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Concordance of DNA methylation pattern in plasma and tumor DNA of Egyptian hepatocellular carcinoma patients

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the fourth leading cause of cancer mortality globally. HCC incidence has doubled in Egypt in the past 10 years, which could be attributed to the high prevalence of hepatitis C virus (HCV) and hepatitis B virus (HBV), although HBV rates have declined after the introduction of the vaccine in 1992. Aberrant DNA methylation may play an important role in hepatocarcinogenesis. Liver biopsy is the current gold standard for methylation studies; however, imaging techniques often suffice for diagnosis making tissue samples increasingly scarce. The efficacy of conducting DNA methylation studies in molecular epidemiology using plasma DNA is still unclear. We compared tumor methylation profile for the tumor suppressor genes APC, FHIT, p15, p16, and E-cadherin in tumor tissues and plasma to test the concordance between the two types of specimen from the same HCC patients. Twenty-eight HCC patients with matching tissue and plasma DNA were recruited from a case—control study in Gharbiah, Egypt. Concordance between the tissue and plasma was statistically significant in all five genes as follows: APC (23/28, 82.1%, $p=0.001$), FHIT (24/28, 85.7%, $p=0.0001$), p15 (25/28, 89.2%, $p=0.045$), p16 (19/28, 67.9%, $p=0.037$), and E-cadherin (22/28, 78.5%, $p=0.0008$). The average specificity was 90%, 86%, 96%, 86%, and 100%, respectively. There was no significant association between methylation and hepatitis viral infection for any of the genes tested in this study. Plasma DNA can be reliable for testing methylation profile in liver cancer patients in this population. Future studies on a larger sample size should investigate methylation profile in populations with higher rates of HBV, HCV, and other risk factors.

Keywords

Methylation; HCC; Tumor tissue; Plasma; Egypt; Liver

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Introduction

Globally, hepatocellular carcinoma (HCC) is one of the leading causes of cancer related deaths, and is the fifth most commonly diagnosed solid tumor (Alacacioglu et al., 2008). The majority of HCC patients occur in sub-Saharan Africa and parts of South East Asia; however, incidence rates appear to be on the rise in developed nations such as the United States, Japan, and Western Europe (El-Serag and Rudolph, 2007). The lack of HCC biomarkers prevents early detection resulting in a poor prognosis of the disease (Sakamoto et al., 2008).

In Egypt the incidence of HCC has doubled in the past 10 years, and it is now the second most incident and lethal cancer in men (Freedman et al., 2006). The heavy burden of HCC parallels high rates of hepatitis C virus (HCV) while hepatitis B virus (HBV) rates have declined after the introduction of the vaccine in 1992 (Lehman and Wilson, 2009; Lau and Lai, 2008). Nevertheless, the age standardized HBV incidence rate in males (20.6/100,000) is seven times higher than what is found in the Middle East Cancer Consortium, and more than three times the incidence rates reported by US SEER (Freedman et al., 2006).

Abnormal gene expression in cancerous cells often occurs as a result of genetic mutations, but recent studies suggest aberrant DNA methylation as an alternative mechanism of tumor pathogenesis (Martens et al., 2009). It has been shown that hypermethylated promoters often lack transcriptional activity, which could result in gene inactivation (Zhang et al., 2007). DNA methylation typically refers to the addition of a methyl group to the cytosine residue in CpG dinucleotides. Normally, clustered CpG dinucleotides (CpG islands) are not methylated regardless of whether they are transcribed or not. Within tumor cells, methylation of CpG islands in the promoter regions of many tumor suppressor genes effectively silences those genes (Teodoridis et al., 2008). These genetic abnormalities could be important early events in carcinogenesis, and could also be potential biomarkers for early detection (Zhou et al., 2007).

Liver biopsy is the current gold standard for detecting methylation events; however, imaging techniques often suffice for liver cancer diagnostic purposes making biopsy tissue samples increasingly scarce (Bongiovanni and Casana, 2008). In Egypt, tissue samples are currently available from only 20–25% of all HCC patients (Ibrahim et al., 2007); however, blood plasma samples are readily available from every patient. Previous studies with breast cancer patients were able to detect promoter methylation in circulating plasma DNA, suggesting a noninvasive approach for early detection of cancer (Papadopoulou et al., 2006). However, the efficacy of conducting DNA methylation studies using plasma DNA from liver cancer patients still has limitations because of the limited sample size or the lack of matching plasma and tumor tissue from the same HCC patient (Chang et al., 2008; Zhang et al., 2007).

The objective of this study was to compare DNA methylation events in tissue and plasma samples from Egyptian HCC patients for the following five tumor suppressor genes: APC, FHIT, p15, p16, and E-cadherin. We also aimed to investigate the association between DNA methylation in tissues and plasma with respect to hepatitis virus status. Due to the steady decline in tissue available from liver cancer patients, the productivity level of future DNA

methylation studies will be greatly enhanced if the efficacy of using plasma is equivalent to tumor tissue.

Materials and methods

Study design

This study was part of a case–control study conducted at the Tanta Cancer Center and the Gharbiah Cancer Society Hospital of the Population-Based Cancer Registry in the city of Tanta in the Gharbiah province of Egypt recruited from December 2007 through November 2008. The case–control study included 150 HCC patients and 150 matched controls on age (± 5 years) and sex. In addition to questionnaire data, blood samples were also collected from all patients and controls. Gharbiah province is a predominantly rural area located in the Nile delta region of Egypt. Only patients who had diagnostic biopsy or resection were included in this study ($n=28$ patients, 21% of all study patients). Participant ages ranged from 17–75 and were predominantly males (20 males and 8 females).

Twenty-eight paraffin embedded tumor tissue samples were collected from all the HCC patients included in this study. Twenty samples were collected and stored at the Tanta Cancer Center in Tanta, Egypt. The remaining eight tissue samples were collected from various private laboratories in Egypt. Two of the tissue samples were derived from surgical resection, and the remaining 26 were derived from biopsy procedures. Matching plasma samples for all 28 HCC patients were sent to the National Cancer Institute, Cairo University in Cairo, Egypt.

Data collection

Blood samples were collected from each participant in order to extract DNA from the plasma. HBV and HCV viral status was determined by two diagnostic methods (serologically and molecularly). Tumor tissue was collected from patients who received a liver biopsy for medical diagnostic purposes.

DNA extraction from tissue

DNA was extracted from the paraffin embedded tissue samples. Briefly, three sections of 10 μm were immersed in 300 μl of lysis buffer containing 10 mM Tris–HCl (pH8.0), 50 mM KCl, 2.5 mM MgCl_2 , 0.01% (w/v) gelatin, and 1% Tween 80. Following addition of proteinase K (200 mg/l), the solution was incubated at 55 $^{\circ}\text{C}$ overnight and appeared clear. DNA was further purified using phenol/chloroform, precipitated with cold alcohol, and dissolved in ion-free water. The concentration and quality of the extracted DNA was determined from its optical density, and PCR amplification with the β -actin primer: 5'GGC GGC ACC ACC ATG TAC CCT 3' and 5' AGG GGC CGG ACT CGT CAT ACT.

DNA extraction from plasma

DNA was extracted through a phenol/chloroform treatment. Briefly, equal volume of buffer equilibrated phenol (pH 7.0–7.5) was added to samples and vortexed. The upper aqueous layer was removed with a “cut down” pipette tip, and an equal volume of phenol/chloroform (1:1) was then added to the aqueous supernatant and vortexed. The upper aqueous layer was

removed again in a similar fashion, and an equal volume of chloroform/isoamyl (24:1) was then added and vortexed. Sodium acetate (3 M) (pH 4.7–5.2) was added to the aqueous supernatant, followed with ice-cold ethanol. Samples were then incubated overnight at -8°C . After decantation of the liquid, the DNA pellet was recovered and dissolved in sterile water. The purity and integrity of the DNA was confirmed by carrying out β -actin gene amplification.

Methylation treatment

Methylation treatment was according to EZ DNA Methylation-Direct TM Kit. The sodium bisulfate treatment converts non-methylated cytosine residues to uracil, while methylated cytosine within CpG islands remains unaffected. Methylation specific polymerase chain reaction was used to determine the methylation status of CpG islands after bisulfate treatment. The method consisted of two steps: amplification and detection. The methylation profile was studied for five genes (APC, FHIT, p15, p16 and E-cadherin). Each PCR product was loaded directly onto 2.5% agarose gels, stained with ethidium bromide, and visualized under ultraviolet illumination. The expected methylated bands for the five genes of interest were as follows: APC (98 bp), FHIT (140–170 bp), p15 (148 bp), p16 (150 bp), and E-cadherin (116 bp).

Statistical methods and statistical analysis

For each gene, the mean age among patients with a methylated promoter was compared to the mean age among patients with a non-methylated promoter using a two sample t-test. The methylation status was assessed using tumor tissue DNA. Results of these analysis are displayed in Table 1.

The association between gender and methylation status was evaluated, for each gene, by comparing the proportion of methylation in males and females. This comparison was carried out using Fisher's exact test. The same test was also used for evaluating the association between residence (urban or rural) and methylation status for each gene.

Concordance in methylation status between tumor and plasma DNA was also evaluated using Fisher's exact test for each gene. Results are displayed in Table 2.

The association between viral hepatitis status (HBV or HCV), including HBV infection type (past infection or immune), and methylation status was also evaluated using Fisher's exact test for each gene. The methylation status was assessed using tumor tissue DNA. Results are displayed in Tables 3 and 4).

Results

Demographic and clinical data

The details of patient demographics and laboratory results from the study subjects are provided in Table 1. We did not observe a significant association between promoter methylation and age for any of the genes as shown in Table 2. The same was true for residence (rural or urban). However, a significant association was observed in the p15 gene between gender and promoter methylation. The proportion of males with methylated p15

promoters was greater than the proportion of females with p15 promoter methylation. The frequency of hepatitis B and C virus was 14.3% ($n=4$) and 78.5% ($n=20$), respectively.

Plasma/tissue concordance

The frequency of promoter methylation in tissue and plasma samples for the five tumor suppressor genes was as follows: APC [18/28, 64.2% for tissue and 15/28, 53.5% for plasma], FHIT [21/28, 75.0% for tissue and 19/28, 67.8% for plasma], p15 [4/28, 14.2% for tissue and 3/28, 10.7% for plasma], p16 [20/28, 71.4% for tissue and 13/28, 46.4% for plasma], and E-cadherin [19/28, 67.8% for tissue and 13/28, 46.4% for plasma]. Please refer to Table 1.

The methylation profile for APC, FHIT, p15, p16, and E-cadherin was compared between DNA extracted from tissue and plasma (results are highlighted in Table 1 and Fig. 1). We observed significant concordance between plasma and tissue DNA for all five genes. The specificity of plasma DNA was higher than the sensitivity for all five genes. The positive predictive power was higher than the negative predictive power for APC, FHIT, p16, and E-cadherin. The negative predictive value was higher for p15.

Methylation profile and viral status

The correlation between HBV or HCV infection and promoter methylation was examined to determine whether the viral status could predict methylation and subsequent gene silencing for the five tumor suppressor genes. A significant relationship was not observed for cases with HBV or HCV. A significant association also lacked between HBV infection type (past infection or immune) and promoter methylation among HCC cases with hepatitis infection. There was no clinical data examining infection type in HCV patients.

Discussion

This study revealed two interesting observations. First, we observed a statistically significant concordance between plasma and tissue methylation profiles. Promoter methylation in plasma DNA was highly specific, though not as sensitive, for the matching change in tissue DNA. This suggests that methylated tissue DNA might originate in tumor cells before appearing in vascular spaces. The positive predictive value (PPV) of plasma DNA was greater than 90% for four out of the five genes tested. The p15 promoter was rarely methylated in tissue and even less frequently in plasma and this resulted in a low PPV and high negative predictive value (NPV).

Second, there was no significant association between DNA tissue methylation and hepatitis viral status for all five tumor suppressor genes examined. Stratification based on infection type – past infection or immune – among HBV patients was also not significantly associated with promoter methylation. Therefore, plasma DNA could be used as a reliable resource for methylation studies in the future, irrespective of the type of hepatitis infection.

Several cancer studies in the current literature suggest that plasma DNA can predict epigenetic alterations in tissue DNA, while others have not found conclusive data. According to Takaei et al. (2006), aberrant methylation for p16 and p15 in serum DNA was

correlated with HCC, and present for a period of 1–9 years before clinical HCC diagnosis. Wong et al. (2000) have found that circulating tumor DNA and HCC cells were detectable in the peripheral blood in patients when examining p15 methylation. Zhang et al. (2007) observed identical epigenetic changes between the tissue and plasma/serum in a statistically significant proportion ($P<.005$) of the patients in association with tumor methylation status. Our results were consistent with these findings; hypermethylation at the promoter site in plasma was significantly concordant with tumor tissue.

Chang et al. (2008), however, failed to find agreement between plasma and tissue DNA. The study used RT-PCR to compare DNA methylation in plasma and tissue from eight HCC patients. The resulting lack of concordance might be attributable to the small sample size and plasma DNA degradation during RT-PCR.

Viral infections have been shown to induce DNA methylation. Host DNA was often methylated in Epstein–Barr virus infected cells (Ambinder et al., 1999) and adenovirus infected tumors (Doerfler, 2009). Increased methylation of the IFN-gene promoter was also observed in patients with human immunodeficiency virus type 1 (Mikovits et al., 1998).

Hepatitis B and C virus infection might also promote methylation of certain genes in HCC patients. Jicai et al. (2006) indicate that chronic HBV infection could be associated with high rate of p16 methylation, and involved in hepatocarcinogenesis. Expression of E-cadherin in tissue culture and in vivo showed an inverse relationship with HBV antigen; however, the precise mechanism is unknown (Liu et al., 2006). Also, increased promoter methylation of p15 was observed among HCC patients with HCV infection (Edamoto et al., 2003).

The strengths of this study are twofold. Unlike some previous studies, we examined methylation status in paired plasma and tissue samples from the same patient. Additionally, our study participants were recruited from the Population-Based Cancer Registry in Gharbiah, Egypt. The availability of viral, clinical, and epidemiological registry data for all the patients makes this study more epidemiologically sound. Although this study was relatively large compared to previous methylation studies, the main limitation in this study was sample size. As mentioned earlier, tumor tissue samples from HCC patients are becoming increasingly scarce.

Conclusions

This study demonstrated that plasma DNA reliably predicted methylation events in tissue DNA in the following tumor suppressor genes: APC, FHIT, p15, p16, and E-cadherin. The specificity of plasma was high among all five tumor suppressor genes; however, the sensitivity was low. With the exception of p15, the positive predictive value of plasma DNA was high. We did not observe a significant association between methylation status and HBV or HCV infection. Therefore, plasma DNA could be used as a reliable resource for methylation studies in the future, irrespective of the type of hepatitis infection.

Due to the high prevalence of HCV and the low prevalence of HBV in Egypt, future studies should examine our findings in other populations with variable prevalence of HBV and

HCV. Additionally, the association between environmental/occupational exposures and methylation of tumor suppressor genes should also be examined.

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sample No.	APC		FHIT		P15		P16		E-cad	
	P	T	P	T	P	T	P	T	P	T
2										
5										
7										
11										
12										
13										
22										
24										
26										
33										
36										
37										
40										
44										
46										
47										
56										
62										
63										
64										
65										
67										
69										
70										
72										
73										
79										
84										

Fig. 1.

Summary of methylation analysis of APC, FHIT, p15, p16, and E-cadherin in 28 HCC samples and the corresponding plasma. Filled boxes indicate the presence of methylation and open boxes indicate the absence of methylation. T, tumor tissue; P, plasma.

Table 1

Clinical and methylation profile for each patient.

Sample #	Age	Sex	HBV status	HCV status	APC		FHIT		p15		p16		E-cadherin	
					Plas	Tis	Plas	Tis	Plas	Tis	Plas	Tis	Plas	Tis
1	47	M	Negative	Positive	P	P	P	P	N	N	N	N	N	P
2	51	M	Negative	Positive	P	P	P	P	N	N	P	P	P	P
3	67	M	Negative	Positive	P	P	N	N	N	N	P	P	N	P
4	65	F	Negative	Positive	P	P	N	N	N	N	N	N	N	N
5	73	F	Negative	Positive	N	P	N	P	N	P	N	N	N	P
6	68	M	Negative	Positive	N	N	N	N	N	N	N	N	N	N
7	40	F	Negative	Positive	P	P	N	N	N	N	N	N	N	N
8	54	M	Negative	Positive	P	P	P	P	N	N	P	P	P	P
9	61	M	Negative	Positive	N	P	P	P	N	N	P	N	N	P
10	17	F	Negative	Negative	P	P	P	P	P	P	P	P	P	P
11	48	M	Negative	Negative	P	P	P	P	P	N	N	N	P	P
12	70	M	Negative	Positive	N	N	P	P	N	N	N	N	N	N
13	50	F	Negative	Negative	N	N	P	P	N	N	N	N	N	N
14	50	M	Negative	Positive	P	P	P	P	N	N	N	N	N	N
15	75	M	Negative	Positive	N	N	N	P	N	N	N	N	N	P
16	50	M	Negative	Positive	P	P	P	P	N	N	P	P	P	P
17	46	F	Negative	Negative	N	N	N	N	N	N	P	P	P	P
18	41	F	Positive	Negative	P	P	P	P	N	N	P	P	P	P
19	65	M	Positive	Positive	P	P	P	P	P	P	N	P	P	P
20	63	M	Negative	Positive	N	N	P	P	N	N	N	N	N	N
21	51	M	Negative	Positive	P	N	P	N	N	N	N	N	N	N
22	70	M	Positive	Positive	P	P	P	P	N	N	N	N	P	P
23	68	M	Negative	Positive	N	N	P	P	N	N	P	P	P	P
24	68	M	Negative	Positive	N	N	P	P	N	N	N	N	N	P
25	45	F	Negative	Negative	N	N	P	P	N	N	P	P	P	P
26	85	M	Negative	Positive	N	P	N	N	N	N	P	P	P	P
27	47	M	Negative	Positive	N	P	N	P	N	N	P	P	N	N

Sample #	Age	Sex	HBV status	HCV status	APC		FHIT		p15		p16		E-cadherin	
					Plas	Tis	Plas	Tis	Plas	Tis	Plas	Tis	Plas	Tis
28	44	M	Positive	Positive	P	P	P	P	N	N	P	P	P	P

P, positive for promoter methylation; N, negative for promoter methylation. Plas, plasma DNA; Tis, tissue DNA.

Table 2

Association between age and promoter methylation.

	Methylated promoter (mean age)	Non-methylated promoter (mean age)	<i>p</i> -value
APC	54.21 (std.=15.40, <i>n</i> =18)	60.40 (std.=11.19, <i>n</i> =10)	0.275
FHIT	55.13 (std.=13.83, <i>n</i> =21)	60.29 (std.=15.49, <i>n</i> =7)	0.414
p15	55.00 (std.=25.61, <i>n</i> =4)	56.66 (std.=12.20, <i>n</i> =24)	0.833
p16	54.94 (std.=15.78, <i>n</i> =20)	60.13 (std.=8.61, <i>n</i> =8)	0.391
E-cadherin	56.62 (std.=15.81, <i>n</i> =19)	56.00 (std.=10.63, <i>n</i> =9)	0.916

The methylation profile for the five tumor suppressor genes was not significantly associated with mean age.

Table 3

The concordance between plasma DNA and tissue DNA.

	Sensitivity	Specificity	PPV	NPV	p-value
APC	77.8%	90.0%	93.3%	69.2%	0.001*
FHIT	85.7%	85.7%	90.5%	66.7%	0.001*
p15	50.0%	95.8%	66.7%	92.0%	0.045*
p16	60.0%	87.5%	92.3%	46.7%	0.037*
E-cadherin	68.4%	100.0%	100.0%	60.0%	0.0008*

PPV, positive predictive value; NPV, negative predictive value.

* The concordance was significant* for all five genes.

Table 4

Statistical association of hepatitis viral status and promoter methylation.

	HBV	HCV	HBV Infection infection type [*]
APC	0.107	0.634	0.508
FHIT	0.545	1	0.508
p15	0.481	1	0.288
p16	0.295	0.639	1
E-cadherin	0.273	0.629	0.66

The methylation profile was not significantly associated with the HBV, HCV, or HBV infection type.

* Infection type: past infection or immune.