

# Increased Plasma DNA Integrity in Cancer Patients<sup>1</sup>

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## ABSTRACT

Tumor-released DNA in blood represents a promising biomarker for cancer detection. It has been postulated that tumor necrosis causes release of DNA of varying sizes, which contrasts apoptosis in normal tissue that releases smaller and more uniform DNA fragments. To test the hypothesis that increased DNA integrity, *i.e.*, a longer DNA strand, is a tumor-associated marker in plasma, we determined the genomic DNA integrity index in plasma DNA using real-time PCR assays. A DNA integrity index and DNA concentration in plasma were determined in 61 patients with gynecological and breast cancers and 65 female patients without neoplastic diseases. We found that the area under the receiver-operating characteristic curve for DNA integrity index was 0.911 for cancer *versus* non-neoplastic patients. Given 100% specificity, the highest sensitivity achieved in detecting the cancer group was 62% (95% confidence interval = 0.50–0.74) at the index cutoff of 0.59. Fifty percent of stage I cancers had a DNA integrity index above this cutoff. All 11 patients with benign adnexal masses that clinically can be confused with malignant gynecological neoplasms demonstrated DNA integrity index < 0.59. Our findings suggest that increased DNA integrity in plasma DNA is associated with cancer, and measurement of DNA integrity may provide a simple and inexpensive measure for cancer detection.

## INTRODUCTION

Tumor-released DNA in blood represents a promising biomarker for cancer diagnosis. It is well recognized that solid malignant tumors release a significant amount of genomic DNA into the systemic circulation probably through cellular necrosis and apoptosis (1–3). Tumor-released DNA can be detected as a result of specific genetic alterations, including microsatellite alterations, allelic imbalance, translocation, mutations, and presence of viral genes (4–8). Although these genetic aberrations have been suggested to have clinical utility, there are several technical difficulties challenging their practical application in cancer screening. A combination of multiple markers and techniques needs to be used because most genetic changes such as mutations in KRAS and BRAF genes and microsatellite instability are not present in the majority of carcinomas (9–11). Although mutations in p53 gene and mitochondria DNA appear more prevalent in many cancer types, simple and reliable techniques are not yet available to detect these mutations in blood because of the lack of hot spot mutations to facilitate their detection. Recently, we have demonstrated that allelic imbalance in plasma DNA as detected by digital single nucleotide polymorphism analysis held promise for early detection of cancer (5); however, this new technology is not yet applicable for a population-based cancer screening. This is mainly because of the technical complexity and high cost currently associated with this analysis (5). Therefore, a simple and inexpensive assay is desirable for the purposes of early cancer detection in mass screenings.

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In an effort to achieve this objective, we have developed a real-time PCR-based assay to assess the DNA strand integrity of plasma DNA based on the following hypothesis. Tumor necrosis is a frequent event in solid malignant neoplasms, and it generates a spectrum of DNA fragments with different strand lengths because of random and incomplete digestion of genomic DNA by a variety of deoxyribonucleases. In contrast, cell death in normal tissues is mainly through apoptosis which results in small and uniform DNA fragments with ~185–200 bp (12, 13). To test this hypothesis, we conducted a case control study to measure DNA strand integrity using a real-time PCR-based assay in a total of 126 plasma samples obtained from neoplastic and nonneoplastic patients.

## MATERIALS AND METHODS

**Samples and Genomic DNA Isolation.** The plasma samples were retrieved from the Gynecological Pathology Tumor Bank and the Division of Clinical Chemistry in the Department of Pathology at the Johns Hopkins Hospital. The acquisition of clinical material was approved by the local institutional review board. These samples included 61 female patients with neoplastic diseases and 65 female patients without neoplastic diseases. There was no significant difference in the patients' age between the neoplastic group (51.7 ± 15.4 years) and the control group (50.1 ± 11.6 years). The specific diagnoses of the patients are presented in Table 1. There were 12 neoplastic specimens of stage I diseases, including 4 ovarian malignant tumors, 5 endometrial carcinomas, and 3 breast carcinomas. After centrifugation of blood (5–10 ml) at 2000 × *g* for 10 min, the plasma samples (500 μl) were carefully collected from the very top portion of the plasma (5). DNA was purified from 200 μl of plasma with a QIAamp DNA Blood Kit (Qiagen, Valencia, CA). Genomic DNA was also extracted from the OVCAR3 cell line (American Type Culture Collection, Manassas, VA) as a control. The PicoGreen dsDNA quantitation kit (Molecular Probes, Inc.) was used to measure DNA concentration following the manufacturer's instruction (5).

**DNA Strand Integrity Analysis.** The DNA strand integrity was measured by a semiquantitative real-time PCR using the i-Cycler (Bio-Rad, Hercules, CA) to determine the integrity index, which was defined as the ratio in relative abundance of 400 *versus* 100 bp PCR products of the β-actin gene that was likely present in all normal and neoplastic cells. Two μl of DNA were used in a 10-μl PCR mixture containing PCR buffer, 3 mM MgCl<sub>2</sub>, 2 mM deoxynucleotide triphosphate, and 0.07 units/μl Taq (Invitrogen, Carlsbad, CA). Amplification was performed in duplicates with primer sets manufactured by Genelink (Hawthorne, NY). Both 100- and 400-bp PCR fragments were amplified using the same forward primer: 5'-GCACCACACCTTCTCAATGA-3'. The nested reverse primer for 100-bp product was 5'-GT-CATCTTCTCGCGTTGGC-3' and for 400-bp was 5'-TGTCACGCACGATTTC-3'. The PCR conditions were: denaturation for 3 min at 95°C followed by 45 cycles of denaturation at 95°C for 30 s; annealing at 57°C (100 bp) or 56°C (400 bp) for 30 s; and extension at 72°C for 10 s (100 bp) and 15 s (400 bp). The Bio-Rad i-Cycler software monitored the changes in fluorescence of Sybr Green I dye (Molecular Probes, Inc., Eugene, OR) in each cycle. The genomic DNA isolated from the OVCAR3 cells in culture was used as reference to determine the relative DNA strand integrity in plasma DNA because culture cells contained highly intact genomic DNA. The threshold (Ct) value for each reaction was calculated by the i-Cycler software package. The Ct value of the 400 bp for a sample was subtracted from that for the OVCA3 control to obtain a ΔCt value for 400 bp. Likewise, the Ct value of a 100 bp for the sample was subtracted from that for the OVCA3 control to obtain a ΔCt value for 100 bp. The ΔCt value for 400 bp was subtracted from that for 100 bp to obtain a ΔΔCt value. The integrity index was calculated as exponential

Table 1 Patients' profile included in this study

Diagnosis	Patient no.
Nonneoplastic diseases	65
Gynecological diseases	14
Status after kidney transplant	8
Cardiovascular diseases	6
Respiratory diseases	6
Autoimmune diseases	7
Gastrointestinal diseases	6
Trauma	2
Diabetes	2
Others	4
No apparent diseases	10
Neoplastic diseases	61
Ovarian tumor	40
Endometrium/cervix carcinoma	9
Peritoneal serous carcinoma	5
Breast carcinoma	7

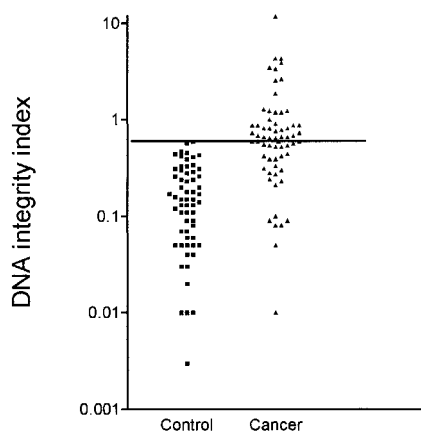


Fig. 1. A scatter plot of the DNA integrity index in plasma samples from patients with neoplastic diseases and nonneoplastic controls. Quantitative real-time PCR was performed to determine the DNA integrity index as defined by the ratio in relative abundance of 400- versus 100-bp PCR products of the  $\beta$ -actin gene. A cutoff (the transverse line) of 0.59 represents the highest index number in the controls.

of  $(-\Delta\Delta Ct \times LN 2)$ . All samples were analyzed blindly without prior knowledge of the specimen identity.

**Statistical Analysis.** Wilcoxon's rank-sum test was used to compare the difference in DNA integrity index between neoplastic and nonneoplastic groups. ROC<sup>3</sup> curves were used to assess the feasibility of using plasma DNA strand integrity and DNA concentrations as diagnostic tools for detecting ovarian and other cancers. A ROC curve is a graph of the sensitivity against the false positive rate (1-specificity), which is used to evaluate test performance at different thresholds of a diagnostic measure.

## RESULTS AND DISCUSSION

The DNA integrity index in all specimens was demonstrated in Fig. 1. The integrity index in both neoplastic and nonneoplastic control specimens varied widely. The median DNA integrity index was 0.66 (interquartile range = 0.42–0.90) in the neoplastic group, which was significantly higher than 0.14 (interquartile range = 0.06–0.28) in the nonneoplastic group ( $P < 0.0001$ , Wilcoxon's rank-sum test). The gel view of PCR products from 10 representative samples was illustrated in Fig. 2. There were detectable bands of 100- and 400-bp PCR products with comparable intensity in the neoplastic samples. In contrast, the intensity of 100-bp bands was much stronger than 400-bp bands in the nonneoplastic controls, indicating the relatively lower abundance of longer DNA fragments in control plasma samples. Both 100- and 400-bp PCR products contained the correct nucleotide

sequences, demonstrating the specificity of the PCR assays. Although the number of patients in each disease category was small, no association was noted between DNA integrity index and a particular benign disease or a specific type of cancer.

ROC curve analysis was used to assess the performance of DNA integrity analysis in diagnosing malignant neoplastic diseases. As shown in Fig. 3, the area under the ROC curve for DNA integrity index was 0.911 for neoplastic versus nonneoplastic patients. In contrast, although there was a difference in plasma DNA concentration between benign and cancer groups ( $P = 0.015$ , Wilcoxon Rank Sum test), the area under ROC curve for plasma DNA concentration was only 0.71. DNA concentrations added little information to the DNA integrity index as it increased the area under ROC curve from 0.911 to 0.912. Similar to our previous report (5), these findings indicated that alterations in the DNA quality rather than quantity better characterized tumor-released DNA in plasma. The highest sensitivity and specificity were obtained at a threshold level for integrity index of 0.59. When the index cutoff of 0.59 was applied to define the positivity for the test, none of the 65 patients with nonneoplastic diseases was positive, whereas 38 of 61 neoplastic patients were detected positive [sensitivity = 0.62 (95% CI = 0.50–0.74)]. With this cutoff, 50% (6 of 12) of stage I cancers were positive, and all 11 patients with benign adnexal masses that clinically could be confused with malignant gynecological neoplasms were negative for the test. The DNA integrity assay was reproducible with  $r = 0.965$  (linear correlation) when the same samples were analyzed independently by two investigators (Fig. 4). The percent agreement was 95.5% when the index of 0.59 was applied as the cutoff to define positivity. Among 40 ovarian neoplasms, there were corresponding serum

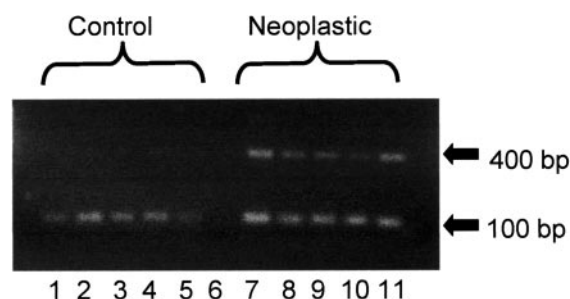


Fig. 2. A gel view of 100- and 400-bp PCR products from five nonneoplastic controls (Lanes 1–5) and five neoplastic specimens (Lanes 7–11). The PCR products were obtained at the exponential stage during the real-time PCR analysis (at the 27<sup>th</sup> PCR cycle for 100 bp and at the 28<sup>th</sup> PCR cycle for 400 bp). All of the specimens contain detectable 100-bp products. The band intensity of 400-bp products is much stronger in neoplastic specimens than in nonneoplastic controls. Lane 6 indicates the negative control.

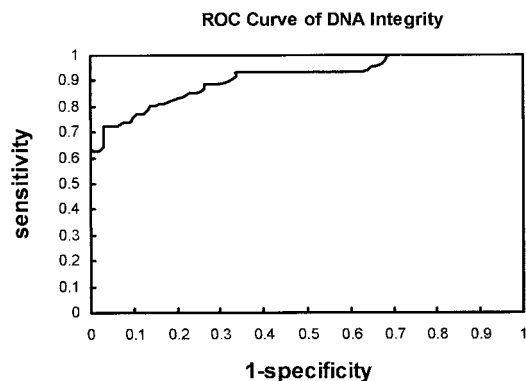


Fig. 3. A ROC curve analysis of the DNA integrity index in detecting cancer. The area under the ROC curve for DNA integrity index was 0.911 in distinguishing neoplastic versus nonneoplastic samples.

<sup>3</sup> The abbreviation used is: ROC, receiver-operating-characteristic.

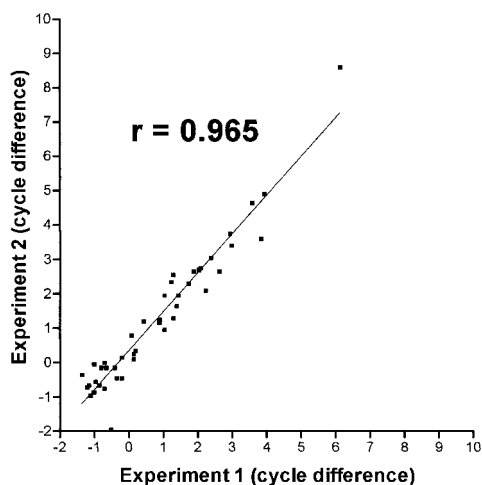


Fig. 4. Interobserver variation in the measurements of the DNA integrity index. The same plasma DNA samples were analyzed by two investigators (experiments 1 and 2) using the same technique and protocol. There is a correlation between the index obtained from experiment 1 and experiment 2 with  $r = 0.965$ . The percentage agreement is 95.5% when the index of 0.59 was applied as the cutoff to define positivity of the test.

CA125 levels available in 25 cases. Although the sensitivity of CA125 (>35 units/ml, the clinical cutoff value) in detecting ovarian cancer was 80%, which was similar to previous reports (14), 4 of 5 CA125-negative cases had integrity index higher than 0.59, suggesting that the DNA integrity test complemented the CA125 levels in detecting ovarian neoplasms. More importantly, the very high specificity of DNA integrity assay using the index of 0.59 as the cutoff is of particular interest given the fact that CA125 has a low specificity in detecting ovarian cancer because elevated CA125 levels are associated with a variety of benign diseases (14).

This article provides cogent evidence that increased DNA integrity index in plasma DNA can be detected in a substantial percentage of patients with potentially curable neoplastic diseases at an apparently high specificity. This assay system, in contrast to other more sophisticated techniques such as Digital single nucleotide polymorphism analysis, may offer a relatively simple and less expensive tool for cancer detection in the general population, although it requires to be demonstrated that increased DNA strand integrity is also observed in other types of carcinomas. DNA integrity analysis, as demonstrated in this study, may be applicable to other body fluids and in the differential diagnosis of malignant *versus* benign tumors. Before the DNA integrity analysis becomes a clinical test, several issues need to be addressed. In this study, the sensitivity of the DNA integrity analysis in diagnosing cancer was 62% at a specificity of 100%. Sensitivity might be improved by analyzing the 100- and 400-bp PCR products amplified from different chromosomal regions in which the region for 400 bp is frequently amplified, and the region for 100 bp is commonly deleted in cancer. As the integrity index is defined as the relative abundance of 400 bp *versus* that of 100 bp, this approach would likely generate a higher index number in cancer group and enhance the

validity of the assay. Results of DNA integrity analysis could also be combined with preexisting (*e.g.*, CA125) or emerging tumor-associated biomarkers in serum that have high sensitivity, yet their specificity is too low for them to be used as stand-alone screening tests for cancer (14–16). It should be noted that the specificity and the sensitivity obtained with the optimal cutoff value from this study might not be as high in other sets of patients and it will be important to assess the test performance with more representative control subjects of different age groups and disease entities including inflammatory, autoimmune, and infectious disease in prospective cohort studies.

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