

## ARTICLE

# Deoxyribonuclease I Activity, Cell-Free DNA, and Risk of Liver Cancer in a Prospective Cohort

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## Abstract

**Background:** Circulating cell-free DNA (cfDNA) is a proposed latent biomarker for several cancers, including liver cancer. Deoxyribonucleases (DNases) facilitate the timely and efficient degradation of cfDNA, leading us to hypothesize that DNase I and/or II might be a more sensitive early biomarker than cfDNA. To test this hypothesis, a study was conducted in a large, prospective cohort.

**Methods:** A nested case-control study (224 liver cancer case patients and 224 matched control subjects) was conducted in a cohort of Finnish male smokers, followed from baseline (1985–1988) to 2014. The associations among DNase I activity, cfDNA, and the risk of liver cancer were assessed using multivariable-adjusted conditional logistic regression.

**Results:** DNase I activity, whether measured as radius (mm) or as units per milliliter, was statistically significantly associated with increased risk of liver cancer ( $P_{\text{trend}} < .01$ ). DNase I activity in the highest quartile was associated with a greater than threefold risk of developing liver cancer (DNase I activity radius  $>2.7$  mm, hazard ratio [HR] = 3.03, 95% confidence interval [CI] = 1.59 to 5.77; DNase I activity  $>2.72$  units/mL, HR = 3.30, 95% CI = 1.64 to 6.65). The strength of this association was not substantially altered by exclusion of cases diagnosed within the first five years of follow-up or those with hepatitis C virus (HCV) infection. In contrast, cfDNA and DNase II was not statistically significantly associated with risk of liver cancer.

**Conclusions:** DNase I activity was a superior latent biomarker of liver cancer than cfDNA. These findings advance the goal of developing a means to detect liver cancer years well before the development of clinical manifestations.

Liver cancer is the sixth most commonly occurring cancer in the world and the second most frequent cause of cancer-related mortality (1). The dominant histological type of liver cancer is hepatocellular carcinoma (HCC), which accounts for approximately 85% of cases (1). Major risk factors for HCC, including chronic hepatitis B and C virus infections, consumption of aflatoxin-contaminated foods, excessive alcohol intake, cigarette smoking, obesity, and diabetes are associated with chronic hepatic inflammation, suggesting that development of HCC progresses from inflammation to liver disease to cancer (2,3). Even though the process of hepatocarcinogenesis can take years to decades (2), early detection remains difficult because of the lack

of effective biomarkers. Serum  $\alpha$ -fetoprotein, for instance, is a widely used biomarker for HCC but its predictive value is limited by low sensitivity (3), thus underscoring the urgent need for novel and more effective biomarkers.

Another potential biomarker for HCC is circulating tumor DNA (ctDNA), which has been used to diagnose patients with advanced neoplasia and to monitor tumor progression (4–6). Similarly, accumulating evidence suggests that elevated levels of circulating cell-free DNA (cfDNA) could be a promising latent biomarker that might predict cancer development (7). Unlike cfDNA, ctDNA has somatic mutations, i.e. methylation at CpG sites (4, 8), and ctDNA is released only from apoptotic tumor

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cells (9), thus representing only a small fraction of total cfDNA (10). In contrast, elevated levels of total cfDNA could be derived from nuclear or mitochondrial DNA that has leaked from dead cells (11), from neutrophil extracellular traps (NETs) (12, 13), or from translocated gut bacterial DNA (14). Irrespective of the cfDNA source, inadequate clearance of cfDNA is likely to be pathological by acting as an autoantigen in autoimmunity (15) or in potentiating inflammation via toll-like receptor 9 (TLR9) (16–18), which could further promote cancer development.

Several studies have reported increased levels of cfDNA in patients with ovarian cancer (19, 20), breast cancer (21), and HCC (22, 23). Although cancer patients, in general, tend to have high levels of cfDNA, prospective cohort studies have suggested that the utility of this biomarker is limited. One such report, from the European Prospective Investigation into Cancer and Nutrition (EPIC), found only weak associations between cfDNA and bladder, lung, and head and neck cancer, whereas the association with leukemia was somewhat stronger (24). Another study observed that cfDNA was a marker for prostate disease; however, cfDNA failed to discriminate between prostate cancer and benign prostate disease (25).

We hypothesized that one reason why cfDNA may not be consistently elevated in cancer is that levels of cfDNA might be kept in check by elevated expression of deoxyribonucleases (DNases), which function as “waste management” enzymes that facilitate the timely and efficient degradation of cfDNA (26). There are two major DNase families: DNase I, which requires divalent cations (eg,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) for its enzymatic activity, peaks at neutral pH, and leaves 5'-phosphates after DNA cleavage (27); and DNase II, which, in contrast, does not require divalent cations, peaks at acidic pH, and leaves 3'-phosphates (27). Considering that DNase I is found in exocrine gland secretions and blood, whereas DNase II is found in lysosomes/phagolysosomes (27), we hypothesized that upregulation of DNase I, but not DNase II, might occur in response to increased release of cfDNA. This would minimize elevations in steady state levels of cfDNA but might result in detectably higher levels of DNase I activity, which could potentially serve as a biomarker of early stage cancer. To date, DNase I has been successfully utilized as an anticancer agent (26, 28–30) and studied as a prognostic marker during cancer therapy (31–34). Hence, the goal of this study was to evaluate the ability of levels of serum cfDNA and DNase I enzymatic activity to predict the development of HCC.

## Methods

### Study Design

The Alpha-Tocopherol Beta-Carotene (ATBC) study was a randomized, controlled trial to test the effects of  $\alpha$ -tocopherol and  $\beta$ -carotene on lung cancer incidence among male smokers in Finland (35). At trial baseline (1985–1988), 29 133 men aged 50–69 years, who smoked at least five cigarettes per day, were randomly assigned to intervention or placebo. Potential study participants who self-reported prevalent cancer (other than nonmelanoma skin cancer), cirrhosis, chronic alcoholism, or other conditions that would limit their participation in the trial were excluded from the study (35). At enrollment, participants provided a blood sample after a fast of at least 12 hours and completed a questionnaire that collected information on demographics, medical, dietary, and lifestyle factors. The trial ended in 1993 and participants have been followed annually to ascertain cancer incidence. This study was approved by the

Institutional Review Boards of both the US National Institutes of Health and the National Public Health Institute of Finland.

For this analysis, all cases of primary liver cancer (defined based on the International Classification of Diseases [ICD], version 9; topography codes 155.0 and 155.1, ie, malignant neoplasms of the liver and intrahepatic bile ducts) diagnosed through December 31, 2014, were identified through linkage to the Finnish Cancer Registry. Three cases were excluded because they did not have a stored serum sample. Among all cases of primary liver cancer, we additionally identified those with HCC histology using International Classification of Diseases for Oncology (ICD-O) morphology codes 8170–8175. Controls were selected from living individuals with an available serum sample and were free of liver cancer at the time of the case's diagnosis. The control participants were matched, pairwise, to cases on age at random assignment and date of blood collection. The final analytic cohort included 224 primary liver cancer cases (including 157 HCC cases) and 224 matched controls. The median length of time from study enrollment to liver cancer diagnosis was 15.6 years, with a range of 8.6–21.2 years.

### Laboratory Analysis

Baseline serum samples were collected, aliquoted, and stored at  $-70^{\circ}\text{C}$ . DNase I and II activity and cfDNA were measured at the University of Toledo College of Medicine and Life Sciences in a blinded fashion. Unless specified, all chemicals were supplied by MilliporeSigma (Burlington, MA).

#### DNase I Activity.

Serum DNase I activity was measured by Single Radial Enzyme Diffusion (SRED). Briefly, to determine serum DNase I activity, 4  $\mu\text{L}$  of sera was diluted with 2  $\mu\text{L}$  of 1 X PBS and applied to a 1.0% agarose gel embedded with magnesium chloride (20 mM), calcium chloride (2 mM), and ethidium bromide (0.01 mg/mL)-intercalated calf thymus DNA (0.2 mg/mL) on petri plates. DNase I in the sample kinks the ethidium bromide-intercalated calf thymus DNA in the gel, forming a halo (zone of clearance) of DNA degradation. After incubating the plates at  $37^{\circ}\text{C}$  for 24 hours, the radiuses of the halos were measured using a Vernier caliper. The radiuses are directly proportional to the amount of DNase I in the sample (36). DNase I activity was calculated using a standard curve generated from known concentrations of bovine DNase I (Thermo Fisher Scientific, Waltham, MA). The within-batch coefficient of variation (CV) for DNase I activity measured in units per milliliter was 20.2 and measured as radius in millimeters was 9.03.

#### DNase II Activity.

Serum DNase II activity was also measured by SRED. In contrast to DNase I, to determine serum DNase II activity, 4  $\mu\text{L}$  of sera was diluted with 2  $\mu\text{L}$  of 1 X PBS and applied to a 1.0% agarose gel embedded with EDTA (20 mM), sodium acetate (100 mM), and ethidium bromide (0.01 mg/mL)-intercalated calf thymus DNA (0.2 mg/mL) on petri plates. DNase II activity was calculated using a standard curve generated from known concentrations of bovine spleen DNase II (MilliporeSigma). Because there was undetectable DNase II activity by the SRED method, no statistical analysis was possible.

### Circulating Cell-Free DNA.

cfDNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. The within-batch CV was 9.2.

### Statistical Analysis

Conditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between each biomarker and liver cancer risk. Biomarker levels were batch-adjusted using a normalization factor derived from quality control samples repeatedly measured across all plates (ie, the difference between the mean values of quality control samples on an individual plate vs all plates combined) and categorized into quartiles based on the distribution among controls. In addition to matching on age and time of blood draw, the following confounders were also included, based on a priori knowledge: body mass index (BMI), education, smoking intensity (pack-years), alcohol intake, and history of diabetes or hypertension at baseline. Missing values for alcohol intake (15 cases and 9 controls) were imputed using the PROC MI procedure (SAS Institute Inc., Cary, NC), using case status and adjustment factors (ie, age, BMI, education, vocational training, smoking, diabetes, hypertension, and marital status). Coffee intake was also evaluated as a potential covariate but did not substantially alter the estimates, thus it was not included in the final models. Furthermore, effect modifications by the following factors were evaluated, using likelihood ratio tests comparing models with and without the interaction term: age, BMI, smoking intensity, and alcohol intake. Several sensitivity analyses were also conducted, including (1) restricting the analytical cohort to histologically confirmed HCC cases and their matched controls; (2) excluding cases diagnosed within the first five years of follow-up; and (3) excluding cases with HCV infection. As a secondary cross-sectional analysis, the mean (SD) of the level of each biomarker was stratified by selected baseline characteristics of participants, including age, BMI, education, smoking intensity, alcohol intake, and coffee consumption. As levels of the biomarkers were roughly normally distributed by visual inspection of the data, natural logarithm transformation was not done when calculating the means. All analyses were performed in SAS, version 9.3 (SAS Institute Inc.). Statistical tests were two-sided.

### Results

Selected characteristics of cases and controls are presented in Table 1. Compared to controls, cases were more likely to be better educated, obese, smoke, drink heavily, consume less coffee, and have a history of diabetes, hypertension, and chronic HCV infection.

Table 2 depicts the mean value of each biomarker according to selected characteristics among controls, including age at random assignment, BMI, education, intensity of cigarette smoking (pack-years), drinks of alcohol per day, and coffee consumption (grams per day). Overall, there were no clear associations among DNase I activity, cfDNA, and the lifestyle variables. In addition, an examination of the correlations among the biomarkers found that the two measurements of DNase I activity were highly correlated (Pearson  $r = .84$ ), but neither DNase I activity expressed as a radius ( $r = .06$ ) or as units per milliliter ( $r = .03$ ) were associated with cfDNA.

**Table 1.** Participant characteristics in a nested case-control study of liver cancer in the Alpha-Tocopherol Beta-Carotene (ATBC) study

Characteristic	Cases No. (%) (n = 224)	Controls No. (%) (n = 224)
Age at random assignment, mean (SD), y	57.4 (4.7)	56.8 (4.2)
Body mass index, kg/m <sup>2</sup>		
<25.0	63 (28.1)	90 (40.2)
25.0–<30.0	111 (49.6)	104 (46.4)
≥30.0	50 (22.3)	30 (13.4)
Education*		
1	47 (21.0)	71 (31.7)
2	114 (50.9)	105 (46.9)
3	63 (28.1)	48 (21.4)
Cigarette smoking, pack-years		
>0–24	53 (23.7)	71 (31.7)
25–34	43 (19.2)	54 (24.1)
35–44	50 (22.3)	52 (23.2)
≥45	78 (34.8)	47 (21.0)
Drinks of alcohol, per day		
0	14 (6.7)	15 (7.0)
>0–<1.0	70 (33.5)	108 (50.2)
1.0–<2.0	59 (28.2)	54 (25.1)
≥2.0	66 (31.6)	38 (17.7)
Coffee consumption, g/day		
0–<200	29 (13.9)	13 (6.0)
200–<500	80 (38.3)	78 (36.3)
500–<1000	83 (39.7)	90 (41.9)
≥1000	17 (8.1)	34 (15.8)
Diabetes		
Yes	22 (9.8)	5 (2.2)
No	202 (90.2)	219 (97.8)
Hypertension		
Yes	56 (25.0)	44 (19.6)
No	168 (75.0)	180 (80.4)
Hepatitis C virus infection		
Yes	10 (4.5)	1 (0.4)
No	214 (95.5)	223 (99.6)
Randomization arm		
Placebo	55 (24.6)	60 (26.8)
α-Tocopherol	63 (28.1)	63 (28.1)
β-Carotene	54 (24.1)	60 (26.8)
α-Tocopherol/β-Carotene	52 (23.2)	41 (18.3)

\*Levels of education: 1 = elementary school or less, no vocation training; 2 = elementary school or less, vocational training; 3 = more than elementary school. Statistical tests were two-sided.

Table 3 shows the associations between each biomarker (categorized in quartiles) and the risk of primary liver cancer. In multivariable-adjusted models, higher levels of DNase I activity were statistically significantly associated with increased risk of liver cancer ( $P_{\text{trend}} < .01$ ). Compared to quartile 1, in both quartiles 3 (DNase activity radius >2.3–2.7: OR = 1.89, 95% CI = 1.01 to 3.53) and 4 (DNase I activity radius >2.72: OR = 3.03, 95% CI = 1.59 to 5.77) there were increased risks of liver cancer associated with DNase I activity expressed as a radius. Similarly, compared to quartile 1, in quartiles 2 (DNase I activity >0.92–1.76 units/mL: OR = 1.98, 95% CI = 1.01 to 3.90), 3 (DNase I activity >1.76–2.72 units/mL: OR = 2.00, 95% CI = 1.00 to 3.98), and 4 (DNase I activity >2.72 units/mL: OR = 3.30, 95% CI = 1.64 to 6.65), there was a statistically significant increased risk of liver cancer when examining DNase I activity expressed as units per milliliter. In contrast to DNase I activity, there was no

**Table 2.** Mean (SD) levels of deoxyribonuclease (DNase I) activity (radius and units per milliliter) and cfDNA according to selected characteristics among controls in the Alpha-Tocopherol Beta-Carotene (ATBC) study

Characteristic	DNase I activity		cfDNA, ng/mL
	radius, mm	units/mL	
Age at random assignment, y			
≤54	2.31 (.065)	1.84 (1.26)	2170.0 (407.9)
55–59	2.39 (.061)	2.10 (1.47)	2230.3 (399.0)
60–64	2.05 (.068)	1.50 (1.14)	2198.0 (340.0)
≥65	2.53 (.038)	2.15 (1.18)	2065.9 (336.3)
Body mass index, kg/m <sup>2</sup>			
<25.0	2.24 (.065)	1.76 (1.34)	2195.7 (413.1)
25.0–<30.0	2.35 (.059)	1.98 (1.25)	2211.8 (358.4)
≥30.0	2.43 (.074)	2.28 (1.63)	2101.9 (403.9)
Education*			
1	2.35 (.069)	2.00 (1.22)	2227.7 (403.1)
2	2.34 (.067)	1.96 (1.47)	2124.3 (367.3)
3	2.21 (.046)	1.76 (1.23)	2281.7 (386.6)
Cigarette smoking, pack-years			
>0–24	2.34 (.063)	1.98 (1.26)	2148.8 (342.8)
25–34	2.23 (.061)	1.82 (1.54)	2171.2 (419.8)
35–44	2.36 (.056)	1.84 (1.24)	2237.3 (429.1)
≥45	2.33 (.076)	2.09 (1.36)	2224.7 (364.1)
Drinks of alcohol, per day			
0	2.17 (.063)	1.44 (.089)	2097.4 (363.1)
>0–<1.0	2.40 (.056)	2.08 (1.34)	2163.7 (392.9)
1.0–<2.0	2.29 (.078)	2.03 (1.46)	2204.9 (386.6)
≥2.0	2.17 (.061)	1.60 (1.38)	2279.8 (395.5)
Coffee consumption, g/day			
0–<200	2.42 (.057)	2.29 (1.81)	2114.3 (253.0)
200–<500	2.31 (.062)	1.87 (1.37)	2225.5 (415.5)
500–<1000	2.32 (.070)	1.96 (1.31)	2195.3 (385.1)
≥1000	2.27 (.059)	1.88 (1.33)	2126.8 (390.3)

\*Levels of education:1 = elementary school or less, no vocation training; 2 = elementary school or less, vocational training; 3 = more than elementary school. Statistical tests were two-sided. cfDNA = circulating cell-free DNA.

association between cfDNA (nanogram per milliliter) and development of liver cancer.

In the sensitivity analyses, results were not substantially altered when restricting the analyses to HCC cases (Supplementary Table 1, available online), excluding cases diagnosed within the first five years of follow-up (Supplementary Table 2, available online), or excluding persons with HCV infection (Supplementary Table 3, available online). There was also no evidence of interaction between the serum biomarkers and age, BMI, smoking pack-years, and alcohol consumption ( $P_{\text{interaction}} > .05$ ).

## Discussion

This is the first study to examine potential associations of DNase I activity and cfDNA with liver cancer risk in a large prospective cohort. We observed that levels of serum DNase I activity were associated with a threefold increased risk of developing liver cancer. This association suggests that, in addition to being a prognostic marker for patients undergoing chemotherapy (31–34), DNase I activity might serve as a latent biomarker of early stage cancer development. In contrast, we did not observe an association between cfDNA and liver cancer risk. Lack of association, however, does not challenge the notion that release of

**Table 3.** Associations between deoxyribonuclease (DNase I) activity (radius and units/mL), circulating cell-free DNA (cfDNA), and liver cancer risk in the Alpha-Tocopherol Beta-Carotene (ATBC) study

Assay	Cases	Controls	Age-adjusted	Fully adjusted
			model*	model†
			HR (95% CI)	HR (95% CI)
DNase I activity, radius in mm				
0–1.9	42	71	1.00 (Referent)	1.00 (Referent)
>1.9–2.3	41	40	1.83 (0.98 to 3.40)	1.65 (0.81 to 3.34)
>2.3–2.7	57	56	1.86 (1.06 to 3.27)	1.89 (1.01 to 3.53)
>2.7	82	54	2.90 (1.64 to 5.13)	3.03 (1.59 to 5.77)
$P_{\text{trend}}$			<.01	<.01
DNase I activity, units/mL				
0–0.92	33	59	1.00 (Referent)	1.00 (Referent)
>0.92–1.76	55	52	1.80 (1.01 to 3.22)	1.98 (1.01 to 3.90)
>1.76–2.72	54	57	1.83 (1.00 to 3.33)	2.00 (1.00 to 3.98)
>2.72	80	53	3.04 (1.66 to 5.58)	3.30 (1.64 to 6.65)
$P_{\text{trend}}$			<.01	<.01
cfDNA, ng/mL				
0–1904.28	64	56	1.00 (Referent)	1.00 (Referent)
>1904.28–2128.48	48	55	0.76 (0.44 to 1.30)	0.64 (0.35 to 1.17)
>2128.48–2413.52	53	55	0.82 (0.48 to 1.43)	0.88 (0.47 to 1.65)
>2413.52	57	55	0.86 (0.43 to 1.74)	0.80 (0.36 to 1.80)
$P_{\text{trend}}$			.15	.05

\*Age-adjusted model accounts for age at study randomization and date of blood collection. Statistical tests were two-sided.

†Fully adjusted model accounts for age and blood collection date and adjusts for body mass index, education, smoking intensity, alcohol intake, diabetes, and hypertension.

cfDNA is enhanced in a protumorigenic environment but rather may reflect that the elevated levels of DNase I keep cfDNA levels partially in check.

Increased levels of cfDNA observed in cancer patients might reflect release of DNA by dividing tumor cells, tumor lysis, or necrotic cells (28), or excessive generation of NETs (13). Regardless of source, the presence of cfDNA in systemic circulation is widely regarded to be tumor-promoting because, in part, of its ability to form an extracellular matrix that facilitates adherence and growth of primary and metastatic cancer cells (37–40). One prior study examined the association between cfDNA and liver cancer risk (41), but that study collected blood samples at diagnosis and, unlike the present study, did not examine DNase I activity. Importantly, the current study analyzed serum samples that were collected prior to diagnosis, thus suggesting that an elevation in serum DNase I activity could be an early stage biomarker.

We hypothesize that elevations in DNase I activity are driven by increased release of cfDNA and serve as a compensatory mechanism to keep cfDNA levels in check and thereby mitigate promotion of inflammation and cancer. The transition from having high DNase I (preclinical) to low DNase I (active disease) in sera may allow cfDNA levels to be left unchecked and result in substantial loss of antitumor surveillance. Accordingly, we envision that the disparity between the cfDNA and DNase I profiles during preclinical and active disease (28) not only may be early biomarkers of liver cancer risk but also may distinguish individuals with occult liver cancer from those with diagnosed disease. Additionally, treating cancer cells with DNase I ex vivo has been reported to substantially impede their metastatic potential (42–45). This antagonistic relationship between cfDNA and DNase I has led several studies to examine their interplay in cancer patients (46, 47), thus raising the prospect that both

can be harnessed as complementary biomarkers to assess active disease. Further prospective studies are clearly warranted to determine the predictive value in screening for DNase I and cfDNA in tandem.

Strengths of the current study include the prospective design, large sample size, long follow-up period (up to 29 years), detailed information on potential confounders (eg, HCV infection), and availability of histology data to identify HCC for a sensitivity analysis. A limitation of the study was that the ATBC study was conducted as a lung cancer prevention trial and, thus, was not specifically designed to assess liver cancer and preexisting liver disease. Because underlying disease processes could affect the concentrations of systemic DNases and cfDNA, we conducted a sensitivity analysis whereby we excluded the first five years of follow-up. Results were not substantially altered, however, suggesting that preexisting liver disease did not substantially affect the findings.

In summary, we report that serum DNase I activity is statistically significantly associated with an increased risk of liver cancer. Thus, serum DNase I activity may have prognostic value in helping identify persons in early stages of developing HCC and perhaps other malignancies. Future studies should explore whether the other member of the DNase I family, DNase1L3 (DNase gamma), which targets extracellular DNA protein complexes (e.g. nucleosomes) for degradation (27), could act as another biomarker or an anticancer agent. Further assessment of endogenous DNase I inhibitors (eg, G-actin, somatostatin) (27, 48) in serum should also be considered in future studies, because these factors are very likely to impact measurements of DNase I activity.

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## Notes

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