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Investigating Predictive Models Based on Circulating Cell-free *INK4A* DNA Methylation, Alpha-fetoprotein, Platelet Count, and Age for Diagnosis and Prognosis of Hepatocellular Carcinoma

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Abstract

Background: As Hepatocellular Carcinoma (HCC) rates continue to rise in the U.S., management remains limited with lack of adequate markers to diagnose patients at early stage when there is curative therapy. Hyper methylation of CpG dinucleotides in the promoter region of inhibitor cyclin-dependent kinase 4A (*INK4A*) has been shown to be a potential biomarker associated with HCC. Additional laboratory measurements including total gamma globulin, alpha-fetoprotein (AFP), and platelet count levels have been suggested as potential markers for HCC. In this study, we examined the combined use of these markers in HCC and non-HCC patients to improve the diagnosis and prognosis of HCC.

Design: We examined *INK4A* promoter methylation using circulating cell-free DNA in serum specimens from patients with HCC and without HCC. Methylation for seven CpG sites was examined using pyrosequencing. The following additional sets of data were recorded: patient age, sex, AFP level, total gamma globulin level, platelet count, and presence or absence of viral hepatitis. Statistical analysis was performed using logistic regression procedure in Statistical Analysis System (SAS) 8.0.

Results: In our study, a total of 153 serum specimens were analyzed, all from different subjects (74 HCC and 79 non-HCC patients), 136 (89%) were male, and 17 (11%) were female. The average age was 54.5 years (standard deviation 8.68). Of the 74 subjects with HCC, 24 (32.44%) were stage 1, 18 (24.32%) were stage 2, 16 (21.62%) were stage 3, and 16 (21.62%) were stage 4. Older subjects were more likely to have HCC than the younger subjects group (means in HCC and non-HCC groups were 56 years and 52 years, respectively, $p = 0.03$). High AFP values were associated with HCC (means in HCC and non-HCC groups were 1583 and 318, respectively, $p < 0.01$) as were low platelet values (means in HCC and non-HCC groups were 131 and 151, respectively, $p = 0.03$). *INK4A* methylation was significantly associated with stage 3 and 4 HCC (odds ratio > 1000, $p = 0.02$). Among *INK4A*, AFP, platelets, gamma globulin, and age, only *INK4A* and AFP were statistically significant when comparing stages 1 and 2 with stages 3 and 4 [probability of stages 1 and 2 = 1/ (1 + exp(- (1.44 - 10.84* *INK4A*-0.00003*AFP)].

Conclusions: Older age, high AFP, and low platelet are associated HCC. Similarly, *INK4A* promoter methylation rates and AFP are associated with advanced HCC stage.

Introduction

Hepatocellular Carcinoma (HCC) is one of the most common causes of cancer-related deaths in the world[1]. As HCC rates continue to rise in the U.S., management has remained limited with lack of adequate diagnostic markers. Also, despite the various treatment strategies available for treatment, the survival rate remains low because of the limitations in early diagnosis. Thus, an urgent need exists to develop a useful diagnostic strategy for early identification of HCC.

Several changes/markers have been identified that have an integral role in the induction and progression of HCC[2,3]. Hyper methylation of CpG dinucleotides in the promoter region of inhibitor cyclin-dependent kinase (CDK) 4A (*INK4A*) has been reported in 60 - 80% of HCC[4-8]. *INK4A* encodes p16 protein, an important cell cycle regulator and inhibitor of cell cycle proliferation. The hyper methylation of *INK4A* ultimately results in blocked and decreased *INK4A* RNA in HCC specimens[9-11], resulting in reduced p16 expression and tumour cell progression. Research has demonstrated potential utility in the "liquid biopsy" for solid tumours. Data has demonstrated that increasing amounts of cell-free tumour DNA and also methylated *INK4A* DNA are released into the blood as HCC progresses[12-15]. This "liquid biopsy" may provide a non-invasive alternative to traditional surgical biopsy procedures for cancer monitoring and detection in high-risk patients. It may also yield a better means of early cancer detection in high risk patients, identify resistance mutations, and monitor response to therapy.

In addition to circulating cell-free *INK4A* DNA methylation status, other laboratory measurements including total gamma globulin, alpha-fetoprotein (AFP), and platelet count have been suggested as potential markers for HCC[16]. In this study, we examined *INK4A* methylation using circulating cell-free DNA and the laboratory profiles of several markers of both HCC and non-HCC patients. Our objective was to create a predictive model for diagnosis and prognosis of HCC.

Materials and Methods

Blood specimens

This study included 153 blood specimens collected during the period of May 2013 to May 2014 from 74 patients with HCC and 79 with benign liver diseases (chronic hepatitis and liver cirrhosis). Samples were processed as described previously[17]. Briefly, whole blood samples were collected without preservatives and stored at 4°C, serum was then separated within 72 h and stored at −70°C until DNA extraction[18]. The diagnoses of HCC and benign liver disease (hepatitis and cirrhosis) were based on imaging characteristics, AFP, pathology evaluation, and past medical history following established practice guidelines[19,20]. The following additional sets of data were recorded: patient age, sex, AFP level, total gamma globulin level, platelet count, and presence or absence of viral hepatitis. The study was approved by the UTMB Institutional Review Board (IRB).

INK4A DNA methylation assay

For the INK4A methylation assay, the QIA vac 24 Plus and QIA amp Circulating Nucleic Acid Kit (Qiagen Inc., Valencia, CA, USA) was used to extract and purify genomic DNA from 1 - 2 ml of serum according to the manufacturer's instructions. Sodium bisulfite treatment with the EpiTect Bisulfite Kit (Qiagen Inc., Valencia, CA, USA) was used for the conversion of unmethylated cytosine to uracil in the genomic DNA according to the manufacturer's instructions. After the conversion reaction and a cleanup step, bisulfite-converted DNA was eluted in a 30 µL elution buffer and used for methylation analysis. Bisulfite-treated genomic DNA was PCR amplified using P16 Pyro-Mark PCR Kit (Qiagen Inc., Valencia, CA, USA). The biotinylated PCR amplicons from 10 µL of PCR reaction mix was captured on streptavidin-coated beads and underwent pyrosequencing using PyroMarkQ24 CpG p16 Kit (Qiagen Inc., Valencia, CA, USA). The methylated fraction (%) of each of the seven CpG sites was measured by pyro-sequencing as described previously[21].

Statistical analysis

Statistical analysis was performed using logistic regression procedure in Statistical Analysis System (SAS) 8.0. Differences were considered significant at the α level of 0.05 for two-sided tests.

Results

We analyzed a total of 153 serum specimens from different subjects (74 HCC patients and 79 Non-HCC patients). The baseline characteristics are summarized in Table 1. One hundred and thirty-six of the subjects (89%) were male and 17 (11%) were female. Average age was 54.5 years (standard deviation 8.68). Of the 153 subjects, 142 (93%) were infected with hepatitis C virus (HCV). Of the remaining 11 subjects (7%), there were 2 subjects with hepatitis B virus (HBV), 3 with alcohol-induced liver disease, 3 with autoimmune hepatitis, and 3 with liver disease from unknown etiology.

Table 1: Clinical characteristics of 153 subjects with and without HCC.

Characteristics	N (%) or mean (SD)
Total	153
Male	136 (89)
Female	17 (11)
Age	54.5 (8.69)
HCV	142 (93)
HBV	2 (1)
Alcohol-induced liver disease	3 (2)
Autoimmune liver disease	3 (2)
Unknown etiology	3 (2)
Hepatitis/cirrhosis	79 (52)
HCC	74 (48)

Table 2 represents the average *INK4A* methylation levels of the individual seven CpG sites assessed using serum samples from patients diagnosed with HCC (74 samples from 74 patients) and benign chronic liver diseases (79 samples from 79 patients). Methylation of the individual seven specific CpG sites was quantified using pyro-sequencing. The highest value of methylation of the seven CpG sites was used for each of the samples. Table 3 summarizes the laboratory results and age distribution of subjects from both the HCC and non-HCC groups. Mean AFP values were higher in HCC group than non-HCC

group (means in HCC and non-HCC groups were 1583 and 318, respectively, $p < 0.01$), as were *INK4A* methylation rate (means in HCC and non-HCC groups were 9.8 and 5.1, respectively, $p < 0.01$). Subjects from HCC group were older (means in HCC and non-HCC groups were 56 years and 52 years, respectively, $p < 0.01$) and had lower mean albumin levels (means in HCC and non-HCC groups were 3.3 and 3.7, respectively, $p < 0.01$). There was no difference in the average platelet and total globulin levels between the HCC and non-HCC groups.

Table 2: Average INK4A methylation rates of 7 CPG sites analyzed.

	CPG site 1	CPG site 2	CPG site 3	CPG site 4	CPG site 5	CPG site 6	CPG site 7
HCC	5.34%	7.22%	4.83%	5.34%	7.56%	4.9%	4.68%
No HCC	1.90%	3.15%	1.25%	2.30%	2.75%	1.44%	1.59%

Table 3: The laboratory results and ages for subjects with and without HCC. Univariate analysis.

		HCC		Non-HCC	P-value
Variables	**Number of subjects**	**Mean (minimum, maximum)**	**Number of subjects**	**Mean (minimum, maximum)**	
AFP (IU/ml)	74	15863.3 (3.9, 217000.0)	79	317.9 (1.0, 5060.0)	< 0.01
Platelet (10^3/microliter)	72	131.3 (34.0, 422.0)	72	151.7 (45.0, 516.0)	0.12
Albumin (g/dL)	72	3.3 (1.7, 4.7)	72	3.7 (2.3, 5.0)	< 0.01
Total globulin (g/dL)	72	4.0 (1.9, 6.2)	72	3.8 (2.7, 5.9)	0.30
Age (yrs.)	72	56.8 (27.0, 89.0)	78	52.4 (21.0, 67.0)	< 0.01
***INK4A* methylation (%)**	72	9.8 (0, 0.8)	79	5.1(0, 21)	< 0.01

Results from multivariate analysis for determining independent factors associated with HCC were summarized in Table 4. Overall, older age ($p = 0.02$) and high AFP values ($p = 0.01$) were both associated with HCC. None of the remaining laboratory values (platelets, total globulin, albumin, and *INK4A* methylation rate) were independent factors associated with HCC. Table 5 shows a summary of the stages of HCC in 73 of 74 subjects. Of the 74 subjects with HCC, 1 subject did not have a listed stage. Approximately 56% (41 of 73) had stages 1 and 2, and 44% (32 of 73) had advanced stages (3 and 4). Positive methylation status at 5% and 10% was seen in 46% (19 of the 41) and 15% (6 out of 41) of the early stage group, respectively. Much higher rates were seen in the advanced stage group, with 91% (29 out of 32) and 47% (15 out of 32) at 5% and 10% limits of detection, respectively.

Table 4: Multivariate analysis for determining independent factors associated with HCC.

Variables	Odds ratio	P-value
AFP (IU/ml)	1.001	0.01
Platelet (10^3/microliter)	0.995	0.18
Albumin (g/dL)	0.652	0.22
Totalglobulin (g/dL)	0.893	0.70
Age (yrs.)	1.067	0.02
***INK4A* methylation (%)**	62.542	0.30

Table 5: Summary of the stages of 73 subjects with stages.

Stages	N (%)	INK4A methylation (5% LOD)	INK4A methylation (10% LOD)
1	24 (33)	-	-
2	17 (23)	-	-
Early stage (I and II)	41 (56)	46% (19/41)	15% (6/41)
3	16 (22)	-	-
4	16 (22)	-	-
Late stage (III and IV)	32 (44)	91% **(29/32)**	47% **(15/32)**

Note: One subject with HCC had no listed stage, and is not summarized in this table.

Laboratory results and age distribution of subjects from both the early and late stages of HCC were summarized in Table 6. Mean AFP values were higher in the late stage group (means in early and late stage groups were 3670.9 and 31968.8, respectively, $p = 0.03$), as were *INK4A* methylation rates (means in early and late stage groups were 5.9 and 14.7, respectively, $p = 0.01$). Subjects from late stage of HCC were older (means in early and late stage groups were 55.9 years and 58.1 years, respectively, $p = 0.03$) and had lower mean albumin levels (means in early and late stage groups were 3.5 and 3.1, respectively, $p = 0.01$). There was no difference in mean platelet levels or mean total globulin levels between the early and late stage HCC groups. Results from multivariate analysis for determining independent factors associated with late stage HCC were shown in Table 7. High AFP values were associated with late stage HCC (p value = 0.04), as were *INK4A* methylation rates (p value = 0.03). Low platelet values ($p = 0.03$) and low albumin levels ($p = 0.02$) were also associated with late stage HCC. Age or the total globulin levels were not independent factor associated with late stage HCC when compared to early stage HCC.

Table 6: The laboratory results and age distribution for subjects with HCC by HCC stage. Univariate analysis.

Variables	Early (stages I & II)		Late (stages III & IV)		P-value
	Number of subjects	Mean (minimum, maximum)	Number of subjects	Mean (minimum, maximum)	
AFP (IU/ml)	41	3670.9 (3.9, 98600.0)	32	31968.8 (18.0	0.03
Platelet (10^3/microliter)	40	118.1 (34.0, 284.0)	31	147.9 (57.0, 422.0)	0.11
Albumin (g/dL)	40	3.5 (1.7, 4.7)	31	3.1 (2.1, 4.3)	0.01
Total globulin (g/dL)	40	4.0 (2.1, 6.2)	31	4.0 (1.9, 5.9)	0.84
Age (yrs.)	39	55.9 (40.0, 69.0)	32	58.1 (27.0, 89.0)	0.03
***INK4A* methylation (%)**	39	5.9 (0, 25)	32	14.7 (2.0, 77.0)	0.01
***INK4A* methylation, > 10%**	41	6 (14.6)	32	15 (46.9)	< 0.01

Table 7: Multivariate analysis for determining independent factors associated with late stage of HCC.

Variables	Odds ratio	P-value
AFP (IU/ml)	1.00003	0.04
Platelet (10^3/microliter)	1.015	0.03
Albumin (g/dL)	0.254	0.02
Total globulin (g/dL)	0.825	0.64
Age (yrs.)	0.983	0.64
INK4A methylation (%)	92836.9	0.03

Note: The reference stage is early stage.

Discussion

In this study, age, AFP, total globulin, albumin, platelet value, and circulating cell-free *INK4A* promoter methylation were evaluated retrospectively in patients with HCC and benign liver diseases. To our knowledge, our study is the first to investigate a combination of circulating *INK4A* methylation status with laboratory values (AFP, platelet values, total gamma globulin) in subjects with liver disease. Older age and high AFP levels were associated with HCC. Similarly, *INK4A* promoter methylation rates, high AFP, low platelet, and low albumin levels were associated with advanced HCC stage. Our findings suggest that these measurements may provide clinical utility as future model for monitoring patients with HCC.

Despite the current recommendation for all cirrhotic patients to undergo consistent surveillance for HCC, the 5 year survival rate for HCC remains low. Small liver nodules (less than 2 cm in size) cannot be accurately diagnosed with imaging studies, and this prompts clinicians to monitor them until they increase in size and reach a definitive diagnosis of HCC. Thus, many of these patients are diagnosed after the cancer has reached an advanced stage. Another problem with this surveillance model is that it is expensive, cumbersome, and it fails to properly separate patients with low vs. high likelihood of developing HCC. Under-surveillance of high-risk patients leads to late stage tumor detection, and over-surveillance of patients with a low risk causes unnecessary expenses and potential harm from unnecessary procedures[22,23]. Therefore, accurately predicting HCC by initiating detection and chemo preventive efforts in high-risk patients and avoiding these efforts in low-risk patients may potentially reduce the cost burden while providing personalized care.

Several variables have been previously described by different authors as being significantly associated with HCC development, including portal hypertension[24], older age[24,25], diabetes[26], alkaline phosphatase[24], lower platelet counts[24,25], albumin[27], and higher baseline values of AFP[28], and gamma globulin[29]. Gavilan and colleagues found similar results when they followed and evaluated 829 patients with hepatitis C virus (HCV) infection for approximately 82 months[30] A total of 58 (7%) of the 829 developed HCC. By constructing a risk score using multivariate analysis, they concluded that advanced age, elevated AFP and gamma globulin, and low platelet counts (< 150,000) were independent predictors of HCC. They also discovered that the average time to develop HCC was significantly shorter in the high-risk group compared to the low- and intermediate-risk groups. The authors concluded that their predictive model could be used to identify a high-risk subgroup of patients with an annual HCC incidence of 2.6%, in which the screening would be cost-effective. Our study had similar findings to previous studies in that advanced age and elevated AFP were associated with HCC. Unlike these studies, however, none of platelet, albumin or total globulin levels were associated with HCC. The reason of the difference is not clear. It may be attributed to Gavilan's larger subject size, and consequently, higher numbers of subjects with more advanced liver disease compared to our study. Our study also evaluated and incorporated *INK4A* methylation status as a variable, which was not examined previously[30].

INK4A hyper methylation has been identified in several studies as a risk factor for HCC[31-38], however, all of these studies used methylation specific PCR (MSP) to evaluate methylation status. MSP is an allele-specific PCR assay that depends on sodium bisulfite converting cytosine to uracil[12]. This method is problematic for several reasons. One problem is the heterozygous epi-alleles that exist after CpG sequence conversion with sodium bisulfite[38]. Additional disadvantages include the assay's inability to quantify levels of methylation and identify specific CpG dinucleotides that are methylated. Establishing quantification levels of methylation is important because benign liver diseases can express low levels of methylation. Many of these levels may not be biologically significant; therefore, establishing a lower limit of quantification (LLOQ) provides a more accurate assessment of tumor-specific methylation. Pyro-sequencing is a semi-quantitative method for evaluating *INK4A* hyper methylation because it measures the percent of methylation from its calculated C:T (methylated: unmethylated) ratio at specific CpG sites. This quantitative ability adds value because a percentage of methylation can be established.

In a previous study[21], we examined *INK4A* promoter methylation by pyrosequencing using circulating cell-free DNA (ccfDNA) in 109 patients, including 66 with HCC and 43 with benign chronic liver disease. The results found significantly

higher levels of methylated *INK4A* in HCC specimens than the non-HCC group. The area under receiver operating characteristic (ROC) curve was 0.82, with 65.3% sensitivity and 87.2% specificity at 5% limit of detection (LOD), 39.0% sensitivity and 96.5% specificity at 7% LOD, and 20.3% sensitivity and 98.8% specificity at 10% LOD, respectively. The results showed that *INK4A* methylation testing of ccfDNA could be a valuable biomarker with regards to diagnostic, predictive, and prognostic characteristics in HCC patients. In this study, ccfDNA was examined by pyro-sequencing as in our previous study[39], and other factors, including age, platelet count, and AFP value were examined using multivariate analysis. In addition to *INK4A* methylation, AFP was also identified as a prognostic indicator of early vs. advanced stage. This suggests that both AFP and *INK4A* methylation levels from circulating cell free DNA may be useful predictors of advanced HCC. Gavilan and colleagues concluded in their study that older age, elevated AFP and gamma globulin, and low platelet levels were independent predictors of HCC[12]. Therefore, these variables may indeed be valuable measurements in diagnosing early HCC.

Our data reflects that levels of circulating methylated *INK4A* correlate with and may also complement levels of AFP to improve the positivity in advanced stage HCC; the positive rates were 96.9%, 91.0%, and 100% in the 32 HCC cases for AFP, *INK4A* methylation, and in combination, respectively, using LOD cut-offs of 200 µg/L for AFP[12] and 5% for INK4A methylation[21]. Similarly, the positive rates were 96.9%, 46.9%, and 96.9% in the 32 cases for AFP, *INK4A* methylation, and in combination, respectively, using LOD cut-offs of 200 µg/L for AFP and 10% for *INK4A* methylation. Supporting this notion, it has been reported that adding *INK4A* methylation status to AFP can increase the diagnostic sensitivity of HCC from 60% to 80%[39], however, no previous studies have examined this relationship to advanced-stage HCC. Additional investigations including prospective studies and more sophisticated *INK4A* methylation assay with higher sensitivity and specificity are needed to examine the clinical performance of the *INK4A* assay for HCC diagnosis, prognosis, and monitoring treatment.

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