SPANDIDOS PUBLICATIONS

Relation between serum levels of cell-free DNA and inflammation status in hepatitis C virus-related hepatocellular carcinoma

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Abstract. Our study revealed that the level of circulating cell-free DNA (cfDNA) is increased in the serum of patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC). To gain insight into the mechanism underlying this phenomenon, we examined the association between cfDNA levels and various clinicopathological factors in 96 patients with HCV-related HCC and 99 non-HCC patients with HCV. Using pooled DNA microarray data, we profiled the expression patterns of inflammatory cytokine genes in 14 primary tumors from the group of HCC patients. We found that there were positive associations between the cfDNA level, aspartate aminotransferase levels and the number of leukocytes and neutrophils in patients with HCV-related HCC but not in non-HCC patients with HCV. The serum cfDNA level was not associated with other clinicopathological factors in HCC or non-HCC patients. A cluster analysis based on the inflammatory cytokine gene data revealed that HCCs with a high serum cfDNA level had increased levels of several inflammatory cytokine genes, suggesting that the serum cfDNA level is associated with the inflammatory status in primary tumors in HCV-related HCC.

Introduction

Since the initial description of circulating nucleic acids in 1948 (1), many investigators have reported on the association between circulating nucleic acids and various human diseases (2). The ability to detect tumor-derived cell-free DNA (cfDNA) in the bloodstream has paved the way for predictive oncology (3-5). This method provides a non-invasive and easy-to-use tool for screening for malignancies and predicting cancer outcomes (2). However, there is an increasing debate regarding the measurement of circulating cfDNA in the bloodstream. It has been suggested that the major source of cfDNA is leukocytes destroyed in serum samples upon blood clotting (5). It remains unclear why broad-ranging cfDNA and cfDNA specific for cancer cells increases in the blood of cancer patients.

Our previous studies showed that the cfDNA level is significantly higher in serum from patients with hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) than in serum from HCV carriers without known HCC (6,7). Using those data (7), the present study was conducted to clarify whether the increased level of cfDNA in the serum of patients with HCV-related HCC is an artifact due to destroyed leukocytes in the blood clotting step, and if not, which factors contribute to the increased level of serum cfDNA in these patients. This is the first study to examine the relationship between cfDNA circulating in the blood and the inflammatory status of primary malignant tumors.

Materials and methods

Serum cfDNA data. We used data of the serum cfDNA level and clinicopathological factors from 196 HCV-infected patients enrolled in our previous study (7). Of the 196 patients, 96 had received a diagnosis of HCC, and the remaining 100 patients had no diagnosis of HCC. The present study revealed that none of the 100 HCV carriers developed HCC within 1 year of cfDNA analysis. We pooled data for the number of leukocytes, neutrophils, lymphocytes, erythrocytes and platelets, and the levels of C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase

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Table I. Association between serum cell-free DNA (cfDNA) levels and clinicopathological factors in 96 patients with HCV-related HCC and in 99 non-HCC patients with HCV.

	Albumin	Total bilirubin	ALT	AST	Leukocytes	Lymphocytes	Neutrophils	Erythrocytes	Platelets	CRP
HCC										
r	-0.022	-0.031	0.103	0.209	0.305	-0.053	0.325	0.135	0.114	0.087
P-value	0.832	0.763	0.318	0.041	0.003	0.607	0.001	0.189	0.268	0.399
Sample no.	96	96	96	96	96	96	96	96	96	96
Non-HCC										
r	-0.130	0.131	-0.012	0.038	0.104	0.044	0.062	0.028	0.020	0.197
P-value	0.198	0.196	0.907	0.711	0.305	0.663	0.541	0.782	0.841	0.230
Sample no.	99	99	99	99	99	99	99	99	99	39

The number of leukocytes, neutrophils, lymphocytes, erythrocytes and platelets in the peripheral blood were calculated. ALT, alanine aminotransferase; AST, aspartate aminotransferase and CRP, C-reactive protein.

(ALT), albumin (ALB) and total bilirubin (TB) in the peripheral blood in each cohort of 96 HCC and 99 non-HCC patients (no clinicopathological data were obtained for one of the non-HCC patients) (Table I).

The study protocol was approved by the Institutional Review Board for the Use of Human Subjects at the Yamaguchi University School of Medicine. Written informed consent was obtained from each patient, as previously described (8).

Expression data for inflammatory cytokine genes in primary HCC. For 14 of the 96 patients with HCV-related HCC evaluated in the present study, we had already performed an expression profiling of 12,600 genes (9). Of these, we searched for as many inflammatory genes, such as those listed in the previous report (10), as possible. We found that expression levels of the eight genes sufficient for evaluation were: Interleukin-1α (IL1A) (gene accession number: M28983/probe number: 1076_at), IL-1ß (M15330/39402_at), IL-1RN (X52015/37603_at), IL-4 (M13982/1574_s_at), IL-7 (M29053/33966_at), IL-8 (M28130/1369_s_at), IL-15 (AF031167/38488_s_at) and tumor necrosis factor-α (TNFA) (X02910/1852_at) (Table II). Using Cluster and Tree-View software (11), we performed a hierarchical cluster analysis of the data for these eight genes. Gene abbreviations were used based on the Entrez Gene at the Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez.

Statistical analysis. Correlations between the cfDNA level and clinicopathological factors were assessed by calculating the Pearson coefficient. All analyses were performed with SPSS 11.0J software (SPSS, Inc., Chicago, IL) run on a Windows computer. $P<0.05$ was considered statistically significant.

Results

We found that the serum cfDNA level was positively associated with the number of leukocytes (r=0.305, P=0.003) and neutrophils (r=0.325, P=0.001), and the AST level (r=0.209, P=0.041) in HCC patients (Fig. 1). However, the serum cfDNA level was not associated with the number of lymphocytes, erythrocytes or platelets or the level of CRP, ALT, ALB or TB in HCC patients (Table I). In non-HCC patients with HCV infection, no association between the serum cfDNA level and these factors was observed (Fig. 1, Table I).

A cluster analysis of the inflammatory cytokine genes resulted in the 14 HCCs being categorized into two clusters: one consisting of five HCCs, three of which had high cfDNA levels (≥117.8 ng/ml), which showed increased levels of inflammatory cytokines such as IL-1, -4 and -8, TNF-α and the other consisting of nine HCCs, all of which showed low levels of cfDNA (<117.8 ng/ml) and inflammatory cytokine genes (Fig. 2).

Discussion

Our present study revealed positive associations between an increased level of serum cfDNA and the number of leukocytes and neutrophils in the peripheral blood of patients with

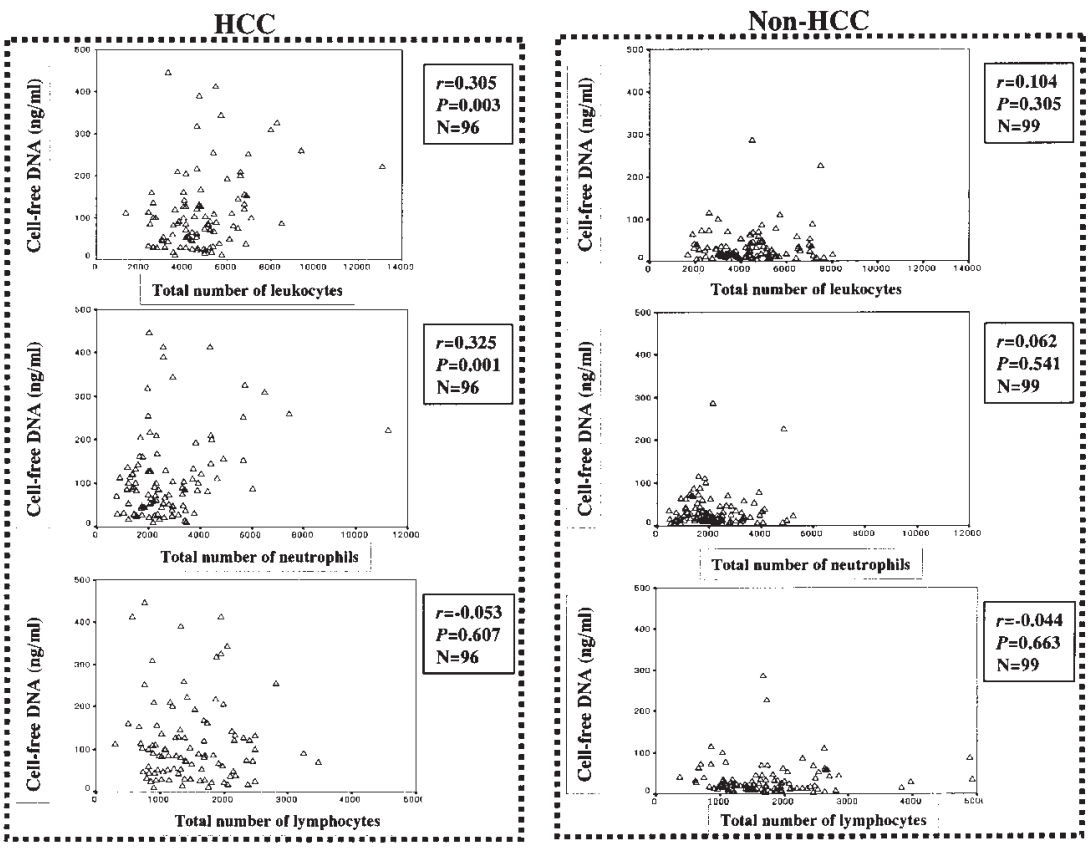

Figure 1. Association between the serum cell-free DNA (cfDNA) level and the number of leukocytes, neutrophils and lymphocytes in 96 patients with HCV-related HCC and in 99 non-HCC patients with HCV.

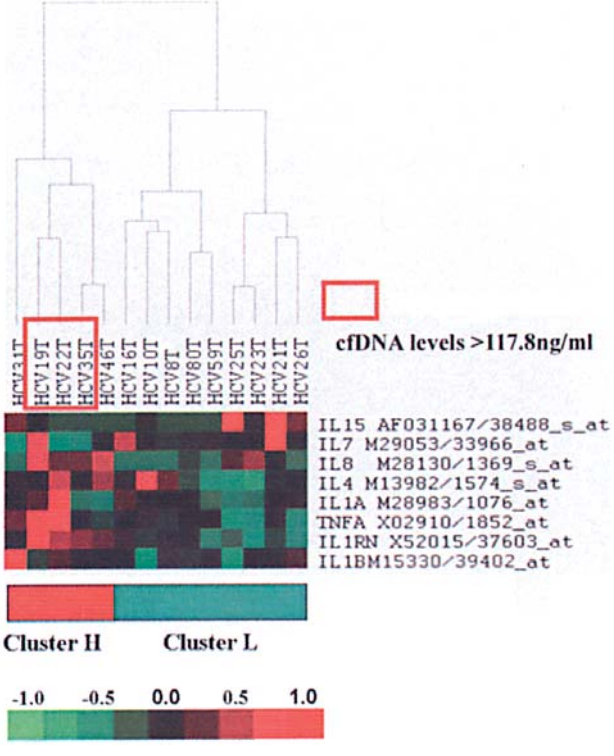

Figure 2. Hierarchical cluster analysis of the eight inflammation-related genes from the microarray platform. The unsupervised algorithm categorized the 14 HCCs into two clusters: cluster H with high levels of the eight genes and cluster L with low levels of the eight genes. Note that cluster H includes three HCCs (HCV19T, HCV22T and HCV35T) with high cfDNA levels (≥117.8 ng/ml^{-1}) and cluster L includes nine HCCs, all of which showed low cfDNA levels (<117.8 ng/ml^{-1}). Gene abbreviations are based on the Entrez Gene at the Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez.

Table II. Cytokine mRNA levels at the primary site of hepatocellular carcinoma.

Sample ID	cfDNA levels (ng/ml)	IL-1A	IL-1B	IL-1RN	IL-4	IL-7	IL-8	IL-15	TNFA
HCV26T	50.9	89.3	198.7	327.6	147.2	95.6	55.9	73.6	85.6
HCV8T	40.0	75.5	181.1	600.1	195.9	64.2	40.0	40.0	128.3
HCV10T	40.0	79.6	40.0	504.9	229.8	73.8	40.0	46.2	194.2
HCV16T	43.4	99.7	135.1	857.6	171.7	125.8	40.0	40.0	145.5
HCV23T	73.5	43.4	154.3	266.2	46.3	68.7	608.5	65.0	79.8
HCV31T	79.8	72.0	326.9	749.2	171.2	40.0	40.0	72.9	220.0
HCV46T	99.0	40.0	135.9	940.7	211.4	70.0	671.5	40.0	116.9
HCV59T	40.0	45.8	120.6	337.1	67.4	97.9	40.0	40.0	142.0
HCV21T	54.0	79.1	166.7	464.6	115.7	147.5	310.5	93.5	188.8
HCV25T	88.6	40.0	40.0	421.8	59.9	84.0	514.8	90.9	70.8
HCV80T	40.0	79.4	113.6	690.2	98.2	67.5	130.2	40.0	177.7
HCV22T	126.4	159.2	200.6	1069.1	223.8	40.0	540.0	40.0	275.7
HCV19T	119.6	100.2	102.2	1303.5	132.3	130.0	690.9	47.6	282.6
HCV35T	1083.8	43.2	153.7	1000.2	134.5	44.6	547.0	40.0	155.3

IL-1A (M28983/1076_at), IL-1B (M15330/39402_at), IL-1RN (X52015/37603_at), IL-4 (M13982/1574_s_at), IL-7 (M29053/33966_at), IL-8 (M28130/1369_s_at), IL-15 (AF031167/38488_s_at), TNFA (X02910/1852_at) and cell-free DNA (cfDNA). Gene abbreviations are based on the Entrez Gene at the Internet address: http://www.ncbi.nlm.nih.gov/sites/entrez.

HCV-related HCC. The lack of association between the cfDNA level and the number of leukocytes and neutrophils in the peripheral blood of non-HCC patients with HCV supports the concept that this phenomenon is specific to cancer and not due to leukocyte damage artifact (12,13). Notably, we found no association between the levels of serum cfDNA and CRP in HCC or non-HCC patients. This result is reasonable considering that CRP is affected largely by liver dysfunction rather than inflammation and all of the patients had HCV-related chronic liver disease.

In 1863, Rudolf Virchow proposed that inflammation is a hallmark of cancer according to the finding of leukocytes within cancer tissues. Since then, it has generally been accepted that if genetic abnormality is the 'match that lights the fire' of cancer, inflammation corresponds to the 'fuel that feeds the flames' (10). This is a likely scenario for hepatocarcinogenesis, as supported by a previous gene-profiling study (14) in which many inflammatory genes were related to the pathogenesis of HCV-related HCC.

Taking together the results of the previous studies (10,14), we aimed to clarify the association between serum cfDNA and inflammation. We initially evaluated the histopathological degree of infiltration of polynuclear and mononuclear leukocytes within the primary HCC tissues of the present study. However, we were unable to identify any relationship between the serum cfDNA level and inflammatory cell infiltration (data not shown). We then used gene profiling data to assess this issue. Our genomic profiling data revealed an association between the increased serum cfDNA level and enhanced expression of inflammatory cytokine genes within the tumor tissue of HCV-related HCCs, suggesting that the serum cfDNA level is associated with the inflammatory status at the primary site of HCV-related HCC. As a result, an increased serum cfDNA level is positively associated with oncogenesis and the progression of HCV-related HCC, as we previously proposed (6,7). The serum cfDNA level may be a potent marker for inflammation-related carcinogenesis.

We found a significant elevation of IL-8 and TNF-α gene expression in the primary tumors of three HCCs with high cfDNA levels (≥117.8 ng/ml). TNF-α acts as a growth factor for cancer cells and promotes DNA damage and inhibits DNA repair (15). Thus, increased levels of TNF-α enable cancer cells to survive and contribute to cancer progression. IL-8 promotes angiogenesis and inflammation (16), and therefore cancer progression. Our present result suggests that inflammation at the primary site of advanced HCV-related HCC destroys cancer cells resulting in the release of cellular DNA into the bloodstream. Further studies are needed to elucidate the molecular basis of cfDNA release into the bloodstream and to evaluate the use of cfDNA as a biomarker for routine clinical use.

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