

Original Article

Cell cycle biology of fibrolamellar hepatocellular carcinoma

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Abstract: Context: Fibrolamellar hepatocellular carcinoma (FLHCC) has a better prognosis than conventional hepatocellular carcinoma. Nevertheless, FLHCC has a propensity to recur with limited responsiveness to chemotherapy. Objective: The purpose of this study was to provide insight into the cell cycle biology of FLHCC, as it relates to FLHCC's relatively indolent nature and lack of chemoresponsiveness. Design: In seven cases of FLHCC, we assessed: 1. immunoreactivity of protein analytes indicating cell cycle progression including Ki-67 (G1, S, G2 and M phases) and S-phase kinase-associated protein (Skp) 2 along with the mitotic index (MI); 2. immunoreactivity for cyclin-dependent kinase inhibitors of cell cycle progression from G1 to S phase, p27Kip1 and p16INK4. Results: The mean percentage of Ki-67 nuclear positivity in neoplastic hepatocytes ranged from 1.0% to 29.7%. Nuclear Skp2 immunoreactivity was not observed in any of the cases. The mitotic index was very low (0-1 mitotic figure / 10 high-power fields). All cases showed moderate to strong nuclear p16INK4 positivity (diffuse in five and focal in two). Contrastively, the adjacent non-neoplastic hepatocytes expressed only mild (2 cases) to no (3 cases) p16INK4. Conclusion: Our analysis has revealed that cell cycle arrest in FLHCC occurs in G0/G1 phase and is associated with overexpression of the cell cycle regulator, p16INK4 in tumoral cell nuclei compared with non-neoplastic hepatocytes. In conjunction with our previous immunohistochemical demonstration of a constitutively activated nuclear factor (NF)-kappaB pathway and stemness characteristics of FLHCC with limited differentiation, this cell cycle arrest elucidates the biology of FLHCC's indolent nature and relative chemoresistance.

Keywords: Cell cycle, liver, fibrolamellar hepatocellular carcinoma, immunohistochemistry, p16INK4, chemoresistance

Introduction

Fibrolamellar hepatocellular carcinoma (FLHCC) is a unique type of primary liver cancer that occurs principally in children and young adults, and is not associated with chronic liver disease [1]. Histologically, it is characterized by large polygonal cells containing abundant eosinophilic cytoplasm, large vesiculated nuclei, prominent nucleoli and surrounded by lamellar bands of fibrosis [1]. Patients with FLHCC have a better prognosis than conventional hepatocellular carcinoma (HCC) that arise in setting of cirrhosis, but similar prognosis as conventional HCC arising in non-cirrhotic livers [1]. Surgical resection is the mainstay of treatment. Compared with conventional HCC, this tumor does not respond well to chemotherapy [2]. Based on these features, FLHCC is now considered as a distinct form of liver cancer.

The molecular profile of FLHCC is largely un-

known. Only a limited number of individual oncogenes and their signaling pathways have been studied in FLHCC and their results indicate that the uniqueness of FLHCC extends to its molecular findings [1]. Our previous study suggests that NF- κ B pathway is constitutively activated in FLHCC and may be involved in development and progression of this tumor [3]. In another study we demonstrated the stemness characteristics in FLHCC and showed contrasts with conventional hepatocellular carcinoma [4]. Involvement of some key molecular signaling pathways and genes that are typically dysregulated in conventional HCC, including tumor protein (TP) 53, alpha-fetoprotein, beta-catenin mutations and survivin, is not consistently found in FLHCC.

Many studies have reported genetic and epigenetic alterations of cell cycle regulators in hepatocellular carcinoma. In particular, the cyclin-dependent kinase (CDK) inhibitors p16INK4,

Table 1. Clinical profile of seven patients

Case	Age (yr)	Race	Sex	Tumor stage	Chronic liver disease	Alpha fetoprotein
1	80	White	Female	T2NOMO	No	Normal
2	23	Asian	Male	T2NOMO	No	Normal
3	42	White	Male	T3N1M1	No	Normal
4	20	White	Female	T2NOMO	No	Normal
5	38	White	Male	T2NOMO	No	Normal
6	27	Hispanic	Female	T2NOMO	No	Normal
7	31	White	Male	T2NOMO	No	Normal

p21(WAF1/CIP1) and p27Kip1 are independently affected and a change in the expression of one or more of these inhibitors contributes to carcinogenesis of the majority (nearly 90%) of HCCs [5]. The CDK inhibitor p16INK4 is predominantly inactivated by post-transcriptional regulation and p16INK4 inactivation contributes to early-stage of hepatocarcinogenesis and in disease progression [5]. In particular, both p16 and p27 are frequently downregulated in HCC, indicating their role in progression of hepatic carcinogenesis [6].

The purpose of this study was to provide insight into the biology of FLHCC, and specifically, its cell cycle biology as it relates to its relatively indolent nature and general lack of chemoresponsiveness, using an immunohistochemical approach.

Materials and methods

After Institutional review board (IRB) approval, paraffin blocks from seven archival cases of FLHCC were retrieved for the study. The age of patients with FLHCC ranged from 20 to 80 years. Of the 7 patients, 4 were men and 3 women. None of the patients had any evidence of chronic liver disease. All patients had negative serology for hepatitis A, B and C infection and normal serum alpha-fetoprotein (AFP). The clinical profile of these patients is listed in **Table 1**. Five of the seven cases had contiguous benign liver in the same slide and these served as internal non-neoplastic controls. Tissue sections from paraffin blocks were cut at 4 µm thickness. Hematoxylin and eosin (H&E) staining was used to assess mitotic index, which was assessed as number of mitotic figures per 10 high power fields (h.p.f.'s). Immunohistochemical probes using standard avidin-biotin technique

was utilized for detection of following cell cycle associated proliferation markers, Ki-67 (DakoCytomation, Carpinteria, CA) and S-phase kinase-associated protein (Skp)2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and the cyclin-dependent kinase (CDK) inhibitors, p16INK4a (BD Pharmingen, San Diego, CA) and p27Kip1 (VisionBiosystems-Novocastra, Newcastle upon Tyne, United Kingdom). Immunoreactivity was assessed, in terms of subcellular compartmentalization, by bright-field microscopy. Absence of staining was considered as negative and presence of distinct nuclear staining was considered positive regardless of the staining intensity. Positive and negative controls were run concurrently and noted to react appropriately. Immunohistochemical expression of the aforementioned analytes in the FLHCC were evaluated and compared with corresponding reactions in benign liver tissue. The percentage of Ki-67 positive nuclei was determined by an automated cellular imaging system (ACIS III by DAKO).

Results

A variable, average percentage of nuclear Ki-67 staining was noted amongst tumors and this ranged from 1.0% to 29.8% with a median of 5.5 % (**Figure 1A**). The tumoral nuclear expression of Skp2 was uniformly absent (<1%) in all of the seven cases (**Figure 1B**). The mitotic index was also very low (0-1 mitotic figure/10hpf). These findings indicate a cell cycle arrest in G0-G1 phase. Moderate to strong nuclear expression of p16INK4 was observed in tumor cells in all seven cases (**Figure 2A**). Two of these seven (29%) cases however showed focal expression. Mild cytoplasmic expression of p16INK4 protein was also noted. The contiguous non-neoplastic liver showed mild nuclear expression of

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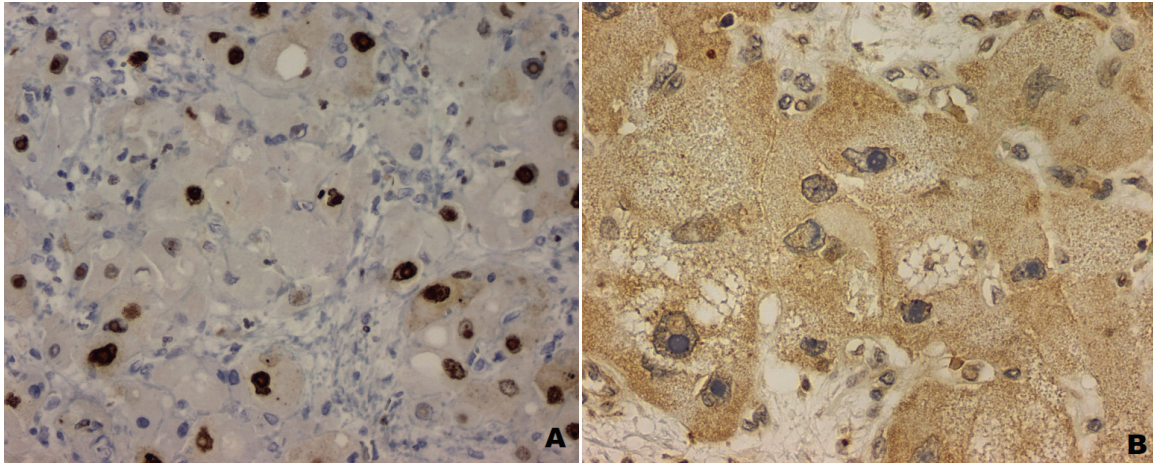


Figure 1. Nuclear Ki-67 immunopositivity in tumor cells of fibrolamellar hepatocellular carcinoma, case with average nuclear percentage of 29.8% (A), original magnification x200). Absence of nuclear immunopositivity of Skp 2 protein with background cytoplasmic staining (B), original magnification x400).

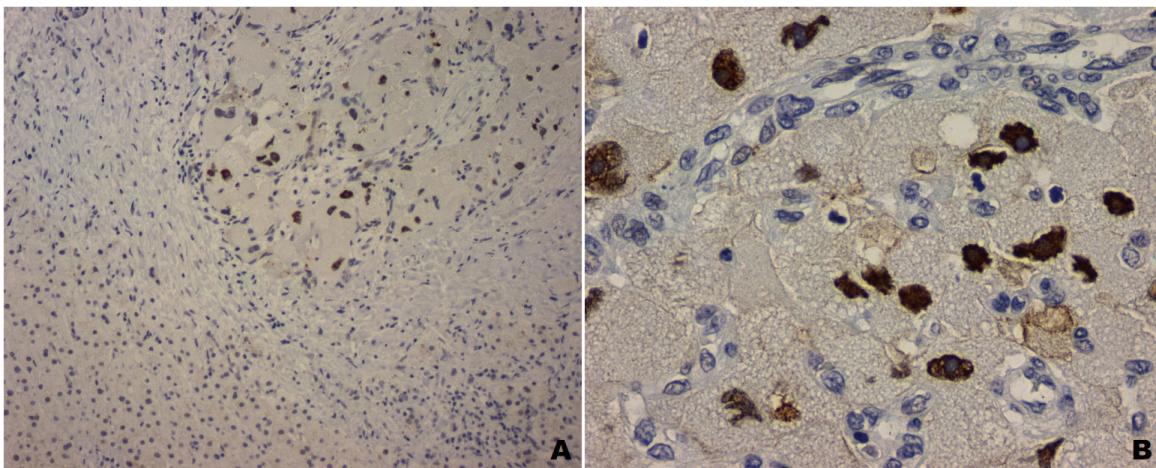


Figure 2. Intense nuclear p16INK4A noted in fibrolamellar hepatocellular carcinoma, upper right contrasts with no expression in adjacent non-neoplastic hepatocytes in lower left (A), original magnification x100). Nuclear localization of p16INK4A with sparing of nucleoli (B), original magnification x 400).

p16INK4 protein in two of the cases and no nuclear expression in three of the cases. This difference in signal intensity and expression frequency of nuclear p16INK4 between tumoral cells and the contiguous non-neoplastic hepatocytes indicates over expression in the tumor per se (Figure 2A). One of the seven (14%) cases also showed mild to moderate nuclear expression of p27kip1 in tumor cells. The expression frequency of these cell cycle analytes is also summarized in Table 2.

Discussion

Our results show that FLHCC has a variable and modest proliferation index (Ki-67 at 1%-29%) with absence of S-phase kinase-associated protein (Skp) 2 expression. Skp 2 defines cells in S (DNA synthesis) phase of the cell cycle [7]. Furthermore, when viewed in the context of a low to absent mitotic index, these findings indicate a proliferative quiescence of tumor cells, consistent with cell-cycle arrest in G0-G1 phase in

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Table 2. Nuclear cell cycle analytes and expression frequency in seven (7) cases of fibrolamellar hepatocellular carcinoma

Analyte	Cell cycle phase	Expression frequency
Ki-67	G1, S,G2, M phase	7/7*
p16INK4	CDKI(Inhibits G1 to S phase)	7/7 (100%)
p27Kip1	CDKI(Inhibits G1 to S phase)	1/7(14%)
Skp2	S phase (Promotes G1 to S phase)	0/7 (0%)
Mitotic Index**	M phase	0-1 mitotic figures /10 hpf

* Ki-67 (proliferation index) with average nuclear percentage range of 1 to 29.8% among the 7 cases (median at 5.5 %). ** Mitotic index range of 0 to 1 mitotic figures per ten high power fields in each of 7 cases.

FLHCC.

Cell cycle progression leading to mitotic division is a precisely orchestrated, sequential event with four major phases G0/G1, S, G2 and M. During these phases the cells are exited from a quiescent state (G0) upon the stimuli by growth factors, entering into an active state(G1); the cells then progress to S phase in which DNA synthesis occurs; following the G1/S phases are the G2 and M phases, in which mitotic events occur. Progression through each cell cycle is tightly controlled by the key cell cycle regulators including cyclins, cyclin-dependent kinases (CDK's) and CDK inhibitors [8]. Cell-cycle entry is mainly promoted by cyclins A, D and E with CDK 2 and 4. The activities of these CDKs are negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs), to include the INK4 family (p16INK4) and the CIP/KIP inhibitors (p27KIP1) [6, 9].

In this study, we observed moderate to strong expression of p16INK4 in the nuclei of tumor cells in all the seven cases. Two of these (29%) cases showed focal nuclear expression of p16INK4. One of the seven (14%) cases also showed p27KIP1 expression. The CDKI, p16INK4 is a G1-specific cell-cycle inhibitor that prevents association of CDK 4 and CDK 6 with cyclin D1 [6, 9]. Similarly, p27Kip1 is also a negative regulator of G1-S phase transition through inhibition of kinase activities of Cdk2/cyclin A and cdk2/cyclin E complexes [6, 9]. Both p16INK4 and p27KIP1 are potent tumor suppressors. Therefore, in our study, p16INK4 immunopositivity coupled with absent nuclear Skp2, low mitotic index and Ki67 indicates that protein p16INK4 is responsible for the observed cell cycle arrest in the G0/G1 phase in FLHCC,

thus holding the cells in a quiescent phase. However the variability of p16INK4 expression, (focal in 2/7 {29%} cases and diffuse in 5/7 {71%} cases) indicates that other cell cycle regulators like p 21(WAF1/CIP1) may play a role in the cell cycle arrest in FLHCC. Future studies will be necessary to confirm this. The presence of mild nuclear p16INK4 expression in the contiguous non-neoplastic liver in two cases, in this study, possibly reflects the transition phenomenon, where these hepatocytes morphologically look non-neoplastic, but have acquired the molecular characteristics of the tumor. We have reported similar phenomenon in our previous study on stemness characteristics of FLHCC, wherein we observed acquisition of CD44 in one of the cases [4].

Several studies in literature have reviewed the cell-cycle deregulation in cancer [10-14]. Cell cycle deregulation associated with cancer occurs through mutation of proteins important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding CDK, cyclins, CDK activating enzymes, CDK inhibitors, CDK substrates and checkpoint proteins [14]. The p16 gene (encodes for p16INK4 CDK inhibitor) is altered in a high percentage of human tumors and can be inactivated by a variety of mechanisms including deletions, point mutations and hypermethylation [15]. Deletions of the p16 gene, resulting in loss of expression of the p16INK4 protein, have been reported in approximately 50% of gliomas and mesotheliomas, 40%-60% of nasopharyngeal, pancreatic and biliary tract tumors and 20%-30% of acute lymphoblastic leukemias [16]. Since p16 is a negative regulator of G0/G1 to S-phase transition and acts as a cell-cycle checkpoint at this level, epigenetic alterations in p16 lead to loss

of this tumor suppressor function, causing enhanced cell proliferation and carcinogenesis [15].

Many studies have shown functional alterations of cell cycle regulators in hepatocellular carcinoma [17-20]. Biden et al [17] reported that p16 was frequently mutated or deleted in HCC in an Australian population while Chaubert et al [18] reported germline mutations of p16 in a subset of patients with familial HCC. Jim et al [19] found that overall frequency of p16 alterations in HCCs, including hypermethylation and homozygous deletions, was 60%. Matsuda et al [20] found hypermethylation of the p16 gene in 45% of HCC cases with loss of p16 immunorepression, indicating that epigenetic alterations induced loss of p16 protein overexpression in HCC. However above mentioned studies are in sharp contrast to our findings of upregulation and overexpression of p16INK4 protein immunorepression in FLHCC, which in the context of limited to no cell cycle progression beyond the G0/G1 phase (*vide supra*) indicates that the protein is functional and is responsible, at least in part, for holding the cells in a quiescent phase. Our findings support uniqueness of FLHCC, as a distinct form of liver cancer.

Although relatively little is known about the molecular biology of FLHCC, several studies [21-25] have demonstrated that these tumors are generally chromosomally stable, a finding distinct from conventional HCC that develop through accumulation of multiple genetic alterations. Kakar et al [22] in their study on chromosomal changes, in 11 cases of FLHCCs, used comparative genomic hybridization, and found chromosomal imbalances in 55% of their cases. They also noticed a trend towards higher 5-year survival in FLHCC with no cytogenetic aberration, thus suggesting that FLHCC with chromosomal aberrations tend to behave more aggressively, than those without aberrations [22]. Extensive computer assisted search of the National Library of Medicine's Medline Database did not reveal any previous literature on cell cycle biology of FLHCC. The literature citing cell cycle deregulation of HCC [5, 6] does not elucidate whether those studies included cases of FLHCC. Our findings of cell cycle arrest in FLHCC possibly relates to the indolent nature of this tumor and its relative chemoresistance. Moreover, our findings also suggest that use of cell cycle-dependent chemotherapeutic agents act-

ing beyond the G0/G1 phase will not be useful strategy for this cancer. However, our study is limited by few numbers of cases owing to the rarity of this tumor. Further studies with more number of cases are needed to validate these findings.

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