Epigenetic instability is rare in fibrolamellar carcinomas but common in viral-associated hepatocellular carcinomas

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Fibrolamellar carcinomas have a unique predilection for younger individuals and arise in livers without recognizable liver disease. In contrast to typical hepatocellular carcinomas, fibrolamellar carcinomas show few chromosomal changes and lack mutation in key genes such as *TP53* and *CTNNB1*. Epigenetic instability, manifesting as methylation of important tumor suppressor gene promoters, has not been investigated in fibrolamellar carcinomas. Thus, the methylation status of 11 tumor suppressor gene promoters was investigated using methylation-specific PCR: *RASSF1*, *CDH1*, *CDKN2B*, *HPP1*, *CDKN2A*, *GSTP1*, *P16*, *RARA*, *FLJ13081*, *SOCS1*, and *TP53*. Nine fibrolamellar carcinomas were studied including primary tumors ($N=5$) and metastatic deposits ($N=4$) along with control groups of typical hepatocellular carcinoma arising in livers with ($N=21$) and without cirrhosis ($N=10$). In fibrolamellar carcinomas, *RASSF1A* and *CDH1* (e-cadherin) were the most commonly methylated genes with 80–100% of tumors methylated. However, overall fibrolamellar carcinomas showed low levels of methylation with no differences between fibrolamellar carcinomas and their paired normal livers. However, fibrolamellar carcinomas showed significantly less methylation than hepatocellular carcinomas that arose in the background of viral cirrhosis. Overall, methylation was most strongly linked to viral cirrhosis. In conclusion, fibrolamellar carcinoma shows low levels of methylation. In contrast, higher levels of promoter methylation are associated with hepatocellular carcinomas that arise in the setting of viral induced cirrhosis.

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Fibrolamellar carcinoma is a variant of hepatocellular carcinoma that is unique at the clinical, histological, and molecular levels. It occurs in children and young adults with no evidence for chronic liver disease and is characterized histologically by a unique pattern of intratumoral fibrosis and large, polygonal and eosinophilic cells. The etiology of fibrolamellar carcinoma is unknown. The prognosis for fibrolamellar carcinoma is similar to hepatocellular carcinomas that arise in non-cirrhotic livers,¹ and both have a better prognosis than hepatocellular carcinomas that arise in cirrhotic livers.

It is generally agreed that hepatocellular carcinoma develops through the accumulation of multiple genetic alterations. In typical hepatocellular

carcinoma, chromosomal instability is thought to play an important role, as are mutations in various genes that regulate normal growth and development, apoptosis, and maintenance of genomic integrity.² In addition, epigenetic instability with methylation of tumor suppressor gene promoters has been reported to significantly contribute to the pathogenesis of hepatocellular carcinoma.³

Relatively little is known about the molecular biology of fibrolamellar carcinoma, but several studies have demonstrated that, while translocations can be found, fibrolamellar carcinomas are generally chromosomally stable.^{4–6} Likewise, mutations in key genes such as *TP53* and *CTNNB1* (beta-catenin), which are routinely identified in a proportion of typical hepatocellular carcinomas, have not been found in fibrolamellar carcinoma.^{7–9} The limited chromosomal instability and the lack of mutations in key genes found in hepatocellular carcinoma raised the possibility that epigenetic changes, such as methylation of tumor suppressor gene promoters, may play a prominent role in the etiology of fibrolamellar carcinomas. Thus, we

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sought to explore the role of methylation by examining a panel of tumor suppressor gene promoters in fibrolamellar carcinomas and comparing the frequency of methylation to two control groups: hepatocellular carcinomas arising in cirrhotic livers and hepatocellular carcinomas arising in non-cirrhotic livers without evidence for chronic liver disease.

Materials and methods

Tissues

After obtaining institutional approval, excess tissues were harvested from surgical pathology specimens and flash frozen in liquid nitrogen and stored at -80°C . Routine light microscopy confirmed the histological diagnosis in all cases.

For the fibrolamellar carcinomas, a total of five primary tumors were studied. Of these five cases, four had available paired non-neoplastic liver tissues. We also studied four metastatic deposits of fibrolamellar carcinoma. One of the metastases had a corresponding primary tumor that was also included in this study, while the remaining three had no primary tumor tissues available for analysis. As control groups, we studied the hepatocellular carcinoma tissues of 17 individuals with cirrhosis from chronic hepatitis, including chronic hepatitis C viral infection ($N=10$) and chronic hepatitis B viral infection ($N=7$). We also studied individuals who had either cryptogenic cirrhosis or cirrhosis secondary to non-alcoholic fatty liver disease ($N=4$). Most cases of cryptogenic cirrhosis in the United States are thought to be related to burned-out fatty liver disease. As another control group, we also studied individuals with typical hepatocellular carcinoma, but whose background livers were non-cirrhotic and who had no identifiable underlying chronic liver disease ($N=10$). This control group is critical in understanding fibrolamellar carcinoma, as the background livers are normal in both cases and the overall clinical course of fibrolamellar carcinoma is most similar to those hepatocellular carcinomas that arise in non-cirrhotic livers.¹

Methylation-Specific PCR Amplification

DNA was extracted from 20 to 25 mg of liver tissues (Dneasy Tissue, Qiagen, Valencia, CA, USA) and DNA bisulfite treatment was performed using EpiTect Bisulfite Kit (Qiagen, Valencia, CA, USA). There is no well-characterized list of tumor suppressor promoters that define a methylator phenotype in hepatocellular carcinoma. Thus, a list of tumor suppressor genes was selected based on a review of the literature and their relevance to hepatocellular carcinoma. Based on the literature and our preliminary data, it seemed unlikely that methylation of any single gene or small number of

genes was likely to be informative in differentiating the role of methylation in typical hepatocellular carcinoma vs fibrolamellar carcinoma. Thus, we choose a panel of eight tumor suppressor genes to examine. This was supplemented by the addition of three genes previously shown by our laboratory¹⁰ to be underexpressed in fibrolamellar carcinomas at the mRNA levels: *RARA*, *HPP1*, and *FLJ13081*. Primers were either selected from the literature (Table 1) or designed using MethPrimer (<http://www.urogene.org/methprimer/>).

Statistical Analysis

The means of three or more groups were compared by ANOVA, while the means of two groups were compared using Student's *t*-tests (Systat version 10).

Results

Demographic and Histological Findings

Of the five primary fibrolamellar carcinomas, three were from women and two from men with an age at diagnosis of 29 ± 14 years. All individuals with primary tumors underwent surgical resection of their tumor. Patients with metastatic disease underwent resection of these sites with curative intent. All of the cases with metastases were in women, averaging 22 ± 9 years at metastatic disease. In all cases, no clinical or histological evidence for background chronic liver disease was seen in the non-neoplastic tissues and the tumors and metastases were histologically typical of fibrolamellar carcinoma. For the viral cirrhosis control group, the average age at diagnosis was 56 ± 14 years and included 12 men and 5 women. For the fatty liver or cryptogenic cirrhosis group, the average age at diagnosis was 59 ± 10 years and included three men and one woman. For the non-viral, non-cirrhotic control group, the average age at diagnosis was 59 ± 17 years and included six men and four women. The ages at resection did not differ significantly between the control groups, $P=0.9$.

Methylation in Fibrolamellar Carcinoma

Of the three genes previously found to be down-regulated in fibrolamellar carcinomas in our laboratory, *RARA* and *FLJ13081* showed low to absent methylation, while *HPP1* was methylated in 2/5 (40%) of cases (Table 1). Likewise, there was either no or rare methylation of genes involved in cell cycle control, including *TP53*, *P14*, *P15*, and *P16*. Of all the promoters studied, methylation of the *RASSF1* promoter stood out as being the most commonly methylated promoter, with methylation seen in 4/5 cases (Figure 1). *E-cadherin* was partially methylated in all fibrolamellar carcinomas but the paired non-neoplastic tissues were also partially

Table 1 Primers used to amplify tumor suppressor gene promoters

Tumor suppressor gene	UF 5' TO 3'	UR 5' TO 3'	MF 5' TO 3'	MR 5' TO 3'
TP53 ¹¹	GTAGTTTGAATGTTTATTATTGTT	CCTACTACACCTCTACAAACA	GTAGTTTGAAGTTTATTATT TGGC	CCTACTACGCTCTACAAACG
CDKN2A (P14) ¹²	TGAGTTGGTTTGGAGGTGG	AACCAACAACAACACCCCT	GTGAGTTGGTTTGGAGG	AAAACCAACAACGACGAACG
CDKN2B (P15) ¹³	TGTCATGTGTTTGTATTTTGTGTT	GCATACAATAACCAACAACCA	GGTTTCGTATTTTGGGTT	GGTACAATAACCGAACGACCGA
P16 ¹²	TATATAGAGGTGGGTGGATGT	CAAGCCCAACCAACCAACATA	TATATAGAGGTGGGGGGATGCG	ACCCGAAACCGGACCGTAA
SOC1 ¹⁴	TATTTGTTGTATGTTGATTG	AACTCAACACACCAACCTC	TCGTTCTAGTCGATTATC	AAAAAATACCCAGGAACCTCG
GSP1 ¹⁵	GATGTTGGGTGTAGTGTGTT	CCACCCCAATACTAAATCACAACA	TTCGGGTCTAGCGGTCTGTC	GCCCAATACTAAATCAGCAGC
RASSF1	GTTGGGAATTAATATATGTTGA	TAAAACAACAATACAAACAAT	GTTGGGAATTTAAATATTATGGG	TAAAACAACAATACGAAACGAT
FL13081	TTAGTAGTTTTTGGTGGTTTTAT	CAACTCTCTTCAACAACCTAAC	TAGTAGTTTTTGGGGGTTTATC	AACTCTCTTCAACGACTCGAC
RARA ¹⁶	GGTTTGTGTGATTTGGTTTTAT	CTCTCACTAACTCAGCTAACATA	GGTTTCGGGGGATTCGGTTTTAC	CTCTCGCTAAAGTCGCCTAACGACG
HPP1 ¹⁷	GTTATGTGTGTTGTTTGTGTTGT	AACAACCTCCAAAACACAAAATCA	GTTATCGTGTGTTTGTGTTGTC	GACTTCGGAACCAACACAAAATCG
CDH1 (E-cadherin) ¹⁸	TAATTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	TTAGGTTAGAGGGTTATCGGT	TAACTAAAATTCACCTACCGAC

methylated in all cases, suggesting that methylation of this gene was not qualitatively different in the tumor vs non-tumor. Overall, the number of methylated genes in fibrolamellar carcinoma did not differ significantly from their paired normal tissues, $P=0.39$.

Methylation in FLC Metastases

Metastatic deposits of fibrolamellar carcinoma have dramatically increased numbers of over- and under-expressed genes compared to the primary tumors.¹⁰ Interestingly, this change in the expression levels of numerous genes does not appear to be associated with increased numbers of methylated genes, $P=0.43$ (Figure 2).

Methylation in Control Groups

Within the group of hepatocellular carcinomas that arose in the setting of cirrhosis, examination of the data (Tables 2 and 3) showed that cirrhotic livers in the setting of cryptogenic liver disease and non-alcoholic fatty liver disease had significantly lower levels of methylation than virus-associated cirrhosis, $P=0.014$, and for subsequent analysis, the cirrhotics were studied as two separate groups: viral cirrhosis and non-viral cirrhosis.

The number of methylated promoters in fibrolamellar carcinomas did not differ significantly with those hepatocellular carcinomas that arose in non-cirrhotic livers, $P=0.26$, as well as hepatocellular carcinomas that arose in the setting of non-viral cirrhosis, $P=0.87$. However, fibrolamellar carcinomas had significantly lower levels of methylation than hepatocellular carcinomas that arose in the setting of viral induced cirrhosis, $P=0.023$. In fact, hepatocellular carcinomas that arose in the setting of viral-induced cirrhosis had higher levels of promoter methylation than all other groups (Figure 2).

Discussion

This study is the first to examine the methylation patterns of tumor suppressor gene promoters in fibrolamellar carcinoma. While a formal definition of a methylator phenotype has not been well defined at this time for hepatocellular carcinomas, fibrolamellar carcinomas do not appear to have a ‘methylator phenotype’. The results from this study show that fibrolamellar carcinomas have lower levels of promoter methylation than hepatocellular carcinomas that arise in the setting of viral cirrhosis and that overall the levels of methylation in fibrolamellar carcinomas are similar to those hepatocellular carcinomas that arise in non-cirrhotic livers. While the number of cases of cryptogenic cirrhotics and cirrhosis due to fatty liver disease was small, this

Table 2 Methylation of tumor suppressor genes in fibrolamellar carcinoma

Tumor suppressor gene	Normal livers ^a (N = 4)	FLC, primary (N = 5)	FLC, metastasis (N = 4)
CDH1 (E-cadherin)	4 (100%)	5 (100%)	3 (75%)
RASSF1	0 (0%)	4 (80%)	2 (50%)
CDKN2B (P15)	1 (25%)	2 (40%)	0 (0%)
HPP1	1 (25%)	2 (40%)	1 (25%)
CDKN2A (P14)	1 (25%)	2 (40%)	1 (25%)
GSTP1	1 (25%)	1 (20%)	0 (0%)
P16	1 (25%)	1 (20%)	0 (0%)
RARA	0 (0%)	1 (20%)	1 (25%)
FLJ13081	1 (25%)	0 (0%)	1 (25%)
SOCS1	2 (50%)	0 (0%)	0 (0%)
TP53	0 (0%)	0 (0%)	0 (0%)

^aFour of the five FLC had paired normal liver available for study.

Table 3 Methylation of tumor suppressor genes in primary fibrolamellar carcinoma and hepatocellular carcinomas

Tumor suppressor gene	HCC, viral cirrhosis (N = 17)	HCC non-viral cirrhosis (N = 4)	HCC, non-cirrhotic (N = 10)	FLC, primary (N = 5)
RASSF1	15 (88%)	2 (50%)	5 (50%)	4 (80%)
CDH1 (E-cadherin)	15 (88%)	2 (50%)	6 (60%)	5 (100%)
GSTP1	13 (76%)	3 (75%)	2 (20%)	1 (20%)
P16	11 (65%)	3 (75%)	2 (20%)	1 (20%)
SOCS1	11 (65%)	0 (0%)	2 (20%)	0 (0%)
FLJ13081	10 (59%)	1 (25%)	3 (30%)	0 (0%)
CDKN2B (P15)	9 (53%)	1 (25%)	2 (20%)	2 (40%)
HPP1	8 (47%)	1 (25%)	2 (20%)	2 (40%)
RARA	3 (18%)	0 (0%)	0 (0%)	1 (20%)
CDKN2A (P14)	2 (12%)	0 (0%)	0 (0%)	2 (40%)
TP53	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Figure 1 A representative example of methylation results for RASSF1A. Case 1 shows methylation in a typical HCC; case 2 shows methylation on a FLC. Hep3B DNA was used as a positive control.

Figure 2 The average number of methylated tumor suppressor gene promoters varies in association with underlying status of the background liver. The first column shows hepatocellular carcinomas associated with viral cirrhosis, the second column shows hepatocellular carcinoma arising in non-cirrhotic livers with no known underlying liver disease, while the third column shows primary fibrolamellar carcinomas and the fourth column metastatic fibrolamellar carcinoma. Differences that were statistically significant are shown by asterisks, * $P < 0.05$; ** $P < 0.001$.

group appears to more closely resemble those hepatocellular carcinomas that arise in non-cirrhotic livers. Together, these data support a paradigm where viral hepatitis-induced cirrhosis gives rise

to tumors with higher levels of tumor suppressor gene methylation, the 'methylator phenotype', whereas hepatocellular carcinomas and fibrolamellar carcinomas that arise in non-cirrhotic livers give

rise to cancers where methylation is less prominent. This model is supported by a previous study that also found a link between viral hepatitis B and the methylator phenotype.¹⁵ The mechanism for this link between viral cirrhosis and the methylator phenotype is not well defined, but it is of interest to note that hepatitis B can directly upregulate DNA methyltransferase type 1.¹⁹ At the individual tumor suppressor gene level, others have also observed a higher frequency of methylation in hepatocellular carcinomas that arise in the setting of cirrhosis compared to those in non-cirrhotic livers for *RASSF1A*, *CIS*, and *SOCS1*.²⁰ Additional cases of non-viral cirrhosis, such as cryptogenic cirrhosis, non-alcoholic fatty liver cirrhosis, hereditary hemochromatosis, and so on, will need to be examined to more fully define the levels of methylation in this subgroup of hepatocellular carcinomas, but the findings in this study suggest that cirrhosis *per se* is not as strongly associated with a methylator phenotype as viral hepatitis induced cirrhosis.

Overall, *RASSF1A* and *e-cadherin* were the two genes most commonly methylated in fibrolamellar carcinomas. None of these are unique to fibrolamellar carcinoma and are in fact commonly methylated in hepatocellular carcinomas of the usual sort in both this and other studies. Two of these promoters, *CDH1* and *SFN*, were also found to be partially methylated in the paired normal livers of cases with fibrolamellar carcinoma. In contrast, *RASSF1A* was methylated only in the fibrolamellar carcinomas and not in the paired non-neoplastic liver tissues. *RASSF1* encodes for a protein with a number of disparate functions, from regulating microtubule assembly to regulation of apoptosis and the cell cycle.²¹ *RASSF1A* promoter methylation has been reported in numerous types of cancer including hepatocellular carcinomas, where typically more than 80% of hepatocellular carcinomas show methylation.^{13,22,23} Thus, based on this study and others reported in the literature, *RASSF1A* appears to be methylated in all known primary liver tumors that show hepatocellular differentiation, including hepatoblastomas,²⁴ hepatic adenomas,²⁵ fibrolamellar carcinomas, and hepatocellular carcinoma.^{13,22,23}

This study is also the first to show a role for methylation of *HPP1* in primary liver tumors. *HPP1* has been previously reported to be methylated in adenocarcinomas from the esophagus, stomach, small bowel and colorectum.^{26–31} A previous study found *HPP1* to be downregulated in fibrolamellar carcinomas at the mRNA level¹⁰ and the current results demonstrate that the downregulation of *HPP1* expression is associated with promoter methylation. The function of this gene is unknown; however, it codes for a transmembrane protein that contains both epidermal growth factor and follistatin-like domains and may play a role in inhibiting serine proteases.

Further studies will be required to understand the significance of methylation in non-neoplastic liver tissues of individuals with fibrolamellar carcinomas. One possibility is that these changes are pre-neoplastic. For example, if one gene copy is inactivated by methylation, then an additional hit from point mutations or deletions may lead to the loss of protein expression. However, given that these changes were observed in histologically normal livers with no morphological features to suggest pre-neoplastic changes, their significance at this time remains unclear. Furthermore, methylation of promoters may be age related in some cases. For example, both *e-cadherin* and *RASSF1A* methylation has been reported to increase with age in normal liver tissues,^{25,32} although the young ages of individuals with fibrolamellar carcinoma suggest that this explanation may not account for our findings.

The low levels of promoter methylation in fibrolamellar carcinomas as well as hepatocellular carcinomas that arise in non-cirrhotic livers are a critical observation that is likely to be important in designing future chemotherapeutic regimens for hepatocellular carcinoma. Demethylating agents that can restore the function of inactivated tumor suppressor genes are an attractive target for new therapeutics for hepatocellular carcinomas.³³ Our findings suggest that they may be applicable to the majority of hepatocellular carcinomas, since approximately 80% arise in the background of viral hepatitis-induced liver cirrhosis. However, our findings also suggest that different treatment strategies may be needed for those primary hepatocellular carcinomas and fibrolamellar carcinomas that arise in non-cirrhotic livers and do not show a 'methylator phenotype'.

Because of the rarity of fibrolamellar carcinomas, this study is limited in part by the relatively few samples that were studied. However, the morphological and genetic homogeneity of this tumor strongly suggests that these findings are broadly applicable to fibrolamellar carcinomas in general. The findings in this study are further strengthened by our ability to use as a control group hepatocellular carcinomas that arose in non-cirrhotic livers. This group of hepatocellular carcinomas represents another uncommon subset of tumors that remains poorly understood at the etiological and molecular levels, but is a critical control group for interpreting the data on fibrolamellar carcinomas.

In conclusion, fibrolamellar carcinomas overall do not show a 'methylator phenotype'. In fact, the strongest association between increased methylation of tumor suppressor gene promoters was seen with viral-induced cirrhosis. Of the genes studied, *RASSF1A* was the most commonly methylated gene in fibrolamellar carcinomas and was seen in 80% of the primary tumors and 50% of metastases.

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