

Cyclin E Expression in Benign and Malignant Epithelial Neoplasms of the Gallbladder

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Cyclins are the regulatory subunits of cyclin-dependent kinase and play an important role in cell proliferation. Many tumors, such as colon, breast and gastric carcinomas are known to be involved in the deregulation or amplification of cyclins, especially cyclin E, which involves the restriction point of G1-S transition. We investigated the expression of cyclin E in benign and malignant epithelial tumors of the gallbladder and compared the results with the activity of cell proliferation by the Ki67 antigen using immunohistochemical staining. Cyclin E was expressed in the adenocarcinoma tissue in 33.3% of patients (4 out of 12 cases), whereas only one out of 8 cases of adenoma expressed cyclin E (12.5%). There was a correlation between cyclin E expression and the Ki67 labeling index. These results suggest that the high expression of cyclin E in adenocarcinoma of the gallbladder is related to a high rate of cell proliferation.

Key Words: Adenocarcinoma, adenoma, cyclin E, Ki67, gallbladder

Cyclins are essential proteins in cell-cycle regulation that are associated with and activate different cyclin-dependent kinases at different stages of the cell cycle and allow them to pass through a specific stage in the cycle (Dutta *et al.* 1995; Wang *et al.* 1996). In mammalian cells, at least five different cyclins were identified (Sherr, 1993). Cyclins D1 and E are known to cooperate with cdk2 and function in the G1 phase and in the G1-S transition, thus they are called G1 cyclins. Cyclins A and B play an important role in the G2-M phase with cdc2 and are referred to as 'mitotic cyclins' (Koff *et al.* 1991; Xiong *et al.* 1992). Because each cyclin marks a different phase of cell-cycle-E for G1 and early S, A for S and G2, B for late G2 (Pines and Hunter,

1991; Dulic *et al.* 1992; Koff *et al.* 1992)- the fraction of cells positive for a given cyclin should represent the fraction in a corresponding phase of the cell cycle. Furthermore, several malignant tumors express different types of cyclins: cyclin D1 in parathyroid adenoma (Motokura *et al.* 1991), cyclin E in breast cancer (Keyomarsi *et al.* 1994), and cyclin B and E in leukemia or solid tumor cell lines (Gong *et al.* 1994). Among the various cyclins, cyclin E seems to have the most important role in tumorigenesis, considering that it is involved in the restriction point of G1-S transition.

Since there have been no reports about the expression of cyclin E in the benign and malignant epithelial neoplasms of the gallbladder, we studied the expression of cyclin E by use of immunohistochemical staining. The results were compared with the cell-proliferation indices using Ki67 antigen immunohistochemical staining.

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into two groups according to the presence or absence of atypia (Table 2).

MATERIALS AND METHODS

Patients selection

Specimens from 20 patients who had undergone cholecystectomy at Ajou University Hospital for benign and malignant epithelial tumors of the gallbladder were selected on the basis of tissue availability. Twelve patients were diagnosed as adenocarcinoma and 8 as adenoma. Clinical information and pathologic characteristics were collected for all cases (Table 1, 2). Hematoxylin and eosin (H&E) stained sections were evaluated and graded to three categories (well, moderately and poorly differentiated) according to the degree of differentiation (Table 1). The cases of adenoma were categorized

Immunohistochemical staining technique

Immunohistochemical staining for cyclin E and Ki67 was performed on formalin-fixed, paraffin-embedded sections by the avidin-biotin peroxidase complex (ABC) method (Hsu *et al.* 1981). The monoclonal antibody NCL-Ki67-MM1 (Novocastra, Newcastle, UK) was used for the studies of Ki67 expression, and HE12 (Pharmingen, San Diego, CA, USA) for cyclin E expression. The primary antibodies were incubated at 4°C for 12-to-16 hours, followed by incubation with biotinylated secondary antibody of LSAB kit (Dako, Carpinteria, CA, USA). Reaction products were visualized by immersion of the sections in 3,3'-diaminobenzidine

Table 1. Clinical data, cyclin E expression and Ki67 labeling indices in adenocarcinoma

Patient no.	Sex/Age	Size(cm)	Gross feature	Differentiation	Extension	LN involvement	Cyclin E	Ki67
1	M/60	4.5×3	Diffuse	Well	Liver	1/8	+	264
2	F/61	3.5×1.5	Fungating	Well	Proper muscle	—	—	73
3	M/73	7×3	Diffuse	Well	Liver	—	+	703
4	F/69	2.5×2.2	Diffuse	Moderate	Liver	—	—	382
5	M/61	8×7	Diffuse	Poor	Liver,colon	1/10	+	268
6	M/44	4.5×3.5	Fungating	Well	Liver	0/9	—	162
7	F/62	5×3	Diffuse	Moderate	Liver	1/7	—	76
8	F/60	4	Diffuse	Well	Perimuscular	—	—	342
9	F/64	3.5×2.5	Fungating	Moderate	Perimuscular	—	+	385
10	F/79	4	Diffuse	Moderate	Perimuscular	—	—	237
11	F/67	3×2.2	Fungating	Well	Mucosa	0/4	—	158
12	M/49	2×2	Fungating	Well	Mucosa	—	—	173

Table 2. Clinical data, cyclin E expression and Ki67 labeling indices in adenoma

Patients no.	Sex/age	Size(cm)	Atypia	Cyclin E	Ki67
1	M/37	0.8×0.7	—	—	122
2	F/46	2.5×2, 0.7, 0.5*	+	—	130
3	F/67	1×0.7	—	—	11
4	F/37	0.5×0.4	—	—	0
5	F/73	1×0.8	—	—	0
6	M/49	2.5×2	+	—	45
7	M/35	1×0.8	+	—	226
8	F/54	3×1.4	+	+	81

*: Three separate adenomas were present in this case.

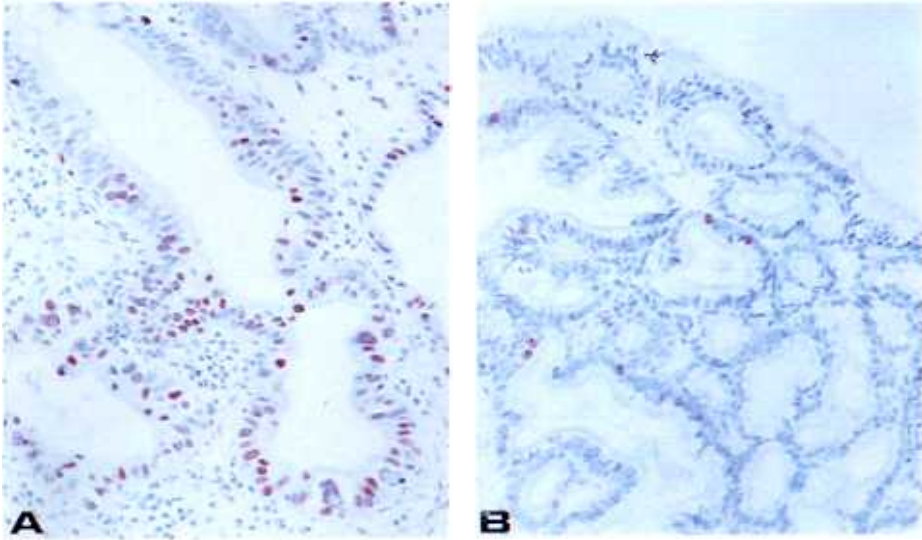


Fig. 1. A: Immunohistochemical staining with anti Ki67 in adenocarcinoma of the gallbladder. Carcinoma nests showing high rate of Ki67 expression ($\times 200$). B: Immunohistochemical staining with anti Ki67 in adenoma showing a few scattered positive reactions ($\times 200$).

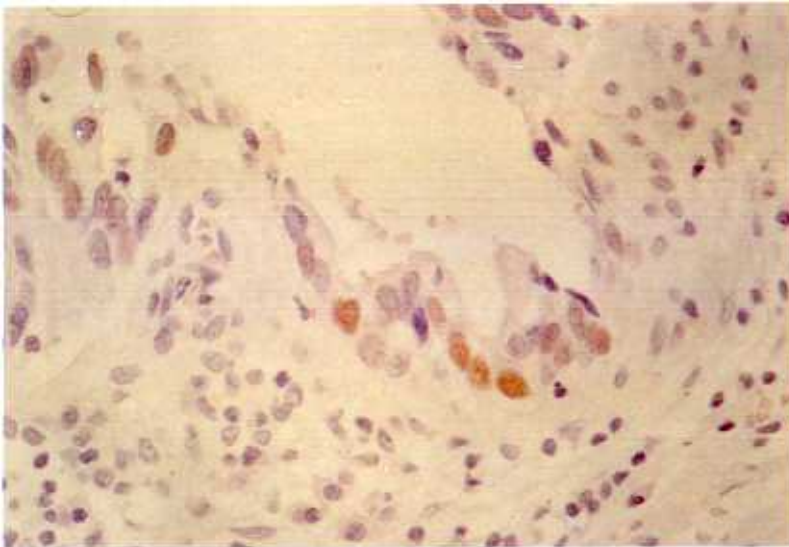


Fig. 2. Cyclin E expression in the nuclei of cells in adenocarcinoma (Immunohistochemical staining, $\times 200$).

tetrahydrochloride solution (Sigma Chemicals, St. Louis, MO, USA) containing hydrogen peroxide. The sections were counterstained with Meyer's hematoxylin. The results for cyclin E were evaluated as positive and negative. Nuclear staining was considered to be positive. The Ki67 labeling indices were determined in both benign and malignant tumors. A total of 500-1000 tumor cells were counted in each cases. Nuclei with well-defined brown granular staining were considered to be positive. Ki67 labeling indices were calculated as the number of positive nuclei per 1000 nuclei counted.

Statistics

Nonparametric tests were used to analyze our data. The Mann-Whitney U test was used to compare data between two groups. A p value of <0.05 was considered statistically significant.

RESULTS

Ki67 labeling indices in adenoma and adenocarcinoma of the gallbladder

The adenocarcinoma tissues possessed much higher labeling indices than the adenoma tissues (268 vs 76, $p < 0.05$, Table 1, 2 and Fig. 1). In the adenoma group, the cases with atypia showed much higher labeling indices than those without atypia.

Cyclin E expression in the adenoma and adenocarcinoma of the gallbladder

Only one of 8 cases of adenoma expressed cyclin E (12.5%, Table 2) and this case also showed significant atypia. Among the cases of adenocarcinoma, 4 out of 12 (33.3%) were stained with cyclin E (Table 1). All the positive staining were in the nuclei (Fig. 2). None of the nearby nonneoplastic biliary epithelium was positive. There was a much higher incidence of cyclin E expression among the cases with extension to the liver or metastasis to the colon than those confined to the gallbladder (50% vs 14.2 %, Table 1).

Correlation between Ki67 labeling indices and cyclin E expression in the adenocarcinoma of the gallbladder

The adenocarcinoma cases with cyclin E expression showed a much higher labelling index than those without cyclin E expression (405 vs 200, $p < 0.05$, Table 1).

DISCUSSION

Gallbladder cancer is a relatively rare neoplasm accounting for about 0.3-0.7% of all cancer cases (Albores-Saavedra and Henson, 1984). The tumor has a peak incidence in the age group from 70-79 years, is more common in females (Albores-Saavedra and Henson, 1984) and is frequently associated with cholelithiasis and cholecystitis.

Multistep genetic changes are associated with the development of human cancer (Fearon and Vogelstein, 1990) and many genetic changes are reported to be associated to the development of cancer, such as p53. However, few are reported to involve the development of gallbladder cancer: only the p53 and c-erbB2 proteins are known to play some role in the neoplastic transformation of gallbladder epithelial cells (Kamel *et al.* 1993).

In attempts to understand the relationship between the cell cycle and cancer, many laboratories have investigated the role of cyclin/cdk complexes in carcinogenesis. The cyclin A gene is the site of integration of a fragment of the hepatitis B virus genome in hepatocellular carcinogenesis (Wang *et al.* 1990). It is also associated with the adenovirus oncoprotein E1A in adenovirus-transformed cell lines (Pines and Hunter, 1990). The cyclin D1 locus (Prad1) is known to be overexpressed due to a chromosomal rearrangement that translocates it to the enhancer of the parathyroid hormone gene (Matsushima *et al.* 1991; Motokura *et al.* 1991; Quelle *et al.* 1993). Prad1 has also been found to reside near the B cell lymphoma-associated chromosome 11 breakpoint (known as BCL1) which can be activated by t(11;14) translocations (Rosenberg *et al.* 1991). The cyclin D1 is also known to be amplified

and overexpressed in some cases of liver cancer, breast cancer and esophageal cancer (Jiang *et al.* 1992; Zhang *et al.* 1993; Bartkova *et al.* 1994a; Bartkova *et al.* 1994b). Cyclin E expression is known to have striking abnormalities in neoplasia. Qualitative and quantitative alteration in cyclin E expression in breast cancer and other solid tumors independent of the S-phase fraction has been documented (Keyomarsi *et al.* 1994) and its expression is associated with breast tumor stage and grade. Cyclin E is also overexpressed in colon and gastric cancer (Akama *et al.* 1995; Wang *et al.* 1996). Cyclin E seems to have the most important role in carcinogenesis considering that it is involved in the restriction point of G1-S transition. In this study, we found that cyclin E was expressed in about 33% of gallbladder cancers in keeping with a high level of Ki67 labeling indices. Expression of the cyclin E protein was specific to neoplastic epithelium and was not seen in adjacent normal tissue, suggesting that expression of cyclin E was a marker for oncogenesis and not just for proliferation. Cyclin E was expressed in one case of adenoma (12.5%). In view of the fact that the latter case exhibited significant atypia, we speculated that adenoma, especially with atypia, is a premalignant condition and supports multistep carcinogenesis in the development of gallbladder cancer. Our results showed that there was a correlation between cyclin E expression and Ki67 labeling indices; this result does not support the notion that deregulation of cyclin E is associated with the development of gallbladder cancer. The positive staining of the cyclin E protein in cancer tissue could be because of the overexpression of this cyclin, due to amplification of this cyclin gene, or due to the failure to appropriately degrade the cyclin messages in the cell cycle (Keyomarsi and Pardee, 1993). Our study does not permit an explanation of the precise mechanism of cyclin E expression in a neoplasm. Further study at the molecular level will be necessary. As expected, the Ki67 labeling indices were much higher in the cases of adenocarcinoma than adenoma.

We conclude that cyclin E expression seems to be one of the factors contributing to the uncontrolled proliferation of gallbladder cancer cells. Since its expression was in keeping with the proliferation rate of tumor cells in our study and other researches

(Dou *et al.* 1993; Jansen-Durr *et al.* 1993; Knoblich *et al.* 1994; Wang *et al.* 1996), we suggest that cyclin E also can be used as a marker of proliferation.

REFERENCES

- Akama Y, Yasui W, Yokozaki H, Kuniyasu H, Kitahara K, Ishikawa T, Tahara E: Frequent amplification of the cyclin E gene in human gastric carcinoma. *Jpn J Cancer Res* 86: 617-621, 1995
- Albores-Saavedra J, Henson DE: Tumors of the gallbladder and extrahepatic bile ducts. Atlas of Tumor Pathology, 2nd series Fascicle 22. Washington, Armed Forces Institute of Pathology(AFIP), 1984, 30
- Bartkova J, Jiri L, Heiko M, Dorrit L, Micheal S, Bartek J: Cyclin D1 protein expression and function in human breast cancer. *Int J Cancer* 57: 353-361, 1994a
- Bartkova J, Lukas J, Strauss M, Bartek J: The PRAD-1/cyclin D1 oncoprotein product accumulates aberrantly in a subset of colorectal carcinoma. *Int J Cancer* 58: 568-573, 1994b
- Dou Q, Levin AH, Zhao S, Pardee AB: Cyclin E and cyclin A as candidates for the restriction point protein. *Cancer Res* 53: 1493-1497, 1993
- Dulic V, Lees E, Reeds SI: Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* 257: 1958-1961, 1992
- Dutta A, Chandra R, Leiter RM, Lester S: Cyclins as markers of tumor proliferation: Immunocytochemical studies in breast cancer. *Proc Natl Acad Sci USA* 92: 5386-5390, 1995
- Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990
- Gong J, Ardelt B, Traganos F, Darzynkiewicz Z: Unscheduled expression of cyclin B1 and cyclin E in several leukemia and solid tumor cell lines. *Cancer Res* 54: 4285-4288, 1994
- Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex(ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody(PAP) procedures. *J Histochem Cytochem* 29: 577-580, 1981
- Jansen-Durr P, Meichle A, Steiner P, Pagano M, Finke K, Nitz J, Wessbecher J, Draetta G, Eilers M: Differential modulation of cyclin gene expression by MYC. *Proc Natl Acad Sci USA* 90: 3685-3689, 1993
- Jiang W, Kahn SM, Tomita N, Zhang YJ, Lu SH, Weinstein IB: Amplification and expression of the human cyclin D gene in esophageal cancer. *Cancer Res* 52: 2980-2983, 1992
- Kamel D, Paakko P, Nuorva K, Vahakangas K, Soini Y: p53 and c-erbB2 protein expression in adenocarcinomas and epithelial dysplasias of the gallbladder.

- J Pathol* 170: 67-72, 1993
- Keyomarsi K, O'Leary N, Molnar G, Lees E, Fingert HJ, Pardee AB: Cyclin E, a potential prognostic marker for breast cancer. *Cancer Res* 54: 380-385, 1994
- Keyomarsi K, Pardee AB: Redundant cyclin overexpression and gene amplification in breast cancer cells. *Proc Natl Acad Sci USA* 90: 1112-1116, 1993
- Knoblich JA, Sauer K, Jones L, Richardson H, Saint R, Lehner CF: Cyclin E controls S phase progression and its downregulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* 77: 107-120, 1994
- Koff A, Cross F, Fisher A, Schumacher J, Legullec K, Philippe M, Roberts JM: Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* 66: 1217-1228, 1991
- Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR, Roberts JM: Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* 257: 1689-1694, 1992
- Matsushime H, Roussel MF, Ashman RA, Sherr CJ: Colony-stimulating factor 1 regulates novel cyclins during G1 phase of the cell cycle. *Cell* 65: 701-713, 1991
- Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM, Arnold A: A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature* 350: 512-515, 1991
- Pines J, Hunter T: Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. *Nature* 346: 760-763, 1990
- Pines J, Hunter T: Human cyclins A and B1 are differentially located in the cell and undergo cell cycle dependent nuclear transport. *J Cell Biol* 115: 1-17, 1991
- Quelle DE, Ashmun RA, Shurleff SA, Kato JY, Bar-Sagi D, Roussel MF, Sherr CJ: Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev* 7: 1559-1571, 1993
- Rosenberg CL, Kim HG, Show TB, Kronenberg HM, Arnold A: Rearrangement and overexpression of D11S287E, a candidate oncogene on chromosome 11q13 in benign parathyroid tumor. *Oncogene* 6: 449-453, 1991
- Sherr CJ: Mammalian G1 cyclins. *Cell* 73: 1059-1065, 1993
- Wang A, Yoshimi N, Suzui M, Yamauchi A, Tarao M, Mori H: Different expression patterns of cyclin A, D1, and E in human colorectal cancer. *J Cancer Res Clin Oncol* 122: 122-126, 1996
- Wang J, Chenivresse X, Henglein B, Brechot C: Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. *Nature(Lond)* 343: 555-557, 1990
- Xiong Y, Connolly T, Futcher B, Beach D: Human D-type cyclin. *Cell* 65: 691-699, 1991
- Zhang YJ, Jiang W, Chen CJ, Lee CS, Kahn SM, Santella RM, Weinstein IB: Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. *Biochem Biophys Res Commun* 196: 1010-1016, 1993