Journal of Genetics and Molecular Biology
Vol. 14, No. 1, 24-29, March 1, 2003

Expression of Cyclin D1 and p16^{INK4} Gene Products in Human Ovarian Cancer

Chin-Hsiang Chien[1], Chin-Tin Chen[2], Chia-Tung Shun[3], Yu-San Lee[2], Song-Nan Chow[4]

1. Department and Institute of Biochemistry, National Yang-Ming University
2. Center for Optoelectronic Biomedicine, National Taiwan University College of Medicine
3. Department of Forensic Medicine, National Taiwan University College of Medicne
4. Department of Obstetrics & Gynecology, National Taiwan University Hospital, Taipei, Taiwan

Several changes have been documented in epithelial ovarian cancer including altered production and response to peptide growth factors, activation of protooncogenes, and inactivation of tumor suppressor genes. It has been observed that alterations of cyclin D1 and cdk inhibitor p16^{INK4} induce the abnormal cell proliferation. In this study, we investigated whether overexpression of cyclin D1 and loss of expression of p16^{INK4} occur in epithelial ovarian cancer. Specimens of normal ovaries and ovarian tumors were obtained and snap frozen at the time of operation. All histological slides were reviewed to confirm the diagnosis and histological subtypes of human ovarian tumors. Immunohistochemical staining for cyclin D1 and p16^{INK4} was performed in freshly frozen samples of ovarian tumors. Specific staining for cyclin D1 was observed in 12 (66.7 %) of 18 ovarian cancers and 2 (40%) of 5 borderline malignant tumors. Loss of expression of p16^{INK4} protein was found in 10 (55.6%) of 18 ovarian cancer and 2 (40%) of 5 borderline tumors. According to the results of our study on limited number of ovarian tumor tissue specimens, there is a correlation between the over-expression of cyclin D1 and loss of expression of p16^{INK4} in human epithelial ovarian cancer, implicating that both of them are important cell cycle regulators relevant to tumorigenesis of human epithelial ovarian cancer.

Key words: ovarian cancer, cyclin D1, p16^{INK4} gene

Introduction

Ovarian cancer is the most important cause of death among patients with gynecological malignancies. Most malignant ovarian tumors arise from the single layer of epithelial cells that cover the ovary or lines inclusion cysts. Epithelial ovarian neoplasms

*Corresponding author: Prof Song-Nan Chow, Head, Department of Obstetrics & Gynecology, National Taiwan University Hospital, 7, Chung-Shan South Road, 100, Taipei, Taiwan
TEL: 886-2-2312-3456 x 5165 FAX: 886-2-2321-1683 e-Mail: snchow@ha.mc.ntu.edu.tw

comprise a wide spectrum including benign, borderline malignant, and invasive tumors. It has been proposed that these pathologic entities may represent sequential stages in the evolution of an ovarian cancer [1]. Several changes have been reported in epithelial ovarian cancer including altered production and response to peptide growth factors, activation of protooncogenes, and inactivation of tumor-suppressor genes [2]. However, there is still no clear understanding of the molecular etiology involved in the tumorigenesis of ovarian cancer.

Recent observations on the sequence of molecular events involved in the progression of cell cycle have increased our understanding about the development and progression of human malignancies. Previous studies indicated that transitions between phases of the cell cycle are controlled by the sequential activation of a family of serine/threonine protein kinases, termed cyclin-dependent kinases (CDKs). Activation of CDKs requires the binding of its partner protein, cyclins. The activities of CDK/cyclin complex are regulated by phosphorylation/dephosphorylation on conserved threonine and tyrosine residues or binding by CDK inhibitors (CDIs) [3-5]. In the G1 phase of cell cycle, the growth-positive and –negative signals determine whether cells progress the G1 phase and then commit itself to another round of DNA replication. During the last few years, attention has focused on the cell cycle regulators during the G1 phase because their deregulation could allow cell growth to become insensitive to the external controls. During this process, D-type cyclins seem to play key roles. In fact, overexpression of D-type cyclin has been strongly implicated in tumorigenesis [6-9]. This connection is strengthened by compelling evidences that D-type cyclins-CDK4,6 complexes are capable of phosphorylating tumor suppressor protein, retinoblastoma gene product (pRB), in vivo and in vitro to control the cell growth [10,11]. One of the inhibitors of cyclin D/CDK4, p16^{INK4}, has been shown to block the phosphorylation of Rb protein which is essential for entering into S phase. Therefore, changes in the amount or composition of the D-type cyclin or p16^{INK4} may lead to uncontrolled cell growth. In fact, these two types of changes have been documented in tumor cells or human neoplasia [12-14].

Convergent results from different studies have indicated that abnormalities of cyclins, cyclin D1 especially, and CDK inhibitors might be involved in tumorigenesis. To determine whether alternations of the cyclin D1 and p16^{INK4} are involved in ovarian carcinogenesis, we have studied the expression level of cyclin D1 and p16^{INK4} immunohistochemically is tissue specimens of 9 normal ovaries, 5 borderline malignant tumors and 18 ovarian cancers. The relationship of cyclin D1 and p16^{INK4} expression was also analyzed.

Materials and Methods

Collection of tissue samples

Normal or malignant ovarian tissues specimens were obtained and snap frozen in embedding medium, and stored a –70°C freezer at the time of surgery in the Department of Obstetrics and Gynecology, National Taiwan University Hospital. All histopathologic slides were reviewed to confirm the diagnosis and the histological subtypes.

Immunohistochemical staining for cyclin D1 and p16^{INK4}

Cryostat sections of 10 μm thickness were picked up onto pre-coated glass slides, and fixed with ice-cold acetone. The sections were incubated with 3% hydrogen peroxide to inactivate the endogenous peroxidase. To inhibit the non-specific binding, slides were incubated with 10% normal goat serum for 30 minutes. Monoclonal mouse anti-human

cyclin D1 or $p16^{INK4}$ antibody (Transduction Laboratory, USA) were applied and incubated at 4°C for overnight. The sections were then washed three times with phosphate-buffer saline (PBS), followed by incubation with biotinylated anti-mouse IgG antibody for 60 minutes. After washing with PBS, peroxidase conjugated streptavidin/ biotin complex was applied. The bound complex was visualized with diaminobenzidine substrate and with a blue haematoxylin counterstain. Positive and negative control sections for cyclin D1 were obtained from two cell lines, the 3T3CYCD and 3T3pCMV. These two cell lines were derived from NIH 3T3 cell line transfected with cyclin D1 expression vector, pCMVcycD1, and a control vector, pCMV, respectively. The 3T3CYCD and 3T3pCMV were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and G418 (400 μg/ml) in a 5% CO_2 atmosphere at 37°C. Negative control experiment for cyclin D1 was performed by incubating the ovarian specimen with phosphate buffer saline. Negative control for $p16^{INK4}$ was also obtained by incubating the specimen slides with PBS instead of monoclonal $p16^{INK4}$ antibody.

Statistical analysis

Data were analyzed by Fisher exact test.

Results

Seven normal ovarian tissues and 23 epithelial ovarian tumors including 5 borderline malignant ovarian tumors and 18 ovarian cancers, were obtained for this study. These ovarian tissue specimens were examined for cyclin D1 and $p16^{INK4}$ expression by immunohistochemical staining using cyclin D1– or $p16^{INK4}$– specific antibody. The representative pictures of positive and negative staining for cyclin D1 protein are shown in Figure 1. The representative immunohistochemical staining for $p16^{INK4}$ is shown in Figure 2. The results of expression of cyclin D1 and $p16^{INK4}$ in relation to histopathologic characteristics of tissue specimens are summarized in Table 1. As notice, overexpression of cyclin D1 was not demonstrated in any of 7 normal ovarian tissues. However, compared to normal ovarian tissues, 2(40%) of the 5 borderline ovarian tumors and 12 (66.7 %) of 18 ovarian cancers exhibited overexpression of cyclin D1 protein. On the other hand, the rates of loss expression of $p16^{INK4}$ were 40% and 55.6 % for borderline ovarian tumors and ovarian cancers, respectively.

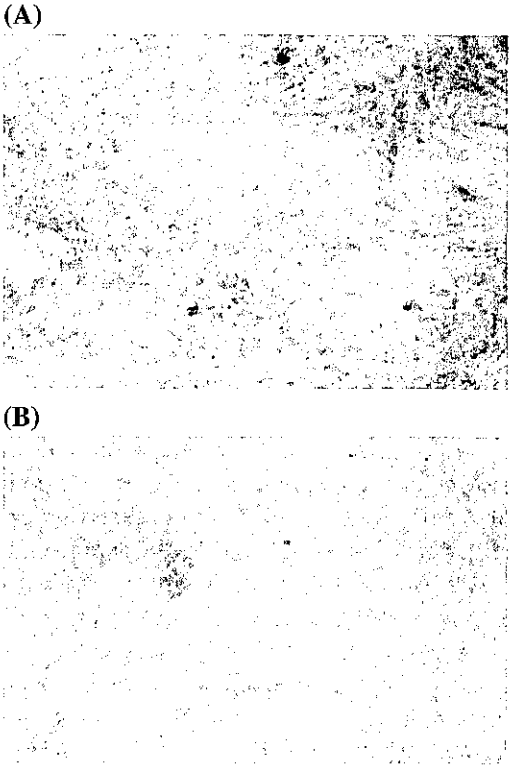

Fig 1. Immunohistochemical staining for cyclin D1 protein in human ovarian cancer. (a) positive staining; (b) negative staining .

Discussion

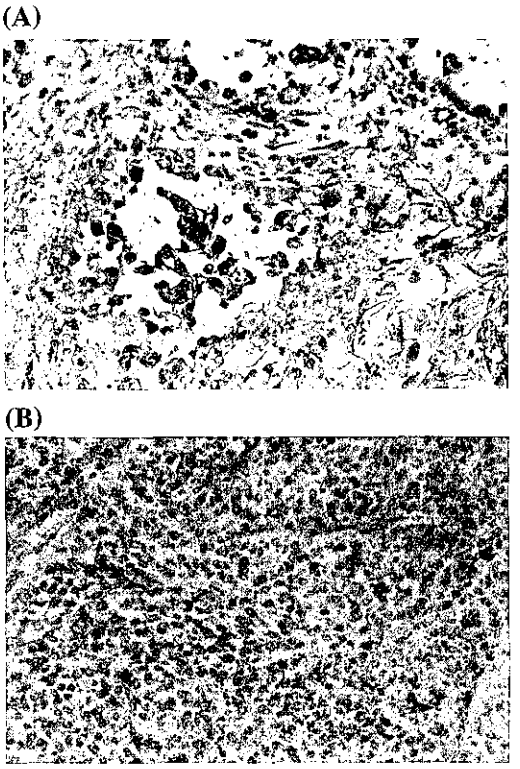

Fig 2. Immunohistochemical staining for p16^{INK4} protein in human ovarian cancer. (a) positive staining; (b) negative staining.

Our present data showed that the gene products of cyclin D1 are over-expressed in human ovarian tumors of borderline malignancy and cancer, with a frequency of 60.9%[14/23]. The rate of loss expression of p16^{INK4} in tumor tissues (borderline malignancy and cancer) was 52.2% (2/23, 2 in borderline and 10 in cancer), suggesting that there is a remarkable alteration of expression of p16^{INK4} in human epithelial ovarian cancer and borderline malignant tumor.

Several lines of evidence have suggested that cyclin D1 is involved in the G1 to S transition of the cell cycle [15,16]. At the biochemical level, the cyclin D1 with CDK4 or CDK6 appear to regulate cell cycle progression by the subsequent phosphorylation of critical substrates Rb and other cell cycle-regulated proteins, such as PCNA and p21 [11,17,18]. However, the p16^{INK4} protein negatively regulates the cell cycle progression by binding to CDK4 and preventing the association of CDK4 with cyclin D1 [19]. If the expression of p16^{INK4} is inactivated, there is no regulatory mechanism to block the cell cycle, and this might lead to uncontrolled cell growth.

Cyclin D1 has been regarded as a prominent oncogene because it is most consistently amplified and overexpressed in several types of tumor [20]. Most normal human tissues including muscle, liver, spleen, prostate and ovary either lacked any detectable cyclin D1 staining or showed only a rare scattered single cell immuno-reactivity [21]. In this study, we found that there was no detectable cyclin D1 expression in normal ovarian epithelium tissues and 66.7 % of ovarian cancer tissues exhibit cyclin D1 overexpression which suggests that cyclin D1 did play an important role in ovarian tumorigenesis.

The p16 gene is located at chromosome 9p21-22 which has been found frequently deleted and associated with development of ovarian cancer. This was supported by the finding of higher frequency of p16 mutations (point mutation or deletion) in cell lines (22). Our present results show that over 55 % of ovarian cancer loss p16^{INK4} expression, which suggests that p16^{INK4} alteration is a frequent molecular event in ovarian cancers. Alterations in expression of cyclin D1 and p16^{INK4} have been reported previously in human ovarian and endometrial cancers [23-27]. Loss of expression of p16^{INK4} mRNA has been observed in the SK-OV-3 ovarian carcinoma cell line [24]. Overexpression of mRNA levels of cyclin D1 was detected in borderline ovarian tumors as well as in ovarian cancer cases [25]. The positive rate of immunostaining of cyclin D1 protein was observed in the range of 56 % to 71 % of ovarian tumors examined [25, 26].

Table 1. Overexpression of cyclin D1 and loss of expression of $p16^{INK4}$ protein in spectrum of normal and malignant ovarian tissues

Type of ovarian tissues	Number of specimens tested	Overexpression of cyclin D1 (%)*	Loss of expression of $p16^{INK4}$ (%)†
Normal	7	0 (0 %)[a]	0 (0 %)[e]
Tumor	23	14 (46.7%)[b]	12 (40 %)[f]
Borderline malignant	5	2 (40 %)[c]	2 (40 %)[g]
Ovarian cancer	18	12 (66.7%)[d]	10 (55.6%)[h]
Total	30	14 (46.7%)	12 (40 %)

* Fisher exact test: [a,b] p=0.0056; [a,c] p=0.15; [c,d] p=0.28; [a,d] p=0.0036

† Fisher exact test: [e,f] p=0.0156; [e,g] p=0.15; [g,h] p=0.45; [e,h] p=0.0134

Cyclin D1 and $p16^{INK4}$ act respectively as a positive and a negative cell-cycle regulator. The perturbations of these two components are likely to occur in human cancers. Thus loss of $p16^{INK4}$ or the overexpression of cyclin D1 will promote the phosphorylation and functional inactivation of pRb, leading to uncontrolled cellular proliferation. In our present study, the abnormal expression of cyclin D1 and $p16^{INK4}$ reinforces the notion that they are components involved in an important pathway to tumorigenesis.

Acknowledgement

This study was supported by a research grant of NSC 86-2314-B-002-338 from the National Science Council, Taipei, Taiwan ROC, and by Global Vista Medical Foundation.

References

1. Berchuck A, Kohler MF, Boente MP, Rodriguez GC, Whitaker RS, Bast RC Jr: Growth regulation and transformation of ovarian epithelium. Cancer, Jan 1993; 15: 545-551.
2. Chow SN et al. Molecular biology of human ovarian cancer. Int Surg.1996; 81: 152-157.
3. Sherr CJ: G1 phase progression: cycling on cue. Cell, 1994; 79: 551-555.
4. Nurse P: Ordering S phase and M phase in the cell cycle. Cell, 1994; 79: 547-550.
5. Morgan DO: Related Articles, Links Principles of CDK regulation. Nature, 1995; 374: 131-134.
6. Rosenberg CL, Kim HG, Shows TB, Kronenberg HM, Arnold A: Related rearrangement and over-expression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumors.Oncogene, 1991; 6: 449-553.
7. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G: Amplification and over-expression of cyclin D1 in breast cancer detected by immuno-histochemical staining.Cancer Res, 1994; 54: 1812-1817.
8. Callender T, el-Naggar AK, Lee MS, Frankenthaler R, Luna MA, Batsakis JG: PRAD-1 (CCND1)/cyclin D1 oncogene amplification in primary head and neck squamous cell carcinoma.Cancer. 1994; 74: 152-158.
9. Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, Amenomori M, Shibagaki I, Nakao K, Ikenaga M, et al: Related articles, links amplification and over-expression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. Cancer Res, 1994; 54: 3107-3110.
10. Matsushima ME, Ewen DK, Strom JY, Kato SK, Hanks MF, Roussel, Sherr CJ: Identification and properties of an atypical catalytic subunit (p34PSK- J3/cdk4) for mammalian type G1 cyclins. Cell, 1992; 71: 323-334.
11. Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI, Weinberg RA: Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell, 1992; 70: 993-1006.
12. Hunter T, Pines J: Related. Cyclins and cancer. II: Cyclin D and CDK inhibitors come

of age.Cell, 1994; 79: 573-582.
13. Cordon-Cardo C: Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. Am J Pathol, 1995; 147: 545-560.
14. Sherr CJ. Cancer cell cycles. Science, 1996; 274: 1672-1677.
15. Xiong Y, Connolly T, Futcher B, Beach D: Human D-type cyclin. Cell, 1991; 65: 691-699.
16. Lew DJ, Dulic V, Reed SI. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell, 1991; 66: 1197-1206.
17. Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell, 1992;71:505-514.
18. Dowdy SF, Hinds PW, Louie K, Reed SI, Arnold A, Weinberg RA: Physical interaction of the retinoblastoma protein with human D cyclins.Cell, 1993; 73: 499-511.
19. Serrano M, Hannon GJ, Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature, 1993; 366: 704-707.
20. Schuuring E, Verhoeven E, Mooi WJ, Michalides RJ: Identification and cloning of two over-expressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene, 1992; 7: 355-361.
21. Bartkova J, Lukas J, Strauss M, Bartek J: Cell cycle-related variation and tissue-restricted expression of human cyclin D1 protein.J Pathol. 1994; 172: 237-245.
22. Kanuma T, Nishida J, Gima T, Barrett JC, Wake N: Alterations of the p16INK4A gene in human ovarian cancers. Mol Carcinog, 1997; 18: 134-141.
23. Schuyer M, van Staveren IL, Klijn JG, vd Burg ME, Stoter G, Henzen-Logmans SC, Foekens JA, Berns EM: Sporadic CDKN2 (MTS1/p 16ink4) gene alterations in human ovarian tumours. Br J Cancer, 1996; 74: 1069-1073.
24. Yaginuma Y, Hayashi H, Kawai K, Kurakane T, Saitoh Y, Kitamura S, Sengoku K, Ishikawa M: Analysis of the Rb gene and cyclin-dependent kinase 4 inhibitor genes (p16INKA and p15INK4B) in human ovarian carcinoma cell lines. Exp Cell Res, 1997; 233: 233-239.
25. Shigemasa K, Tanimoto H, Parham GP, Parmley TH, Ohama K, O'Brien TJ: Cyclin D1 overexpression and p53 mutation status in epithelial ovarian cancer. J Soc Gynecol Investig, 1999; 6: 102-108.
26. Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C: Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. Am J Pathol, 2001; 158: 2097-2106.
27. Ruhul Quddus M, Latkovich P, Castellani WJ, James Sung C, Steinhoff MM, Briggs RC, Miranda RN: Expression of cyclin D1 in normal, metaplastic, hyperplastic endometrium and endomereioid carcinoma suggests a role in endometrial carcinogenesis. Arch Pathol Lab Med, 2002; 126: 459-463.