RESEARCH ARTICLE

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

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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 suppressor genes - hepatobiliary malignancies

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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; Russell 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 p16INK 4a gene belongs to the G1 control gene (a tumor suppressor...
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 throughout 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 than carcinoma of the gallbladder or the stones and had histopathological 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=3) 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% H2O2 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

| Table 1. List of Primary and Secondary Antibody Used |
|----------------------------------------------|
| Primary antibody | Type (clone) | Dilution & Application/ time | Antigen Retrieval (retrieval 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        | Cyclin D1 | P16 | pRb | Pearson Chi-square (P value) | Linear-by-linear Associate correction on (p value) |
|-----------------|-----------|-----|-----|-----------------------------|-----------------------------------------------|
| T stage         | (-) (+)   | (-) (+) | (-) (+) | Cyclin D1                  | pRb                                           |
| T2              | 3 2       | 1 2   | 1 2  1 | 2 1                         | 0.297 0.298 0.116                               |
| T3              | 18 12     | 6 16  | 2 13  5 | 13 5                        | 0.396 0.374 0.104                               |
| T4              | 4 1       | 3 4   | 0 4   | 0                          | 0.461 0.292 0.145                               |
| N Stage         | (-) (+)   | (-) (+) | (-) (+) | Cyclin D1                  | pRb                                           |
| N0              | 19 10     | 9 18  | 1 14  5 | 14 5                        | 0.181 0.162 0.098                               |
| N1              | 6 5       | 1 4   | 2 5   | 1                          | 0.065 0.090 0.035                               |
| M stage         | (-) (+)   | (-) (+) | (-) (+) | Cyclin D1                  | pRb                                           |
| M0              | 22 15     | 7 19  | 3 17  5 | 17 5                        | 0.024 0.013 0.012                               |
| M1              | 3 0       | 3 0   | 2 2   | 2                          | 0.678 0.696 0.351                               |
| Differentiation | (-) (+)   | (-) (+) | (-) (+) | Cyclin D1                  | pRb                                           |
| Well Differentiated | 18 12   | 6 12  | 6 12 | 6                          | 0.302 0.24 0.063                                |
| Mod Differentiated | 5 3     | 2 3   | 2 2   | 2                          | 0.641 0.420 0.154                               |
| Poorly Differentiated | 2 0     | 2 0   | 2 2   | 2                          | 0.604 0.595 0.461                               |

Table 3. Intensity of Staining of the Three Proteins

| Variable        | Group I (n=25) | Group II (n=25) | Group III (n=10) |
|-----------------|----------------|-----------------|------------------|
| Cyclin D1       | No. (%)        | No. (%)         | No. (%)          |
| Expression 0    | 15 (60%)       | 15 (60%)        | 8 (80%)          |
|                | 1 6 7 1        | 1 6 7 1         | 1 6 7 1          |
|                | 2 3 3 1        | 2 3 3 1         | 2 3 3 1          |
|                | 3 1 0 0        | 3 1 0 0         | 3 1 0 0          |
| Total (+)       | 10 (40%)       | 10 (40%)        | 2 (20%)          |
| Rb expression   | 0 19 (76%)     | 17 (64%)        | 5 (50%)          |
|                | 1 5 5 2        | 1 5 5 2         | 1 5 5 2          |
|                | 2 1 2 2        | 2 1 2 2         | 2 1 2 2          |
|                | 3 0 1 1        | 3 0 1 1         | 3 0 1 1          |
| Total (+)       | 6 (24%)        | 9 (36%)         | 5 (50%)          |
| p16 expression  | 0 22 18 10     | 15 10           | 5 (50%)          |
|                | 1 3 7 0        | 1 3 7 0         | 1 3 7 0          |
|                | 2 0 0 0        | 2 0 0 0         | 2 0 0 0          |
|                | 3 0 0 0        | 3 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 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 (χ²=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 biliary 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 cholangitis 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 (x²=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 (x²=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, cholangitis 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.

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