RESEARCH ARTICLE

Cyclin D1, Retinoblastoma and p16 Protein Expression in Carcinoma of the Gallbladder

Vineeta Srivastava, Brijesh Patel, Mohan Kumar, Mridula Shukla, Manoj Pandey*

Abstract

Background: Cancer of the gallbladder is a relatively rare neoplasm with a poor prognosis. The exact mechanisms of its genesis are not known and very little information is available on molecular events leading to labeling this as an orphan cancer. Materials and Methods: In this prospective case control study we evaluated the expression of p16, pRb and cyclin D1 by immunohistochemistry to study the G1-S cell-cycle check point and its possible role in gallbladder carcinogenesis. A total of 25 patients with gallbladder carcinoma (group I), 25 with cholelithiasis (group II) and 10 normal controls. were enrolled Results: Cyclin D1 expression was seen in 10 (40%) patients each with carcinoma and cholelithiasis while only in 2 (20%) of the normal gallbladders but differences were not statistically significant (p value=0.488). p16 was expressed in 12% patients of carcinoma of the gallbladder and 28% of cholelithiasis, however this difference was not statistically significant (p value=0.095). Retinoblastoma protein was found to be expressed in 50% of normal gallbladders and 6 (24%) of carcinoma and 8 (32%) of gallstones. The present study failed to demonstrate any conclusive role of cyclin D1/RB/ p16 pathway in carcinoma of the gallbladder. Conclusions: The positive relation observed between tumor metastasis and cyclinD1 expression and p16 with nodal metastasis suggested that higher cyclin D1/p16 expression may act as a predictive biomarker for aggressive behavior of gallbladder malignancies.

Keywords: Gallbladder - cell-cycle - apoptosis - tumor supressor genes - hepatobiliary malignancies

Asian Pacific J Cancer Prev, **14 (5)**, 2711-2715

Introduction

Gallbladder carcinoma is a highly malignant neoplasm having a poor prognosis that is mostly due to the advanced stage at presentation (Lazcano-ponce et al., 2001; Offerhaus et al., 2002; Misra et al., 2003). Though rare in most countries, it shows wide geographic variation with pockets of high incidence throughout the world and a female preponderance (Rifatbegovic et al., 2007). The causes of gallbladder cancer are poorly understood. Cholelithiasis is commonly implicated as gallstones are found to be associated with nearly 70% of all cholecystectomy specimens. Attempts have been made to define the molecular biology of gallbladder cancer; however, the literature is limited. Most studies have looked at p53 and ras gene mutations that are found in large number of patients. For the cancer to develop it has to bypass the normal cell-cycle controls and gain the capacity for uncontrolled proliferation, ability to proliferate in absence of appropriate signals, and to ignore signals that stop proliferation and induce apoptosis (Hartwell and Kastan, 1994; Michalides, 2002). The cell-cycle check points are one such restriction point. Of these the G1-S check point is controlled by a complex network of protein interactions and phosphorylations, including the pRB, cyclin dependent kinase (CDKs) and Cyclin dependent kinase inhibitors (CDKI). This makes pRB, p16 and Cyclin D major regulators of cell-cycle. Cyclin D1 forms active complexes with CDK4 and CDK6 that promote cell-cycle progression to S-phase by phosphorylating and inactivating the retinoblastoma protein (pRB) (Kato et al., 1993; Weinberg, 1995; Lerma et al., 2002; Sdek et al., 2002). Cyclin D1 over expression is a common event in cancer but does not occur solely as a consequence of gene amplification. Rather, increased levels of Cyclin D1 frequently result from its defective regulation at the post-translational level (Gillett et al., 1994; Rusell et al., 1999). The Cyclin D1 promoter is the link between growth signals conveyed by the mitogen-activated protein kinase (MAPK) pathway and cell proliferation (Li et al 2006; Sauter et al., 2002).

p16, a CDKI, prevents formation of the cyclin-CDK complex required to phosphorylate pRB and consequently S-phase entry (Serrano et al., 1993; Kamb et al., 1994; Li et al., 2006). The $p16^{INK\ 4A}$ gene belongs to the G1 control gene (a tumor suppressor

Department of Surgical Oncology and Pathology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India
**For correspondence: manojpandey@vsnl.com*

gene on chromosome 9p21), a new tumor suppressor gene, identified in 1995 and called as multiple tumor suppressor 1 (MTS1) for its suppressing function on multiple tumors (Serrano et al., 1993). Overexpression in gallbladder dysplasia and adenocarcinoma as compared to normal epithelium had been reported (Lynch et al., 2008; Choi et al., 2010). However, p16 gene inactivation has also been described (Tadokoro et al., 2007) and overexpression of retinoblastoma protein may predict decreased survival and correlate with loss of p16INK4 protein in gallbladder carcinomas (Shi et al., 2000). The retinoblastoma protein is a tumor suppressor protein that is dysfunctional in many types of cancers (Murphree and Benedict, 1984). In humans the protein is encoded by the RB1 gene located on 13q14.1-q14.2. pRB is one of the core effectors of the G1/S transition (Das et al., 2005). pRB is underphosphorylated througout G1 phase and phosphorylated just before S-phase (Yoo et al., 2002; Beasley et al., 2003). Hypophosphorylated pRB arrests cell in G1 phase and phosphorylation relieves this inhibition resulting in S-phase entry (Kang et al., 2002; Yoo et al., 2002). Alteration of both cyclin D1 and other cell-cycle regulated proteins has been described for gallbladder carcinoma (Xuan et al., 2005). Hyperplasia of mucous epithelium caused by gallstones, a well-established risk factor, is reported to be associated with changes in the p16/CyclinD1/CDK4 pathway (Feng et al., 2011). Cyclin D1/P16/pRb pathway has been shown to play a critical role in tumorigenesis as this controls the entry of cells into the S-phase of cell-cycle by regulating the G1-S check point. The present case control study was carried out to evaluate the expression of cyclin D1, p16 and pRB in gallbladder carcinoma and to correlate it with stage of disease and compare their expression with that in cholelithiasis and normal gallbladder.

Materials and Methods

Collection and fixation of the specimen

Between November 2007 and July 2009, a total of 60 patients were recruited in this case control study. Of these, 25 patients had gallbladder carcinoma (group I), 25 had cholelithiasis (group II) and 10 patients were taken as normal controls, these patients had cholecystectomy performed for causes other then carcinoma of the gallbladder or the stones and had histological normal gallbladder. All patients with cancer were staged using AJCC 2002 TNM classification. Majority of the patients (n=17, 68%) were in stage II, while there were 16% patients in stage III (n=4), 12% in stage IV (n=03) and 4% (n=1) in stage I. Histologically, all the patients had adenocarcinoma of the gallbladder. Of these 18 (72%) were well differentiated and 5 (20%) were moderately differentiated.

After obtaining a written informed consent, the tissue specimens were obtained at the time of surgery and were fixed in 10% neutral buffered formalin overnight.

Immunohistochemistry

4 μm sections were cut from the blocks and were deparaffinized in xylene followed by hydration in a graded series of alcohols. Antigen retrieval was performed by immersing material in 0.5 M citrate buffer (pH6), and placing it into a microwave oven for 20 minute. Endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 for 20 minutes at room temperature. After rinsing in TBS buffer (Ph7.4), the sections were incubated with primary antibody against Cyclin D1, P16 and pRB at 4°c overnight. After 3 washing with tris buffer for 5 minute each, covered the sections with secondary antibody. The details of primary and secondary antibodies used are detailed in Table 1. Sections were counterstained with 3-3'-diaminobenzidine (DAB) followed by hematoxylin. Slides were washed in running water and were mounted with DPX.

The protein expression was then scored as: -, <25% positive cells; +, 25-50% positive cells; ++, >50-75% positive cells; and +++, >75% positive cells.

Statistical analysis

The data is expressed as percentages, categorical data was analyzed using chi square test, and correlation was carried out by spearman correlation.

Results

The mean age of the patients was 47.4±8.11 years (range: 25-70) in patients of gallbladder carcinoma

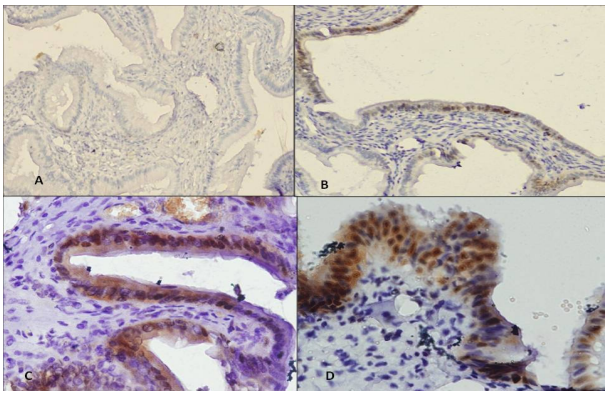

Figure 1. Photomicrograph Showing Staining for Cyclin D-1. A) Negative staining in normal gallbladder, B) 2+ staining in cholelithiasis patient (100X), C) magnified view of staining in cholelithiasis (400X), D) 3+ staining in carcinoma of the gallbladder (400X)

Table 1. List of Primary and Secondary Antibody Used

Primary antibody	Type (clone)	Dilution & Application/ time	Antigen Retrival (retrival solution pH)	Detection system	Source
Cyclin D1	Polyclonal	Prediluted/ 30 min.	Microwave oven (pH 6 citrate)	Supersensitive Multilink, BioGenex	BIOgENEX, San Ramson, CA (USA)
P16	Monoclonal	1:50/ 30 min.	Microwave oven (pH 6 citrate)	Immunocruz System	Santa Cruz, CA (USA)
pRB	Monoclonal	1:50/ 30 min.	Microwave oven (pH 6 citrate)	Immunocruz System	Santa Cruz, CA (USA)

Table 2. Relation of Cyclin D1, p16 and pRb with Various Parameters in Cancer Groups

Variable		N=25	Cycln D1		P16		pRb			Pearson Chi-square (P value)	Linear-by-linear Associate (p value)	Pearson correction on (p value)
			(-)	(+)	(-)	(+)	(-)	(+)				
T stage	T2	3	2	1	2	1	2	1	Cyclin D1	0.297	0.298	0.116
	T3	18	12	6	16	2	13	5	P16	0.396	0.374	0.104
	T4	4	1	3	4	0	4	0	pRB	0.461	0.292	0.145
N Stage									Cyclin D1	0.181	0.162	0.098
	N0	19	10	9	18	1	14	5	P16	0.065	0.090	0.035
	N1	6	5	1	4	2	5	1	pRB	0.629	0.620	0.322
M stage									Cyclin	0.024	0.013	0.012
	M0	22	15	7	19	3	17	5	P16	0.495	0.365	0.258
	M1	3	0	3	3	0	2	2	pRB	0.687	0.696	0.351
Differentiation	Well Differentiated	18	12	6	12	6	12	6	Cyclin D1	0.302	0.24	0.063
	Mod Differentiated	5	3	2	3	2	3	2	P16	0.641	0.420	0.154
	Poorly Differentiated	2	0	2	0	2	0	2	pRb	0.604	0.595	0.461

Table 3. Intensity of Staining of the Three Proteins

		Group I (n=25) No. (%)	II (n=25) No. (%)	III (n=10) No. (%)
Cyclin D1 expression	0	15 (60%)	15 (60%)	8 (80%)
	1	6	7	1
	2	3	3	1
	3	1	0	0
Total (+)		10 (40%)	10 (40%)	2 (20%)
Rb expression	0	19 (76%)	17 (64%)	5 (50%)
	1	5	5	2
	2	1	2	2
	3	0	1	1
Total(+)		6 (24%)	9 (36%)	5 (50%)
p 16 expression	0	22	18	10
	1	3	7	0
	2	0	0	0
	3	0	0	0
Total (+)		3 (12%)	7 (28%)	0 (0%)

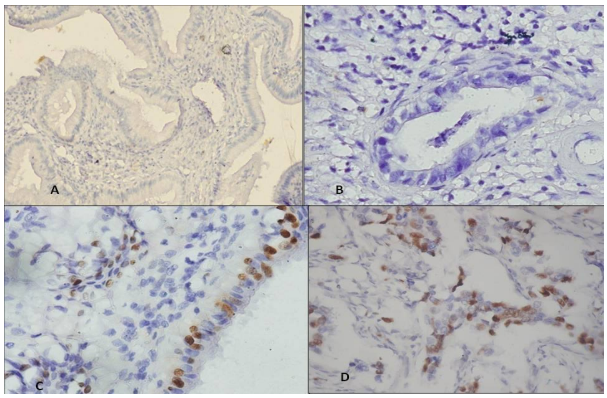

Figure 2. Photomicrograph Showing p16 Immunostaining. A) negative normal control, B) Negative immunostaining in carcinoma of the gallbladder, C) 1+ staining in cholelithiasis, D) 2+ staining in carcinoma of the gallbladder

with 68% being in the 4th and 5th decade of life. Cyclin D1 expression was seen in 10 (40%) patients each with carcinoma and cholelithiasis while only 2 (20%) normal gallbladders expressed it. The differences in Cyclin D1 expression was not statistically significant (p value=0.488) (Table 2). The level of expression of cyclin D1 in all the three groups in majority of the cases was mild (+1) and nuclear (Table 3). However, all the three cases of distant metastasis (stage IV) expressed Cyclin D1 and it significantly correlated with metastatic disease (p

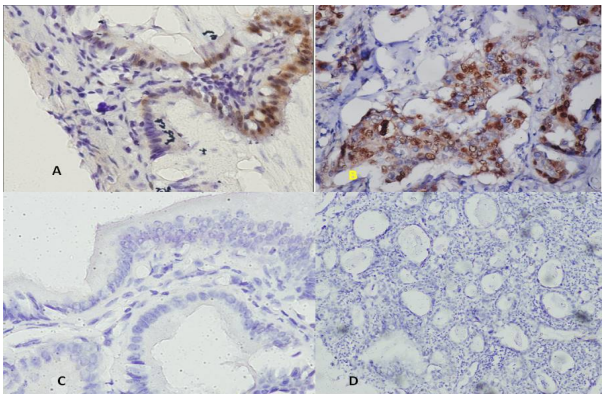

Figure 3. Immunostaining for pRB. A) 2+ staining in cholelithiasis, B) 3+ staining in carcinoma of the gallbladder, C) negative staining in cholelithiasis, D) negative staining in carcinoma of the gallbladder

value=0.012) (Table 2, Figure 1).

p16 was expressed in 12% patients of carcinoma of the gallbladder and 28% of cholelithiasis, however this difference was not statistically significant (p value=0.095). None of the cases with normal gallbladder expressed p16 (Figure 2). The grade of expression is detailed in Table 3. A positive correlation between p16 expression and nodal involvement was observed in carcinoma group (p value=0.035) (Table 2).

Retinoblastoma protein was found to be expressed in 50% of normal gallbladders and 6 (24%) of carcinoma and 8 (32%) of gallstones (Figure 3). The difference in expression was however not significant (p value=0.327). The intensity of staining is detailed in Table 3. Retinoblastoma protein expression also failed to show any correlation with tumors stage or grade (Table 2).

Among the 10 cases of p16-positive gallbladder cancer, 4 were pRb positive, while 7 were Cyclin D1 positive. Tumor suppressor gene p16 expression correlated with Cyclin D1 (χ^2=5.742, p value -0.017; r=0.309, p value -0.016). No correlation was observed between pRb and Cyclin D1, p16 and pRb.

Discussion

Gallbladder carcinoma is the most common malignancy in billiary tract and represents 1% of all the cancers (Jemal et al., 2005). The disease has poor prognosis due to late detection and early metastasis and

invasion of adjacent organs (Fan et al., 2002). Despite recent advances in radiological and surgical techniques, the long term survival of gallbladder carcinoma is poor, with the overall 5 year survival rate ranging from 5% to 13% (Pandey et al., 2001; 2003). Compared with other common cancers, identification of prognostic markers of gallbladder carcinoma has not been extensively studied (Cubertafond et al., 1994; Ito et al., 2004), leading to this tumor being often called as "orphan tumor". Preoperative clinical or radiological staging, an essential process for the prognostic evaluation of gallbladder carcinoma, has been extensively debated by the clinicians, due to its limitations in accurate classification (Levy et al., 2002; Flemming et al., 2007). Therefore, identification of reliable molecular marker may provide important prognostic information and facilitate adequate treatment plans and targets for a novel therapeutic approach.

Cyclin D1/p16/pRb pathway has been shown to play critical role in tumorigenesis (Cho et al., 2002; Hwang et al., 2002; Guner et al., 2003). Cyclins and Cyclin inhibiting proteins are the main regulator in a cell-cycle progression. Progression of cells from G1 to S-phase is controlled via pRB phosphorylation by Cyclin D1 complexed with Cyclin-dependent kinases (CDKs) 4&6, which are in turn regulated by CDK inhibitors, such as p16 protein (Yoo et al., 2002; Beasley et al., 2003).

Several human cancers have been found to have deregulated Cyclin D1 (Brantley and Harbour 2000), however, in the present study no difference expression was observed between carcinoma and cholilithiasis group. Earlier studies have found that Cyclin D1 protein expression significantly correlate with invasion and metastasis. All of the three cases with metastatic disease in our study showed Cyclin D1 expression suggesting a positive relation between tumor metastasis and Cyclin D1 expression (x^2=5.114, p value=0.024, R=0.452, p value=0.012). Our results are in line with those of Said et al. (2012) for Cyclin D1.

The p16 gene, located on chromosome 9p21, encodes a critical negative regulator of cell cycle progression and is inactivated in various cancers. The p16 gene is an important tumor suppressor gene, which interacts strongly with cyclin-dependent kinases 4 and 6, and inhibits their ability to interact with cyclin D (Sherr, 1996). p16 induces cell-cycle arrest at G1 and G2/M checkpoints, which blocks cells from phosphorylating retinoblastoma protein 1, and prevents cells from exiting the G1 phase of the cell-cycle (Weinberg, 1995). p16 can act as a negative regulator of normal cell proliferation. Inactivation of the p16 gene plays an important role in tumorigenesis. In our study there was no significant difference in expression of p16 between the groups (x^2=4.704, p value=0.095). Controversy abound its relation with various clinico-pathological factors. Ma et al. (2005) reported that decreased expression of p16 is correlated with pathological grade and tumor progression in gallbladder carcinoma. However, Shi et al. (2000) and Quan et al. (2001) have reported that loss of p16 protein expression is not significantly associated with any clinico-pathological factors or survival. We also failed to find any association between p16 expression and tumor size, metastasis, stage of the disease and grade of the tumor. However, a positive correlation between p16 expression and nodal involvement was observed in patients with carcinoma of the gallbladder.

RB protein is the centre of several cell-cycle regulatory pathways. Its non-phosphorylated form exists in G0/G1 phase of the cell-cycle and phosphorylated form in S/G2 phase, suggesting it to be an important regulatory gene. RB protein plays an important role in cell growth and differentiation (Dasgupta et al., 2004). In hypophosphorylated state it represses E2F transcription factor activity at the promoter site for the genes required for entry into the S-phase. In its hyperphosphorylated and inactive state which is basically done by Cyclin CDK complexes, it releases E2F transcription factors so the genes are transcribed for entry into the S-phase (Pei et al., 2005; Li et al 2006). Loss of pRB has been demonstrated in a variety of cancers, including gastric, pancreatic and bladder cancers, small cell lung and colorectal carcinoma (Pan et al., 2002; Gregorc et al., 2003; Raghvan et al., 2003; Zhang et al., 2003). In present study no significant difference was found in pRB expression in carcinoma gallbladder, cholilithiasis and normal gallbladder. Also there was no significant correlation between pRB and tumor size, nodal status, metastasis, staging and grading of the tumor.

In conclusion the present study failed to demonstrate any conclusive role of cyclin D1/RB/ p16 pathway in carcinoma of the gallbladder. However, a positive relation was observed between tumor metastasis and cyclinD1 expression and p16 with nodal metastasis suggesting that aggressive behavior for carcinoma of the gallbladder having higher Cyclin D1 /p16 expression and its expression may act as a predictive biomarker for behavior of the gallbladder malignancy. Further studies are required to test and support this hypothesis.

References

Beasley MB, Lantuejoul S, Abbondanzo S, et al (2003). the p16/ cyclin D1/ Rb pathway in neuroendocrine tumors of the lung. *Hum Pathol*, **34**, 136-42.

Brantley MA Jr, Harbour JW (2000). Deregulation of the Rb and p53 pathwaysin uveal melanoma. *Am J Pathol*, **157**, 795-1801.

Cho NH, Kim YT, Kim JW (2002). Alteration of cell-cycle in cervical tumor associated with human papilloma virus: cyclin dependent kinase inhibitor. *Yonsei Med J*, **43**, 722-8.

Choi HJ, Yun SS, Kim HJ, Choi JH (2010). Expression of p16 protein in gallbladder carcinoma and its precancerous conditions. *Hepatogastroenterology*, **57**, 18-21.

Cubertafond P, Gainant A, Cucchiaro G (1994). Surgical treatment of 724 carcinoma of the gallbladder. Results of the French surgical association survey. *Ann Surg*, **219**, 275-80.

Das SK, Hashimoto T, Schimizu K, et al (2005). Fucoxanthin induces cell cycle arrest at G0/G1 phase in human colon carcinoma cells through up-regulation of p21WAF1/Cip1. *Biochim Biophys Acta*, **1726**, 328-35.

Dasgupta P, BettsV, Rastogi S, et al (2004). Direct binding of apoptosis signal-regulating kinase 1 to retinoblastoma protein: novel links between apoptotic signaling and cell cyclr machinery. *J Biol Chem*, **279**, 762-9.

Fan YZ, Zhang JT, Yang HC, Yang YQ (2002). Expression of MMP-2, TIMP-2 protein and the ratio of MMP-2/ TIMP-2

in gallbladder carcinoma and their significance. *World J Gastrointerol*, **8**, 1138-43.
Feng Z, Chen J, Wei H, et al (2011). The risk factor of gallbladder cancer: hyperplasia of mucous epithelium caused by gallstones associates with p16/CyclinD1/CDK4 pathway *Exp Mol Pathol*, , 569-77.
Fleming ID, Cooper JS, Henson DE, et al (1997). AJCC cancer staging manual. 5th ed. JB Lippincott, Philadelphia 1997.
Gillett C, Fantl V, Smith R, et al (1994). Amplification and overexpression of Cylin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res*, **54**, 1812-7.
Gregorc V, Ludovini V, Pistola L, et al (2003). Relevance of p53, bcl-2 and Rb expression on resistance to cisplatin-based chemotherapy in advanced non-small cell lung cancer. *Lung Cancer*, **39**, 41-8.
Guner D, Sturm I, Hemmanti P, et al (2003). Multigene analysis of Rb pathway and apoptosis control in esophageal squamous cell carcinoma identifies patients with good prognosis. *Int J Cancer*, **103**, 445-54.
Hartwell LH, Kastan MB (1994). Cell cycle control and cancer. *Science*, **266**, 1821-8.
Hawang CF, Cho CL, Huang CC, et al (2002). Loss of cyclin D1 and p16 expression correlates with local recurrence of nasopharyngeal carcinoma following radiotherapy. *Ann Oncol*, **13**, 1246-51.
Ito H, Matros E, Brooks DC, et al (2004). Treatment outcomes associated with surgery for gallbladder cancer: a 20-year experience. *J Gastrointest Surg*, **8**, 183-90.
Jemal A, Murray T, Ward E, et al (2005). Cancer Statics. *CA Cancer J Clin*, **55**, 10-30.
Kamb A, Gruis NA, Weaver-Feldhaus J, et al (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science*, **264**, 436-40.
Kang YK, Kim WH, Jang JJ (2002). Expression of G1/S modulators (p53, p16, p27, cyclin D1, Rb) and Smad4/Dpc4 in intrahepatic cholangio carcinoma. *Hum Pathol*, **33**, 877-83.
Kato J, Matsushime H, Heibert SW, EwenME, Sherr CJ (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRB) and pRb phosphorylation by the cyclin D – Dependent kinase CDK4. *Genes Dev*, **7**, 331-42.
Lazcano-ponce EC, Miqual JF, Munoz N, et al (2001). Epidemiology and moleecular pathologyof gallbladder cancer. *CA Cancer J Clin*, **51**, 349-64.
Lerma E, Esteler M, Harman JC, Prat J (2002). Alteration of p16/Rb/cyclin D1 pathway in vulvular carcinoma, vulvular intraepithelial neoplasia and lochen sclerosis. *Hum Pathol*, **33**, 1020-5.
Levy AD, Murakata LA, Rohrmann CA (2001). Gallbladder carcinoma: radiologic- pathologic correlation. *Radiographics*, **21**, 295-314.
Li W, Sanki A, Karim RZ, et al (2006). The role of cell cycle regulatory proteins in the pathogenesis of melanoma. *Pathology*, **38**, 287-301.
Lynch BC, Lathrop SL, Ye D, Ma TY, Cerilli LA (2008). Expression of the p16(INK4a) gene product in premalignant and malignant epithelial lesions of the gallbladder. *Ann Diagn Pathol*, **12**, 161-4.
Ma HB, Hu HT, Di ZL, et al (2005). Association of cyclin D1, p16 1nd retinoblastoma protein expressions with prognosis and metastasis of gallbladder carcinoma. *World J Gastroenterol*, **11**, 744-7.
Michalides R (2002). Cell cycle regulators: role in etiology, prognosis and treatment in cancer. *Ann Oncol*, **13**, 39.
Misra S, Chaturvedi A, Misra NC, Sharma ID (2003). Carcinoma of the gallbladder. *Lancet Oncol*, **4**, 167-76.
Murphree AL, Benedict WF (1984). Retinoblastoma: clues to human oncogenesis. *Science*, **223**, 1028-33.
Offerhaus GTA (2002). Tumors of the gallbladder, extrahepatic bile ducts and ampulla of vater. atlas of tumor pathology. *J Clin Pathol*, **54**, 816.
Pan MH, Chen WJ, Lin-Shiau SY, Ho CT, Lin JK (2002). Tangeretin induces cell-cycle G1 arrest through inhibiting cyclin-dependent kinase 2 and 4 activities as well as elevating Cdk inhibitors p21 and p27 in human colorectal carcinoma cells. *Carcinogenesis*, **23**, 1677-84.
Pei XH, Xiong Y (2005). Biochemical and cellular mechanisms of mammalian CDK inhitors: a few unresolved issue. *Oncogene*, **24**, 2787-95.
Pandey M, Pathak AK, Singh S, Gautam A, Shukla VK (2001). Carcinoma of the gallbladder: a retrospective review of 99 cases. *Dig Dis Sci*, **46**, 1145-51.
Pandey M (2003). Risk factors for gallbladder cancer a reappraisal. *Eur J Cancer Prev*, **12**, 15-24.
Quan Zw, Wu K, Wang J, et al (2001). Association of p53, p16, and vascular endothelial growth factor protein expression with the prognosis and metastasis of gallbladder cancer. *J Am Coll Surg*, **193**, 380-3.
Raghvan D (2003). Molecular targeting and pharmacogenomics in the management of advanced bladder cancer. *Cancer*, **97**, 2083-9.
Rifatbegovic Z, Mesic D, Ljuca F, Zildzic M, Morankic M (2007). Incidence and surgical treatment of cancer in gallbladder. *Med Arh*, **61**, 30-3.
Russell A, Thompson MA, Hendley J, et al (1999). Cyclin D1 and D3 associate with the SCF complex and are coordinately elevated in breast cancer. *Oncogene*, **18**, 1983-91.
Said K, Glaumann H, Björnstedt M, Bergquist A (2012). The value of thioredoxin family proteins and proliferation markers in dysplastic and malignant gallbladders in patients with primary sclerosing cholangitis. *Dig Dis Sci*, **57**, 1163-70.
Sauter ER, Yeo UC, Von Stemm A, et al (2002). Cycin D1 is a candidate oncogene in cutaneous melanoma. *Cancer Res*, **62**, 3200-6.
Sdek P, Zhang Z, Coa J (2002). Influence of HPV 16 on the expression of p16/ cyclin D1/ Rb in oro epithelial cancer. *Zhongua Kouqiang Yixue Zazhi*, **16**, 84-6.
Serrano M, Hannon GJ, Beach D (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*, **366**, 704-7.
Sherr CJ (1996). Cancer cell cycles. *Science*, **274**, 1672-7.
Shi YZ, Hui AM, Li X, Takayama T, Makuuchi M (2000). Overexpression of retinoblastoma protein predicts decreased survival and correlates with loss of p161INK4 protein in gallbladder carcinomas. *Clin Cancer Res*, **6**, 4096-100.
Tadokoro H, Shigihara T, Ikeda T, Takase M, Suyama M (2007). Two distinct pathways of p16 gene inactivation in gallbladder cancer. *World J Gastroenterol*, **13**, 6396-403.
Weinberg RA (1995). The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323-30.
Xuan YH, Choi YL, Shin YK, et al (2005). An immunohistochemical study of the expression of cell-cycle-regulated proteins p53, cyclin D1, RB, p27, Ki67 and MSH2 in gallbladder carcinoma and its precursor lesions. *Histol Histopathol*, **20**, 59-66.
Yoo J, Park SY, Kang SJ, Shim SI, Kim BK (2002). Altered expression of G1 regulatory proteins in human soft tissue sarcomas. *Arch Pathol Lab Med*, **126**, 567-73.
Zhang R, Zhang JJ, He ZC, Cheng SJ, Gao YN (2003). Research advances on bladder cancer associated genes. *Aizheng*, **22**, 104-7.