Review

Cell Cycle Genes in Ovarian Cancer: Steps Toward Earlier Diagnosis and Novel Therapies

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ABSTRACT

Human malignant tumors are characterized by abnormal proliferation resulting from alterations in cell cycle-regulatory mechanisms. The regulatory pathways controlling cell cycle phases include several oncogenes and tumor suppressor genes that display a range of abnormalities with potential usefulness as markers of evolution or treatment response in ovarian cancer. This review summarizes the current knowledge about these aberrations in malignant tumors of the ovary. We sought to divide cell cycle-regulatory genes into four subgroups on the basis of their predominant role in a specific phase or during the transition between two phases of the cell cycle.

INTRODUCTION

Cancer is frequently considered to be a disease of the cell cycle; alterations in different families of cell cycle regulators cooperate in tumor development. Molecular analysis of human tumors has shown that cell cycle regulators are frequently mutated in human neoplasms, which underscores how important the maintenance of cell cycle commitment is in the prevention of human cancer. Mammalian cell division is precisely regulated in a timely manner by a family of protein kinases, the cyclin-dependent kinases (CDKs), a group of serine/threonine kinases that form active heterodimeric complexes after binding to cyclins, their regulatory subunits. Regulation of CDK activity occurs at multiple levels, including cyclin synthesis and degradation, phosphorylation and dephosphorylation, CDK inhibitor (CKI) protein synthesis, binding and degradation, and subcellular localization. Orderly progression through the cell cycle involves coordinated activation of the CDK protein by binding to the cyclin partner. A succession of kinases (CDK4, CDK6, CDK2, and CDC2) are expressed along with a succession of cyclins (cyclins D, E, A, and B) as cells go from G_1 to S to G_2 to M phase (Fig. 1*A*).

Different CDK-cyclin complexes operate during different phases of the cell cycle. Active CDK-cyclin complexes phosphorylate target substrates, including members of the "pocket protein" family (pRb, p107, and pRb2/p130; refs. 1–3). G_1-S–phase transition in normal cells requires phosphorylation of the retinoblastoma protein pRb and the related proteins pRb2/p130 and p107 by CDKs, which causes the release of E2F transcription factors controlling various genes required for DNA synthesis and cell cycle control.

Endogenous inhibition of CDKs is also caused by two families of regulatory proteins induced under mitogenic stimuli: the INK4 family, comprising $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, which specifically inhibit CDK4 and CDK6 (4); and the CIP/KIP family, including $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which causes a broader range of inhibition and acts in a concentration-dependent manner (5). All CKIs cause G_1 arrest when overexpressed in cells by association and inhibition of the CDKs. INK4 proteins dissociate cyclin D-CDK complexes and redistribute the CIP/KIP proteins to CDK2, producing a double inhibition. At low concentrations, CIP/KIP family proteins enhance CDK4 association with cyclin D, increasing the activity of the complex, whereas at high concentrations, they inhibit kinase activity, presumably by increasing the stechiometry in the CDK complexes (6). The best studied events of the cell cycle are the G_1 phase preceding the DNA synthesis (S) phase and the mechanism that drives the cell across the restriction (R) point in late G_1, which is crucial for the cell's destiny toward division, differentiation, senescence, or apoptosis. Several studies suggest that traversion of the restriction point within the G_1 phase is the key event in cell cycle regulation and that the rest of cell cycle progression occurs almost automatically once the R point has been overcome (7). Several proteins can inhibit the cell cycle in G_1 phase; if DNA damage occurs, p53 accumulates in the cell and induces the p21-mediated inhibition of cyclin D-CDK. The frequent loss of G_1 regulation in human cancer has revealed targets for possible therapeutic intervention. In contrast to G_1 regulators, less is known about the genes that regulate the S, G_2, and M phases of the cell cycle such as cyclin A- and cyclin B-kinase complexes and their inhibitors. The significance of cell cycle-regulatory genes in carcinogenesis is underlined by the fact that most of them have been identified as proto-oncogenes or tumor suppressor genes.

OVARIAN CANCER: BACKGROUND

Ovarian cancer remains a highly lethal disease. In developed countries, ovarian cancer accounts for more deaths than all

Received 5/5/04; revised 9/10/04; accepted 9/10/04.
Grant support: Sbarro Health Research Organization and National Institutes of Health grants (A. Giordano).
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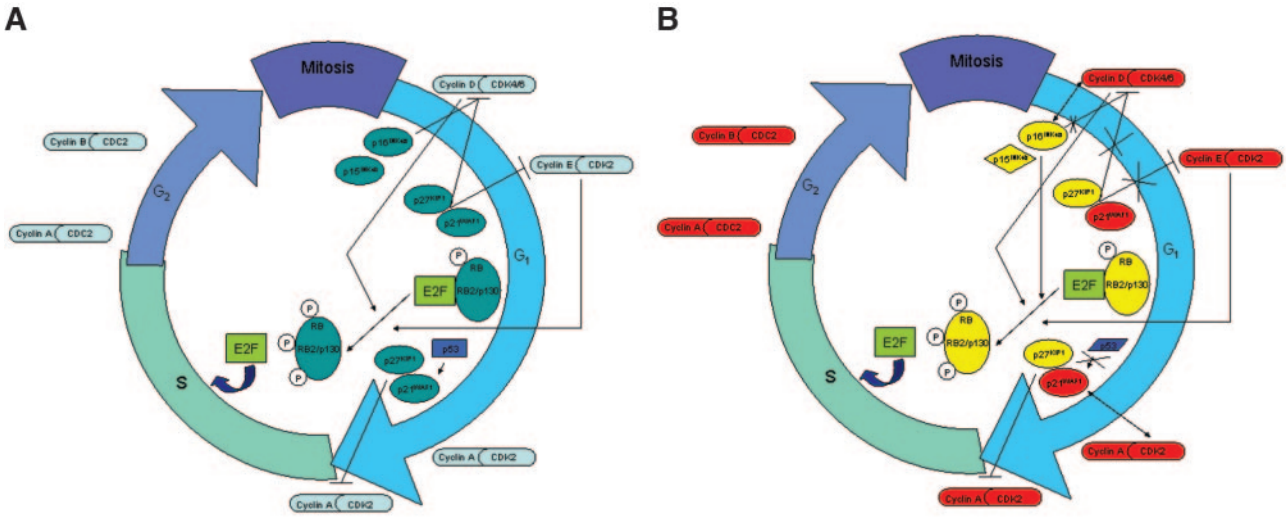

Fig. 1 *A*, schematic model of the normal mammalian cell cycle. Negative regulators of the cell cycle are indicated in *dark blue*, whereas positive controllers are shown in *light blue*. G_1-S transition in normal cells requires phosphorylation of the retinoblastoma proteins by CDKs, which causes the release of E2F transcription factors controlling various genes required for DNA synthesis during S phase. The CKIs $p21^{WAF1}$ and $p27^{KIP1}$ act by binding to cyclin-CDK2 complexes to inhibit their catalytic activity and induce cell cycle arrest, whereas $p16^{INK4a}$ inhibits CDK4/6. Wild-type *p53* activates transcription of the *p21* gene. *B*, deregulation of cell cycle machinery in ovarian carcinomas. Down-regulated genes are indicated in *yellow*, whereas up-regulated ones are shown in *red*. CDK4 and cyclin D overexpression has been reported to be associated with low p16 expression (see the *arrow* with *double heads*). The up-regulation of cyclin D and the down-regulation of p16 lead to enhanced pRb phosphorylation, thus inducing its inactivation. p27 down-regulation leads to an increase in cyclin E-CDK2 activity that contributes to ovarian carcinoma development. p15 is frequently affected by homozygous deletion in ovarian carcinoma (p15 deletion is indicated by a *diamond shape*). High p21 expression is associated with higher CDK2 levels (see the *arrow* with *double heads*). Mutated *p53* affects the p21 pathway.

other gynecological malignancies combined; it occurs in 1 of 57 women in the United States, and it is expected that 25,580 women will be diagnosed with the disease in the year 2004. As the result of advances in surgical management and chemotherapeutic options over the last three decades, the median survival for ovarian cancer patients has improved. However, overall survival has not been significantly altered. Although susceptibility genes such as *BRCA1* and *BRCA2* have been identified, a majority of ovarian cancers occur sporadically without known risk factors. In addition, most patients present with advanced disease, for which highly effective curative therapy is currently unavailable. Depending on the cell of origin, ovarian neoplasms can be divided into epithelial tumors that account for 90% of the malignant tumors, sex chord-stromal tumors, and germ cell tumors. The epithelial tumors are further divided into histologic subgroups with different malignant potential (namely, endometrioid, mucinous, serous, clear cell, and undifferentiated carcinomas).

In follow-up studies, mucinous and endometrioid carcinomas have a less aggressive behavior and a better overall survival than serous tumors. A characteristic of serous, mucinous, and endometrioid ovarian carcinomas is the low malignant potential or the presence of borderline tumors having a low risk of invasion. It is hoped that a better understanding of the molecular mechanisms underlying the tumorigenic process of ovarian carcinoma will lead to earlier diagnosis, novel therapies, and, ultimately, better outcomes.

We sought to classify the cell cycle-regulatory genes as G_1, G_1-S, S, and G_2-M regulators on the basis of their specific role during cell cycle progression.

G_1 REGULATORS

D-type cyclins are transcribed in the G_1 phase of the cell cycle. The isoforms D1, D2, and D3 are functionally equivalent, and they are expressed in a tissue-specific manner. CDK4 and CDK6 are activated by D cyclins to phosphorylate the retinoblastoma protein pRb, a known cell proliferation regulator. The members of the INK4 family exert their inhibitory activity by binding to the CDK4 and CDK6 kinases and preventing their association with D-type cyclins.

Cyclin D1. In contrast to other tumor types, the *cyclin D1* gene (*CCND1*) is rarely amplified in ovarian carcinomas. In 65 specimens analyzed by Masciullo *et al.* (8), the frequency of the overexpression of the gene was estimated to be 18%, but none of the tumors showed amplification of *cyclin D1*. No difference in *cyclin D1* mRNA levels was found between primary and recurrent disease. A statistically significant correlation was found between elevated levels of cyclin D1 and well to moderately differentiated grade (grade 1 to grade 2; $P = 0.005$), but no association with clinical outcome was found (8).

Overexpression of cyclin D1 was also observed by Dhar *et al.* (9) in 89% of 81 epithelial ovarian tumor sections in both borderline and invasive tumors. There was no association between protein overexpression and tumor stage or grade of differentiation. Furthermore, no correlation between cyclin D1 expression and clinical outcome was observed. Amplification of the *cyclin D1* gene was detected in only 1 of a subset of 29 tumors showing overexpression of cyclin D1 protein. The authors concluded that deregulation of *CCND1* expression leading to both cytoplasmic and nuclear protein localization is a fre-

quent event in ovarian cancer and occurs mainly in the absence of gene amplification (9).

Barbieri *et al.* (10) defined the pattern of cyclin D1 expression in the development of ovarian cancer in 55 cases of benign ovarian tumors, 12 borderline cases, and 37 ovarian carcinomas. A statistically significant increase in median cyclin D1 values was observed from benign to borderline to malignant to recurrent tumors. The authors found a significant relationship between cyclin D1 expression and progression-free survival ($P = 0.031$; ref. 10).

Cyclin D1 was found to be overexpressed mainly in borderline and low-grade ovarian tumors, in contrast with high-grade tumors, by Sui *et al.* (11). Within the malignant tumor group, the authors found an association between higher cyclin D1 expression and a well-differentiated phenotype (grades 1 and 2). The reason why cyclin D1 was decreased in the high-grade (grades 2 and 3) tumors is not clear. Some possible explanations can be made. First, there may be multiple pathways in the development of ovarian tumors. The authors hypothesized that *cyclin E* rather than *cyclin D1* plays a predominant regulatory role in the progression of ovarian carcinomas. Second, the tumor cellular origins of borderline and low-grade tumors may be different from those of high-grade tumors (11). These observations are similar to those obtained with breast cancer studies, in which cyclin D1 overexpression is associated with a favorable prognosis (12).

Cyclin D2. Similar to *cyclin D1*, the *cyclin D2* gene is only sporadically amplified in ovarian tumors. Courjal *et al.* (13) investigated *cyclin D2* expression in 237 ovarian tumors. Only on rare occasions did *cyclin D2* show increased DNA copy numbers, and it was never found to be overexpressed at the RNA level (13).

Expression studies on various tumor types have shown that more than 80% of the granulosa cell tumors, but only single epithelial tumors, express high levels of *cyclin D2* mRNA (14).

Milde-Langosch *et al.* (15, 16) detected cyclin D2 protein expression in all analyzed granulosa cell tumors ($n = 7$) but in only 23% of 93 ovarian epithelial carcinomas. Thus, cyclin D2 overexpression is characteristic of a single histologic tumor type that accounts for 6% of all ovarian malignancies.

Cyclin D3. Like the other D-type cyclins, *cyclin D3* is expressed during the G_1 phase in dividing cells, but much less is known regarding its expression, activity, and regulation. In human ovarian tumors, *cyclin D3* amplification has been sporadically reported, but up to now, cyclin D3 overexpression has not been described (13).

CDK4. Overexpression of *CDK4* has been found in 14% to 15% of a relatively large number of ovarian tumors on mRNA and protein levels (8), although gene amplification has not been demonstrated thus far (8). *CDK4* status has not been associated with clinical outcome (17).

CDK4 overexpression has been reported to be associated with an increased expression of *cyclin D1* (8) and low *pl6* expression (18). Sui *et al.* (18) described a significant increase of CDK4 activity in malignant ovarian tumors in contrast with benign tumors ($P < 0.01$), suggesting that CDK4 activity may play an important role in ovarian carcinogenesis.

$p16^{INK4a}$. *p16* has been widely investigated in ovarian tumors on the DNA, RNA, and protein levels. *p16* deletion occurred at a rate of 50% in 12 ovarian cancer cell lines analyzed by Fang *et al.* (19), despite the lack of such a frequency of mutation in primary ovarian cancer cells (20). They also observed that *p16* expression induced transcriptional down-regulation of the *RB* gene (19). Ovarian cancer cell lines coexpressing *p16* and *RB* are insensitive to *p16* overexpression, suggesting that tumors that express both genes may be unresponsive to *p16* gene therapy (21). Loss of expression of the *p16* tumor suppressor occurs more often in ovarian cancers lacking *p53* mutations (22), consistent with the paradigm that inactivation of *p53* is less important in ovarian carcinogenesis when another G_1 regulatory gene has already been inactivated. The growth-inhibitory effect of *p16* has been confirmed in a study carried out with the two ovarian cancer cell lines SKOV3 and OVCA-420 (23). In SKOV3 cells, G_1 arrest induced by *p16* transduction prevents paclitaxel- and vindesine-induced cell death (24). High-level *p16* expression was observed in serous and endometrioid phenotypes, with a positive relation to high levels of both cell proliferation and *p53* abnormalities. There was no association between mRNA and protein levels detected by immunohistochemical and Western blot analyses. None of 131 cases showed a methylation status of the *p16* gene promoter (25). Two different studies also revealed no evidence of methylation and low levels of mutations (26, 27). However, there are some data supporting *p16* promoter hypermethylation as a mechanism underlying the down-regulation of the gene. Milde-Langosch *et al.* (28) found hypermethylation in 12 of 19 negative cases, most of them moucinous and endometrioid carcinomas. Suh *et al.* (29) demonstrated that both promoter methylation and aberrant mRNA processing may interfere with *p16* expression in ovarian tumors. Kudoh *et al.* (30) found homozygous deletion in 18% of 45 patients analyzed and suggested that deletion of *p16* is a potential indicator for poor chemotherapy response and adverse prognosis in ovarian cancer patients. In a study carried out on 190 epithelial ovarian tumors, Dong *et al.* (31) found that a high number of p16-positive tumor cells was associated with advanced stage and grade and with poor prognosis. On the other hand, in a recent study, a significant influence of p16 expression on overall survival was not confirmed (15). Higher levels of p16 are expressed in retinoic acid-sensitive CAOV3 cells compared with retinoic acid-resistant SKOV3 cells (32).

$p15^{INK4b}$. The *p15* gene, which is located on 9p, contains sequences highly homologous to exon 2 of *p16*. The *p15* gene also inhibits both CDK4 and CDK6 kinase activities (33, 34). Little is known about the potential role of this gene in ovarian epithelial tumors. Ichikawa *et al.* (35) investigated the involvement of *p15* inactivation in ovarian tumorigenesis with 49 primary ovarian tumors and 6 ovarian cancer cell lines. Homozygous deletion was found in 10% of primary tumors, but mutation of *p15* was not detected in any sample. Alterations in *p15* were observed in serous, endometrioid, and clear cell carcinomas, but not in mucinous carcinomas, suggesting that inactivation of *p15* may be the histologic type-specific event in ovarian tumorigenesis (35). In contrast to this study, among 70 ovarian epithelial tumors, a *p15* mutation occurred in only a single ovarian tumor, and homozygous deletion of the *p15* gene was observed in only one additional case, suggesting that the *p15* gene may not play an important role in ovarian tumorigen-

esis (36). Kudoh *et al.* (30) found homozygous deletion of *p15* in 33% of 45 cases; moreover, the deletion of the gene was a potential indicator for poor chemotherapy response and a significant poor prognostic factor in advanced ovarian cancer.

A recent study has shown that homozygous deletion of *p15* may account for transforming growth factor β resistance in some populations of ovarian cancer cells (37).

G_1-S REGULATORS

Genetic analysis of human tumors has revealed that some of the molecules most often altered in cancer are those involved in the control of the G_1-S transition of the cell cycle, a time when cells become committed to a new round of cell division. During the G_1-S transition, the cyclin E-CDK2 and cyclin D-CDK4 complexes promote progression and are each inhibited by the associated CKI p27. If DNA damage occurs, p53 accumulates in the cells and induces the p21-mediated inhibition of cyclin D-CDK. The transition to S phase is triggered by the activation of the cyclin D-CDK complex, which phosphorylates pRb.

Cyclin E. Marone *et al.* (38) hypothesized that *cyclin E* and *CDK2* are, in part, coregulated and may have a role in ovarian tumor development after finding that *cyclin E* and *CDK2* are regulated in ovarian tumors by gene amplification and at the level of RNA transcriptional control.

Clear cell carcinoma revealed significantly increased cyclin E associated with an increase in p21, compared with the other histologic subtypes (39).

Sui *et al.* (40) found higher levels of cyclin E expression in ovarian carcinomas with respect to benign tumors, gradually increasing from benign (9.1%) to borderline (47.8%) to malignant ovarian tumors (70.2%; $P < 0.0001$). This finding indicated that cyclin E overexpression is closely associated with the malignant biological feature of ovarian tumors. In addition, cyclin E overexpression correlated with advanced clinical stage and the presence of ascites.

High mortality risk was associated with cyclin E overexpression [relative risk (RR), 2.02; $P = 0.034$], suggesting that increased cyclin E expression not only contributed to the development of ovarian malignancy but also correlated with the poor prognosis of ovarian carcinoma patients. Sui *et al.* (40) found that patients with p27(−) cyclin E (++)/CDK2 (++) had an almost 3-fold higher RR of mortality (RR, 2.91; $P = 0.0001$), which was independently associated with poor overall survival ($P = 0.035$). The prognostic relevance of *cyclin E* was also evaluated by Farley *et al.* (41) in 139 cases of primary advanced ovarian cancer. High cyclin E was associated with worse survival only in the subgroup of women who received the combination of cisplatin and Taxol. This may be influenced by the superior efficacy of a Taxol-containing regimen compared with the cytoxan regimen. Conversely, *cyclin E* expression may modulate the cell's sensitivity to Taxol. Cyclin E-associated CDK activity could be an important molecular complex for targeted therapy. It is interesting to hypothesize that effective inhibition of *cyclin E* may enhance ovarian cancer sensitivity to cisplatin and Taxol in combination (41).

CDK2. Gene amplification and overexpression of *CDK2* were found in only 6% of 119 ovarian carcinoma specimens analyzed by Marone *et al.* (38) in a study focusing on the role of both cyclin E and its associated kinase, CDK2. In most cases, CDK2 and cyclin E levels correlated with each other, indicating at least partial coregulation of these two genes. CDK2 expression has also been associated with p21 expression in the IGROV1 ovarian cancer cell line; both genes (CDK2 and p21) are expressed at higher levels with respect to benign ovarian tumors (42). The expression of cyclin E and CDK2 gradually increased from benign to borderline to malignant tumors in a study carried out on 103 cases, suggesting that overexpression of cyclin E or CDK2 was significantly associated with malignancy in ovarian tumors (40).

RB. Alterations in the retinoblastoma gene (*RB*) are common in human neoplasia. Among the RB family members, *RB* is the most investigated gene in ovarian cancer disease. The *RB* gene was found to be abnormal in four of six ovarian cancer cell lines analyzed, suggesting a role for this gene in the carcinogenesis of some human ovarian tumors at the point of *RB* gene inactivation (43). Dong *et al.* (31) showed that most of the malignant ovarian tumors among 125 specimens (71%) had a strong pRb expression compared with normal ovaries, in which the protein is hardly detectable. Reduced pRb expression was the significant predictor for poor prognosis in stage I patients. Moreover, the relationship between the expression of pRb and p16 depended on tumor stage: in stage I tumors, the authors found an inverse correlation, whereas most advanced tumors showed a direct correlation between pRb and p16 (31). It is also reported that Rb protein and mRNA are expressed at higher levels in cell lines lacking *p16* than in those with normal *p16* (19). These findings are in accordance with the knowledge that *RB* and *p16* tumor suppressor genes function in the same pathway of cell cycle control. Investigations regarding the pRb/cyclin D1/p16 pathway showed that coexpression of pRb, p16, and cyclin D1 is present in 82% of ovarian cancer tissues and cell lines, suggesting that defects in the pRb/cyclin D1/p16 pathway, other than the loss of pRb or p16, may play a major role in the development of ovarian cancer (21). Nieman *et al.* (44) showed that for most ovarian carcinomas, *RB* alteration is not necessary for the development of a malignant phenotype, and *RB* mutation, when it does occur, may represent a sporadic event in ovarian carcinogenesis. Ovarian cancer cells with wild-type pRb are sensitive to BRCA1-induced growth suppression, suggesting that pRb is involved in the growth suppressor function of BRCA1 (45). Diminished pRb levels are related to several clinicopathological indicators of aggressiveness in ovarian adenocarcinomas such as increasing grade, advancing stage, and bulk residual disease (46). Critical interactions between p53 and pRb pathways in ovarian carcinoma pathogenesis are emerging from a recent study by Flesken-Nikitin *et al.* (47), who provided direct genetic evidence that defects in p53- and pRb-mediated pathways cooperate in ovarian carcinogenesis.

RB2/p130. In a study in nude mice, Pupa *et al.* (48) showed that ectopic expression of pRb2/p130 suppresses the tumorigenicity of the SKOV3 ovarian cancer cell line overexpressing erb-2 both *in vitro* and *in vivo*. No alterations of the *RB2/p130* gene were found in 43 tumors analyzed by Alvi *et al.* (49). In a recent study on 45 primary ovarian carcinomas, pRb2/p130 protein was lost or decreased in 40% of the specimens, and the enhanced, adenovirus-mediated pRb2/p130 syn-

thesis leads to a drastic growth arrest in the G_1 phase of the cell cycle in ovarian cancer cell lines, suggesting its tumor suppressor function in ovarian cancer (50).

$p21^{CIP1/WAF1}$. *p21* is a CKI whose expression is usually induced by *p53* and that is responsible for the p53-dependent G_1 arrest in response to DNA damage.

Barboule *et al.* (42) showed that *p21* is able to inhibit CDK2-kinase activity and is therefore functional in the IGROV1 ovarian carcinoma cell line. This CDK inhibitory activity is bypassed at least by overexpression of CDK2 and cyclin A and perhaps also by proliferating cell nuclear antigen overexpression (42). Among 106 patients with epithelial ovarian cancer, 61% showed p21 expression associated with early tumor stage and no residual disease after primary resection. Unexpectedly, no association with tumor grade was found. High p21 expression, which was observed in only 11% of all cases, was related to a good prognosis. The clinical follow-up showed a better overall survival for cases with strong p21 expression *versus* cases with weak expression or no expression ($P = 0.033$; ref. 51). The association of p21 status with clinicopathological parameters and clinical outcome was also investigated in a series of 102 ovarian tissue samples including normal ovary, primary ovarian tumors, omental metastasis, recurrent disease, and residual tumor after chemotherapy exposure. In the group of stage III–IV ovarian cancer patients, p21-positive cases showed a more favorable prognosis than p21-negative cases: the 3-year time to progression rate was 58% for p21-positive cases and 33% for p21-negative cases ($P = 0.036$; ref. 52).

The expression of p21 was also assessed by immunohistochemistry in epithelial ovarian malignancies in relation to p53 status, cell proliferation, and patient survival. Low p21 expression was significantly associated with high-grade tumor ($P = 0.0005$), advanced FIGO (International Federation of Gynecologists and Obstetricians) stage ($P = 0.001$), and primary residual tumor ($P = 0.0001$). Low levels of p21 were also considered a marker of poor overall survival. The combination of p53 expression with the absence of p21 expression was strongly associated with poorer disease-free and overall survival, and p21/p53 expression independently predicted tumor recurrence (53, 54). Conversely, the combination of p21-positive and p53-negative cases was found to be a better independent indicator of prognosis and survival in patients with ovarian carcinoma than either p21 or p53 alone (55). In a recent study on a series of 267 patients, Rose *et al.* (56) surprisingly found, for the first time, compromised survival for patients with p53-null/p21-positive tumors ($P = 0.005$).

p21 was found to be overexpressed in 48% of a series of 185 uniformly treated patients with stage III ovarian cancer but did not show prognostic significance. p21 was not found to be predictive for response to chemotherapy in this large group of patients with advanced ovarian cancer (57). p21 positivity was not a significant predictor of favorable outcome. There was no relationship demonstrated between p21 expression and chemotherapy response in patients treated postsurgically with cisplatin or carboplatin as single agents or together with other chemotherapeutics (58).

By examining the effect of p21 on the response to cisplatin, Lincet *et al.* (59) found that the cytotoxic effect of the drug was enhanced, as demonstrated by the increased rate of cell death.

$p27^{Kip1}$. The *p27* gene is rarely affected by structural alterations in human malignancies. *p27* is a CKI that regulates progression from G_1 into S phase by inhibiting a variety of cyclin-CDK complexes, including cyclin D-CDK4, cyclin E-CDK2, and cyclin A-CDK2. Newcomb *et al.* (60) showed that p27 expression is positively associated with long-term survival in 66% of patients separated into two equal groups, one group of long-term survivors (>5 years) and the other of short-term survivors (<2 years). Baekelandt *et al.* (57), in contrast with the results of Newcomb *et al.* (60), demonstrated only a trend toward reduced survival ($P = 0.092$) in 185 unselected patients. The complete loss of p27 protein expression was a rare event (6% of cases; ref. 57).

Loss of p27 expression (33% of cases) did not correlate with any of the clinicopathological parameters used to predict clinical outcome, but it was associated with short time to progression of the disease in 82 ovarian cancer patients analyzed by Masciullo *et al.* (61). This association was also retained after the exclusion of stage I and stage II tumors, further supporting the hypothesis that loss of p27 confers a more aggressive phenotype to tumor cells and therefore might play an important role in the development of ovarian cancer (61).

Masciullo *et al.* (62) also demonstrated that expression of p27 is a strong predictor of longer time to progression and overall survival in 99 patients with advanced stage ovarian cancer. Moreover, the association between loss of p27 expression and poor survival remained significant after stratification according to the residual tumor at the first surgery, which represents a parameter that plays a major role in affecting response to chemotherapy and survival.

p27-positive cases showed a higher percentage of response to chemotherapy, especially in the group of patients optimally cytoreduced at the first surgery (62).

In another study (40), p27 expression was detected by immunohistochemistry in 75.8%, 78.3%, and 36.2% of cells in benign, borderline, and malignant tumors, respectively. Western blot analysis also showed lower expression of p27 in ovarian carcinomas than in benign tumors. In addition, loss of p27 expression was associated with tumor grade ($P = 0.003$), lymph node metastasis ($P = 0.002$), and residual disease ($P = 0.016$; ref. 40). The discrepancy with the results obtained by Masciullo *et al.* (61) may be explained by different criteria for interpretation of the immunohistochemical pattern of p27 expression as well as for patient selection.

Sui *et al.* (63) examined Jab1, a transcrptional coactivator of AP1 proteins (especially c-Jun and Jun D), and p27 protein expression in 80 ovarian carcinomas. Jab1 expression increased from normal ovarian epithelium to benign tumor to malignant tumor. An inverse statistically significant correlation between Jab1 and p27 expression was found in both benign ($P = 0.003$) and malignant ($P = 0.002$) ovarian tumors. The authors suggested that Jab1 can specifically interact with p27 protein and accelerate its degradation (63). p27 degradation is thought to be the main mechanism responsible for the down-regulation of p27 protein in human tumors (64) because the transcriptional mechanism of this gene is not altered. This suggests that proteins involved in p27 degradation may have oncogenic properties such as Skp2, a protein known to be a component of a ubiquitin ligase complex specific for p27. In fact, increased Skp2 levels

are associated with reduced p27 expression, suggesting that increased Skp2 expression may have a causative role in decreasing p27 expression in epithelial ovarian tumors (65). p27 protein modulation is also likely to be involved in all-*trans*-retinoic acid (ATRA)-induced growth inhibition in ovarian carcinoma cells, given that this protein was found to be up-regulated in these cells after ATRA treatment (66); an ATRA-dependent decrease in Skp2 protein level was also found in the cells, suggesting that Skp2 could play a role in p27 protein up-regulation by inhibiting its degradation by the proteasome.

In a recent study, Plisiecka-Halasa *et al.* (67) performed immunohistochemical analysis of multiple biomarkers in 204 ovarian cancer patients. They demonstrated that overall survival was positively influenced by p21 plus p27 expression only in p53-negative patients. They concluded that, considering multiple parameters, the prognostic value of p27 was strongly determined by p53 status (67).

$p57^{Kip2}$. *p57* is another member of the CIP/KIP family of CKIs that shares structural similarities with *p27*, but its expression is restricted mainly to the gastrointestinal tract. According to the study led by Rosenberg *et al.* (68), more than 95% of the ovarian carcinomas analyzed showed intense nuclear staining, regardless of patient survival data. In this study, p57 was not associated with prognosis, unlike p27 expression, which has previously been shown to be positively associated with long-term survival (60).

In contrast, decreasing p57 expression from benign to borderline to malignant tumors was found by Sui *et al.* (69), and low p57 expression was significantly associated with high tumor grade. When the combined phenotype of p57 and p27 was analyzed, the patients with both p57 and p27 low expression had a lower overall survival rate (69).

p53. Because *p53* has been widely reviewed in the literature, here we will briefly point out its role in ovarian cancer. It is well known that somatic mutation of *p53* represents the most common molecular genetic alteration occurring in epithelial ovarian carcinoma. Inactivation of p53 was detected in 30% to 80% of ovarian carcinoma (70, 71). The frequency of p53 mutation in early-stage ovarian carcinomas of serous histology is comparable with that reported for advanced-stage tumors, and it is therefore likely to occur early in the progression of the most common histologic variant of ovarian carcinoma (72). For invasive carcinomas, the rate of mutation and expression increases with increasing tumor grade and stage, and is more common in tumors of serous histology (73). In addition to germ-line BRCA1 and BRCA2 mutations, somatic p53 alteration leading to p53 accumulation is an important event in hereditary ovarian cancer and is as frequent as in non-BRCA–related ovarian cancer (74). Epithelial ovarian tumors showing p53 alterations are significantly less sensitive to chemotherapy and more aggressive than those with functional p53, and overall survival is shortened in patients with p53 mutations (75, 76).

S REGULATORS

In S phase, phosphorylation of components of the DNA replication machinery by cyclin A-CDK is believed to be important for initiation of DNA replication and to restrict the initiation to only once per cell cycle.

Cyclin A. *Cyclin A* is a particularly interesting member of the cyclin family because it can activate two different CDKs and functions in both S phase and mitosis. In mitosis, the precise role of *cyclin A* is still obscure, but it may contribute to the control of cyclin B stability. Consistent with its role as a key cell cycle regulator, expression of cyclin A is found to be elevated in a variety of tumors. Courjal *et al.* (13) found neither amplification nor mRNA overexpression of *cyclin A* in a set of 237 ovarian tumors. Cyclin A is expressed at higher levels in the IGROV1 ovarian carcinoma cell line than in normal cells (42). In an immunohistochemical study, cyclin A staining was detected in 53% of the serous carcinomas, 40% of the poorly differentiated carcinomas, 29% of the endometrioid carcinomas, but none of the mucinous and clear cell carcinomas (39), and there was a significant association between cyclin A and p53 expression, varying among the different histologic types. Wild-type *p53* is a transcriptional repressor of *cyclin A* (77), and increased cyclin A expression in p53-positive cases might result from *p53* mutations, leading to loss of this repressor activity.

G_2-M REGULATORS

Transition from G_2 to M phase involves destruction of cyclin A and ascendancy of cyclin B. The protein phosphatase cdc25 removes inhibitory phosphates from CDK1-cyclin B complexes. During the normal cell cycle, negative regulation by phosphorylation of cyclin B/cdc2 prevents premature mitotic entry before the completion of S phase.

Cyclin B, cdc25A, cdc25B, and cdc2. Cyclin B1 is the regulatory subunit of the cdc2 kinase and is a protein required for mitotic initiation. The ability of p53 to control mitotic initiation by regulating intracellular cyclin B1 levels suggests that the cyclin B-dependent G_2 checkpoint has a role in preventing neoplastic transformation. The analysis of gene expression profiles in 4 normal and 27 neoplastic ovarian tissues by oligonucleotide microarrays revealed high expression of *cyclin B*, *cdc25B*, and *cdc2* in a subset of tumor samples and most ovarian carcinoma cell lines (78). *cdc25A* overexpression was found in 88% of tumorigenic ovarian cancer cell lines, but in only 20% of nontumorigenic ovarian cancer cell lines (79), and Broggini *et al.* (80) found an association of cdc25A- and cdc25B-positive immunostaining with an unfavorable outcome in 106 patients. These findings suggest that regulators of the G_2-M transition might be useful prognostic indicators in ovarian carcinomas (80).

CONCLUSIONS

Among the G_1 regulators, *cyclin D1*, *CDK4*, and *p16* play a crucial role in ovarian cancer tumorigenesis and development. Cyclin D1 seems to be the D-type cyclin most involved in ovarian tumors. Low expression of cyclin D1 seems to promote the development of ovarian tumors. Cyclin D1 is overexpressed in borderline and invasive tumors, although there is no association with clinical outcome. Cyclin D2 overexpression is characteristic of a single histologic tumor type, the granulosa cell tumor, which accounts for 6% of all ovarian malignancies. On the contrary, cyclin D3 overexpression has not been described in ovarian tumors. CDK4 activity increases in malignant tumors with respect to benign tumors, suggesting its important role in

ovarian carcinogenesis. The tumor suppressor *p16* (*INK4a* gene), like *p15* (*INK4b* gene), when deleted, is an indicator of poor chemotherapy response and unfavorable prognosis. There are controversial results about the methylation status of the *p16* promoter. No evidence of hypermethylation was found in a high percentage of p16-negative cases in a number of different studies; however, there are some reports demonstrating hypermethylation of *p16* promoter in a significant number of negative cases. Also, the prognostic value of the status of *p16* in ovarian carcinomas is controversial. There are some data demonstrating the association between p16 positivity and poor prognosis as well as data demonstrating the lack of correlation between p16 expression and overall survival. Lack of p16 is associated with p53 wild-type and is typical of mucinous and endometrioid tumors. Ovarian tumors that express both *p16* and *RB* are insensitive to *p16* introduction in the ovarian cancer cells, suggesting that tumors expressing both genes may be unresponsive to *p16* gene therapy. Little is known about the role of *p15* in ovarian carcinomas. No mutations have been detected. Its inactivation may be the histologic type-specific event in ovarian tumorigenesis. Alterations in the *p15* gene occur in serous, endometrioid, and clear cell carcinomas, but not in mucinous carcinomas. Most of the G_1-S regulators play an important role in ovarian cancer due to the fact that they control the G_1-S transition, a crucial step in cell cycle control. *Cyclin E* is a key regulator of the G_1-S transition. Abnormalities in *cyclin E* expression have been related to survival in a variety of cancers. In ovarian cancer, cyclin E overexpression is a frequent event and is closely associated with the malignant biological feature of ovarian tumors as suggested by the observation that its expression increases from benign to borderline to malignant tumors and correlates with advanced clinical stage and poor survival. *CDK2*, like *CDK4*, is also associated with the malignancy of ovarian tumors. *p27*, a member of the CIP/KIP family, plays an important role in ovarian cancer development, as suggested by the observation that its loss confers a more aggressive phenotype to tumor cells; in fact, its expression decreases from benign to borderline to malignant tumors. p27 expression is positively associated with long-term survival. The prognostic value of p27 is strongly determined by *p53* status. A better overall survival is linked to *p53* status (p53 negative) in the presence of p21 expression. The role of p21 as a prognostic factor alone or in association with other markers has been widely investigated. Low p21 expression can be considered a marker of poor overall survival. Conversely, patients with strong p21 expression *versus* those with weak or no p21 expression show a better overall survival. p21 does not seem to be predictive for response to chemotherapy. Compromised survival is associated with p53-null/p21-positive tumors, but this combination is a better independent indicator of prognosis and survival than either p21 or p53 alone. The actual mutation of *p53* is the most common molecular alteration occurring in both early-stage and invasive ovarian carcinomas, especially in those of serous histology, and confers resistance to chemotherapy and shortened overall survival. According to one (68) of the only two studies reported in the literature regarding p57 in ovarian tumors, most ovarian cancers show an intense nuclear staining of p57, but it is not associated with prognosis; however, in the second study (69), low p57 expression was significantly associated with high tumor grade, and patients with low expression of both p57 and p27 have a lower overall survival. The retinoblastoma family member *RB* seems to have a role in the carcinogenesis of some human ovarian tumors. Loss of pRb expression might contribute to enhanced proliferation in early ovarian tumorigenesis, but in later stages, the carcinomas might become independent of pRb expression. Because *RB* and *p16* tumor suppressor genes function in the same pathway of cell cycle control, most advanced tumors show a direct correlation between pRb and p16. Diminished pRb levels are related to several clinicopathological indicators of aggressiveness in ovarian adenocarcinomas such as increasing grade, advancing stage, and bulk residual disease. There is direct genetic evidence that defects in p53- and pRb-mediated pathways cooperate in ovarian carcinogenesis. pRb2/p130 is down-regulated in 40% of ovarian carcinomas, and its tumor suppressor function has been demonstrated in ovarian cancer cells, but no mutations of this gene have been detected in ovarian tumors. The expression of the S regulator *cyclin A* varies among different histotypes; its expression decreases from serous to poorly differentiated to endometrioid carcinomas, and it is absent in mucinous and clear cell carcinomas. Increased *cyclin A* expression is reasonably associated with *p53* status because *p53* wild-type is a transcriptional repressor of *cyclin A*. The G_2-M regulator *cyclin B*, together with *cdc25A* and *cdc25B*, is a useful prognostic factor in ovarian cancer; high expression of *cyclin B* is associated with an unfavorable outcome. These findings suggest that regulators of the G_2-M transition might be useful prognostic indicators in ovarian carcinomas.

The described abnormalities of cell cycle regulators in ovarian carcinomas (Fig. 1*B*; Table 1) suggest that most of the cell cycle-regulatory genes play a crucial role in ovarian cancer tumorigenesis and/or development. The main goal in cancer therapy remains an early diagnosis of the disease, and some of the cell cycle genes described could be useful markers for achieving this goal and therefore developing more targeted therapies. In the study of gynecological cancers, we must also take into consideration the involvement of hormones, in partic-

Table 1 Type of alteration found in the cell cycle-regulatory genes involved in ovarian cancer

Gene	Alteration	Reference no.
Cyclin D1	Overexpression	8–11
Cyclin D2	Overexpression (granulosa cell tumor)	13–15
Cyclin D3	No alteration	13
Cdk4	Overexpression	8, 17, and 18
p16	Down-regulation	19, 22, and 30
p15	Homozygous deletion	30, 35, and 36
Cyclin E	Overexpression	38–41
Cdk2	Overexpression	38, 40, and 42
RB	Down-regulation	46
RB2/p130	Down-regulation	50
p21	Overexpression	52 and 53
p27	Down-regulation	57, 61, and 63
p57	No alteration/down-regulation	68 and 69
p53	Mutation	72
Cyclin A	Overexpression	42
Cyclin B	Overexpression	78
Cdc25A	Overexpression	79 and 80
Cdc25B	Overexpression	78 and 80
Cdc2	Overexpression	78

ular estrogen, which is known to stimulate the proliferation of epithelial cells in the female tract and mammary gland. The unanswered question is: are the cell cycle-regulatory genes controlled by estrogens in ovarian cancer? And, if so, in what way? Epidemiologic evidence strongly suggests that steroid hormones, primarily estrogens and progesterone, are involved in ovarian carcinogenesis (81, 82). However, it has proved difficult to fully understand their mechanism of action in the tumorigenic process. Recent data identify *cyclin D1*, *p21*, and *cyclin E-cdk2* as central components of estrogen regulation of cell cycle progression and therefore as potential downstream targets that contribute to the role of estrogen in breast cancer oncogenesis (83).

Estrogen receptor (ER)-α and ER-β, which function as transcription factors to regulate the expression of target genes, carry out and modulate the effects of estrogen. In ovarian cancer cells, it has been shown that expression of ER-α is increased compared with that of ER-β (84). On the other hand, we demonstrated that multimolecular complexes recruited by pRb2/p130 can be key elements in the regulation of ER-α gene expression and may be reviewed as promising targets for the development of novel therapeutic strategies in the treatment of breast cancer (85). More studies are needed to understand where these findings in breast and ovarian cancer can converge toward new therapeutic strategies.

ACKNOWLEDGMENTS

We thank Marie Basso for assistance in editing the text.

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