial tumorigenesis of ovarian cancer, the histological model of progression from adenoma to low malignant potential and carcinoma has been examined. In a previous study (18), we reported a preliminary correlation between cyclin D1 expression, features of transformation, and tumor-proliferative activity in a small number of 33 patients with ovarian tumors.

The aim of the current study was to confirm the oncogenic potential of cyclin D1 in a larger series of benign, borderline, and malignant ovarian tumors by examining the timing and extent of overexpression in cancer development. Moreover, preliminary results regarding the association of cyclin D1 content with clinicopathological characteristics and clinical outcome are shown.

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Table 1 Characteristics of 104 patients with ovarian tumors

Clinical feature	No. of cases
Benign[a]	
All cases	55
Histology	
Serous	16
Mucinous	6
Endometrioid	21
Other	12
Borderline[b]	
All cases	12
Histology	
Serous	7
Mucinous	3
Endometrioid	1
Other	1
Malignant[c]	
All cases	37
Primary	32
Recurrent	5
Histology	
Serous	16
Mucinous	2
Endometrioid	10
Other	9

[a] Median age, 38 years (range, 13–81 years).
[b] Median age, 53 years (range, 16–83 years).
[c] Median age, 58 years (range, 28–81 years).

MATERIALS AND METHODS

Patients and Tumor Samples. A total of 104 patients presenting at the Department of Obstetrics and Gynecology of the University of Genoa entered this study from December 1994 to April 1998. Tissue samples were obtained from the pathologist at the time of surgery, and the ratio of tumor tissue: unaffected tissue always exceeded 80%. Specimens consisted of 55 ovarian benign lesions, 12 borderline tumors, and 37 carcinomas (32 primary carcinomas and 5 recurrent carcinomas at the time of presentation). Histological classification was assessed according to the WHO system, and tumors were graded as well (grade 1), moderately (grade 2), and poorly (grade 3) differentiated. Stage of disease was established according to the Fédération Internationale des Gynaecologistes et Obstetristes staging system. Of the 32 patients with primary untreated malignant disease, 6 received no therapy after surgery, and 26 underwent first-line chemotherapy regimens consisting of a combination chemotherapy with cisplatin, epidoxorubicin, and cyclophosphamide (19 patients); cisplatin and paclitaxel (4 patients); cisplatin, vinblastine, and bleomycin (2 patients); and carboplatin alone (1 patient).

Adequate material from fresh ovarian tissues was immediately processed for cyclin D1 expression analyses. Two samples of normal ovarian tissue, which were used as a control, were taken from the surrounding nonneoplastic tissue during surgical resection. Table 1 summarizes the characteristics of the patients according to tumor type, median age, and histology.

Western Blot Analysis. Cell suspensions were washed twice in cold PBS and dissolved in lysis buffer [1% Triton X-100, 0.15 M NaCl, and 10 mM Tris (pH 7.4)] containing protease inhibitors (50 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) at 4°C for 30 min. The protein concentration was determined by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Equal amounts of total protein (20 μg) were separated on a 12% polyacrylamide gel (SDS-PAGE). A duplicate of the same gel was stained with Coomassie Blue to ascertain that an equal amount of proteins was loaded in each lane. After transfer onto a nitrocellulose membrane (Hybond C-Extra; Amersham Italia Srl, Milan, Italy), protein loading was checked by Ponceau S staining, and nonspecific binding was blocked with BSA (Sigma Chemical Co., St. Louis, MO) in Tris-buffered saline-Tween 20 [0.15 M NaCl, 10 mM Tris (pH 7.4), 0.05% Tween 20] at 4°C overnight. Blots were probed with the anti-cyclin D1 monoclonal antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the horseradish peroxidase-conjugated antimouse IgG (DAKO, Glostrup, Denmark), a cyclin D1 band (M_r 36,000) was visualized by chemiluminescent detection (enhanced chemiluminescence; Amersham) following the supplier's recommended procedures. Prestained molecular weight markers (New England Biolabs, Beverly, MA) were used as a reference.

RT[2]-PCR Analysis. Specific cyclin D1 transcript levels were determined by a semiquantitative RT-PCR amplification. Total RNA from fresh ovarian samples was isolated by the RNAzol B method (Biotecx Laboratories, Houston, TX). RT was carried out on 2 μg of total RNA by the RETROscript first-strand synthesis kit (Ambion, Austin, TX), according to the manufacturer's suggestions.

Synthetic primers were purchased from TIB-MolBiol (Berlin, Germany). Cyclin D1 primers were 5′-GGATG-CTGGAGGTCTGCGAGGAAC-3′ (upstream) and 5′-GAGA-GGAAGCGTGTGAGGCGGTAG-3′ (downstream). β-Actin primers were 5′-GTCGGAAGGTGGACAGCGA-3′ (upstream) and 5′-GGCATCGTGATGGACTCCG-3′ (downstream).

The coamplification of the cyclin D1 and β-actin cDNAs was performed using Taq polymerase (Sigma Chemical Co.) with the following thermocycle parameter: 5 min at 94°C and 30 cycles of 1 min at 94°C, 1.5 min at 65°C, and 1.5 min at 72°C, followed by a final incubation at 72°C. RT-PCR-amplified fragments (cyclin D1, 514 bp; β-actin, 600 bp) were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The ϕX174 DNA marker *Hae*III digest (Sigma Chemical Co.) was loaded as a reference. Densitometric scanning of the bands was carried out on positive/negative instant film (Type 665; Polaroid Co., Cambridge, MA). The resulting bands were quantified, and the relative amount of cyclin D1 mRNA was estimated after normalization with β-actin mRNA detected in the same sample.

Densitometry and Statistical Analysis. Quantitative determination of the cyclin D1 protein and mRNA bands was performed by scanning densitometry using a Ultroscan XL densitometer (Pharmacia, Uppsala, Sweden). The intensities of the bands from films were scanned, and the resulting peak areas related to the absorbance were determined. The densitometric values were classed as undetectable (≤0.001), detectable

[2] The abbreviation used is: RT, reverse transcription.

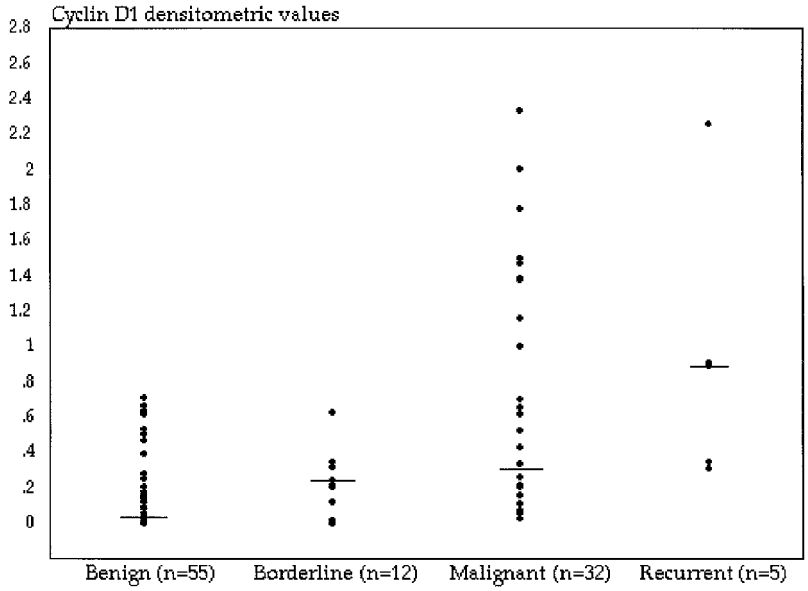

Fig. 1 Cyclin D1 protein expression levels in different types of ovarian tumors. Cyclin D1 values were obtained by densitometric scanning of the Western blot bands. *Bars*, median cyclin D1 value (benign tumors, 0.038; borderline tumors, 0.226; primary malignant lesions, 0.300; recurrent tumors, 0.887). A significant trend over the whole sample was found by the Kruskal-Wallis test ($P < 0.00001$). Comparison of groups: benign *versus* borderline tumors, $P = 0.01$; benign *versus* overall malignant tumors, $P = 0.0001$; borderline *versus* overall malignant tumors, $P = 0.12$.

(0.020–0.20), well detectable (0.21–0.80), and highly detectable (≥0.81).

The correlations between cyclin D1 protein and mRNA levels were evaluated by the Spearman rank test. The association between cyclin D1 densitometric values and tumor types as well as clinicopathological characteristics of patients with malignant diseases was investigated by the nonparametric Kruskal-Wallis test. The survival and progression-free survival analyses of patients with primary ovarian cancer were estimated by the Kaplan-Meier product-limit method. Comparison of survival between cyclin D1-expressing groups was evaluated by the log-rank test. Results were considered significant if P was <0.05. Statistical analyses were carried out using the Statistica 4.1 program (StatSoft, Tulsa, OK).

RESULTS

A highly significant relationship between cyclin D1 protein densitometric values and tumor types was found by the Kruskal-Wallis nonparametric test ($P < 0.000001$). A progressive increase in cyclin D1 expression with the degree of malignancy from benign to recurrent ovarian tumors was reported in the sample population of 104 patients. As shown in Fig. 1, a lower median cyclin D1 value was detected in benign tumors than in borderline diseases and carcinomas: 0.038 (0.001–0.705) *versus* 0.226 (0.001–0.623) *versus* 0.347 (0.027–2.330), respectively. In addition, among the malignant lesions, higher cyclin D1 expression was observed in recurrent tumors than in primary tumors [0.887 (0.309–2.260) *versus* 0.300 (0.027–2.330)].

Seventy-six ovarian samples (43 benign, 5 borderline, and 28 malignant tumors) were evaluable for RT-PCR analysis and categorized according to cyclin D1 signal intensity as described in "Materials and Methods." Representative patterns of cyclin D1 expression from tumor samples (right ovary, left ovary, and omentum) and adjacent unaffected tissue of one patient with a papillary serous carcinoma are shown in Fig. 2. By the Spearman rank test, a significant agreement between cyclin D1 protein and transcript was observed ($P = 0.00001$; $R = 0.554$). Cyclin D1 mRNA expression was lower in benign tumors and increased in malignant lesions; the vast majority of the benign lesions (67.4%) show undetectable levels of the mRNA, whereas all of the borderline tumors and 96% of the carcinomas exhibited detectable to highly detectable cyclin D1 (Fig. 3).

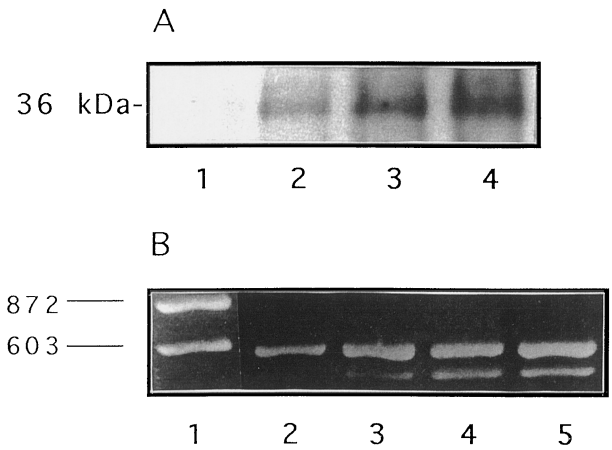

Fig. 2 Expression of cyclin D1 from a case of papillary serous ovarian carcinoma with adjacent unaffected tissue. *A*, representative Western blot. *Lane 1*, unaffected tissue (undetectable); *Lane 2*, left ovary (detectable); *Lane 3*, omentum (well detectable); *Lane 4*, right ovary (highly detectable). The position of the cyclin D1 protein (M_r 36,000) is indicated on the *left*. *B*, representative RT-PCR-amplified fragments (*top band*, β-actin, 600 bp; *bottom band*, cyclin D1, 514 bp). *Lane 1*, DNA molecular size markers (bp); *Lane 2*, unaffected tissue (undetectable); *Lane 3*, left ovary (detectable); *Lane 4*, omentum (well detectable); *Lane 5*, right ovary (highly detectable).

The cyclin D1 expression of the 32 primary carcinomas

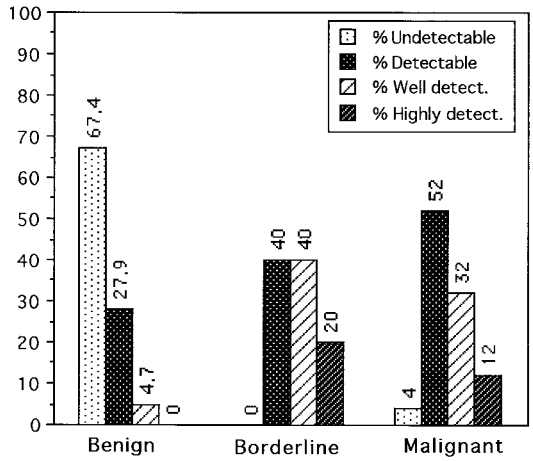

Fig. 3 Distribution of cyclin D1 mRNA expression by RT-PCR among 76 patients with benign, borderline, and malignant ovarian lesions.

was then analyzed in relation to different clinicopathological characteristics and evaluated for potential prognostic significance. Table 2 shows the distribution of the various parameters with the correspondent median cyclin D1 values and statistical analyses. Some of the clinical covariates such as age, performance status, and residual disease after primary surgery did not reveal significant differences in cyclin D1 expression. When the biological covariates of the tumors were taken into account, significantly different patterns of cyclin D1 expression were observed. Increased median cyclin D1 content was reported in serous carcinomas as compared to mucinous tumors ($P = 0.037$) and endometrioid tumors ($P = 0.029$), yielding borderline significance when all of the histotypes were considered together ($P = 0.058$). Moreover, a significantly higher median cyclin D1 value was observed in poorly differentiated lesions than in the well-differentiated lesions ($P = 0.025$), although this significance was not retained after the inclusion of the moderately differentiated tumors in the analysis. In addition, comparison of cyclin D1 content between stage I–II tumors and stage III–IV tumors resulted in a statistically significant difference, with higher cyclin D1 expression in the more advanced tumors ($P = 0.003$).

To examine the impact of cyclin D1 expression on patient outcome, overall survival and progression-free survival analyses were calculated by the method of Kaplan-Meier, and the difference between curves was evaluated by the log-rank test. Patients ($n = 32$) with tumors having detectable or well/highly detectable cyclin D1 levels were selected. As shown in Fig. 4, median survival time was not reached for both the patient groups, and no significant difference in survival was observed between the two curves at 42 months. When the disease-free survival was taken into account, a median progression-free interval of 33 months was reported in the 24 evaluable patients, and a significant increase in the progression-free interval (median not reached) was shown in patients whose tumors exhibited detectable cyclin D1 content ($P = 0.031$) *versus* those with well/high cyclin D1 levels (median progression-free survival, 22 months), as in Fig. 5.

DISCUSSION

The aim of the current study was to assess the role of cyclin D1 expression in the pathogenesis of ovarian tumors and define its prognostic impact in the subset of patients with primary malignant disease. To the best of our knowledge, this is the largest study on cyclin D1 in ovarian tumors carried out at the protein and mRNA levels, analyzing the occurrence of ovarian transformation by altered cyclin D1 expression. Data from Western blotting in this series of 104 patients with benign, borderline, and malignant ovarian lesions revealed a strong correlation between an increase in cyclin D1 expression and a feature of malignancy that was also reflected at the mRNA level. These results are consistent with our preliminary findings obtained in a small series of 33 patients and allow us to draw firmer conclusions than those suggested previously (18). In the present study, the association between cyclin D1 and clinicopathological parameters was also examined, and results from this analysis reported significantly higher cyclin D1 values in serous carcinomas, poorly differentiated tumors, and advanced-stage diseases.

The above-mentioned findings, however, are not fully in agreement with those of Worsley *et al.* (15) and Masciullo *et al.* (16), who evaluated the expression of cyclin D1 in ovarian tumors in relation to histological subtypes and clinicopathological variables, respectively. The first study was carried out by immunohistochemistry on paraffin-embedded blocks of 43 sporadic ovarian carcinomas (including three borderline tumors). The authors reported a 26% overexpression of cyclin D1 that was significantly associated with borderline or well-differentiated tumors. Only nuclear staining for cyclin D1 was scored as positive, and cytoplasmic-stained cells were not considered in the analysis. In the present work, whole cell lysates were processed for Western blot. This might account for the higher cyclin D1 protein expression and might also reflect the different sensitivity between methodologies. The second study also showed a small portion (18%) of cyclin D1-overexpressing tumors in a series of 65 ovarian carcinomas (56 primary and 9 recurrent tumors) analyzed by Northern blot on frozen tissue samples. The ovarian cancer cell line A2780 was considered as a reference, and tumor samples showing a 2-fold increase in cyclin D1 mRNA levels over the A2780 control were scored as positive. Elevated levels of cyclin D1 transcripts were significantly correlated with well-moderately differentiated neoplasms, but significance was not reached with any of the other clinicopathological variables. None of the samples showed amplification of the cyclin D1 gene, and no association between cyclin D1 mRNA levels and clinical outcome was shown. The lower sensitivity of the Northern blot methodology, as compared to RT-PCR, might explain the small portion of cyclin D1-overexpressing tumors in the series studied by Masciullo *et al.* as well as the lack of significance between cyclin D1 and well-known clinical prognostic factors in ovarian cancer. On this basis, it seems clear that the different analytical techniques and, above all, the different scoring systems (*i.e.*, arbitrary definition of the degree of cyclin D1 expression) used do not presently allow valuable comparisons and definite conclusions. In the present study, data on clinical outcome were

Table 2 Cyclin D1 and clinicopathological characteristics of 32 patients with primary ovarian malignant tumors

Covariate	No. of patients	Median cyclin D1 (range)	Kruskal-Wallis statistics	*p*
Overall	32	0.30 (0.03–2.33)		
Age (yrs)				
≤58	16	0.39 (0.03–2.00)	0.000008	1.000
>58	16	0.28 (0.03–2.33)		
ECOG PS[a]				
0	9	0.61 (0.03–1.38)	0.143	0.705
≥1	14	0.30 (0.06–2.33)		
Histology				
Endometrioid	9	0.07 (0.03–2.33)	7.454	0.058
Serous	15	0.61 (0.06–2.00)		
Mucinous	2	0.13 (0.05–0.21)		
Other	5	0.66 (0.16–1.16)		
Residual disease				
≤2 cm	14	0.15 (0.03–1.78)	1.300	0.254
>2 cm	13	0.43 (0.06–2.33)		
Grading				
1	5	0.07 (0.05–0.66)	4.137	0.126
2	10	0.27 (0.03–2.33)		
3	16	0.48 (0.06–1.78)		
FIGO stage[b]				
I–II	7	0.05 (0.03–0.61)	8.382	0.003
III–IV	25	0.43 (0.06–2.33)		

[a] ECOG PS, Eastern Cooperative Oncology Group performance status; unknown cases are not listed.
[b] FIGO, Fédération Internationale des Gynaecologistes et Obstetristes.

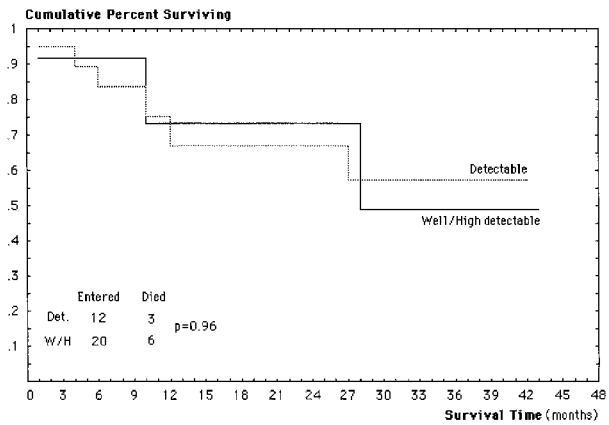

Fig. 4 Relationship between cyclin D1 protein expression and survival in patients with primary ovarian cancer as assessed by the Kaplan-Meier analysis. The statistical significance was estimated by the log-rank test.

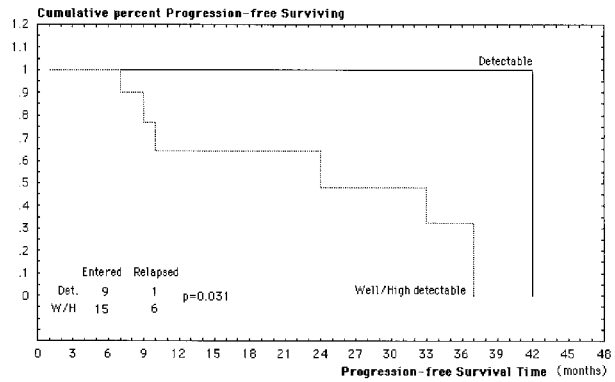

Fig. 5 Relationship between cyclin D1 protein expression and progression-free survival in patients with primary ovarian cancer as assessed by Kaplan-Meier analysis. The statistical significance was estimated by the log-rank test.

collected for all primary carcinomas, and, despite the low number of evaluable patients, analyses of prognosis were attempted. During the follow-up period of this investigation, progression by disease was available in 24 patients, and a significant advantage in disease-free interval for patients with lower levels of cyclin D1 expression was shown. Conversely, the lack of association with survival might be due to the fact that the vast majority of the patients (81%) had been treated with chemotherapy. Previous studies reported a positive association between cyclin D1 overexpression and prognosis, particularly in non-small cell lung cancer (19), pancreatic carcinoma (20), squamous cell carcinoma of the head and neck (21), colorectal adenocarcinoma (22), and soft tissue sarcoma (23). However other authors showed contrasting results, providing shorter survival for tumors expressing negative or low amounts of cyclin D1 such as non-small cell lung cancer (24) and breast cancer (25). These discrepancies suggest that a definite role for cyclin D1 in human carcinogenesis is still unclear and that concurrent genomic abnormalities may play a crucial role in this process.

In conclusion, the present study in a larger series of patients with ovarian tumors confirms that the alteration of cyclin D1 expression may be a critical step and an early event in the development of ovarian cancer. In particular, differences in cyclin D1 expression seem crucial in transitions from benign to borderline or malignant disease. In addition, the prognostic

implications of cyclin D1 expression have been addressed, and although such preliminary results need to be validated in a larger number of tumors and a longer follow-up period, the pathogenetic role of cyclin D1 in ovarian cancer cannot be excluded.

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Clinical Cancer Research

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Federica Barbieri, Monica Cagnoli, Nicola Ragni, et al.

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