Quantitative Analysis of Circulating Plasma DNA as a Tumor Marker in Thoracic Malignancies

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Background: Increased plasma DNA has been found in cancer patients and may have potential as a tumor marker. The objectives of this study were to develop a controlled, quantitative PCR (QPCR) assay to measure plasma DNA and then evaluate plasma DNA concentrations as a tumor marker in patients with thoracic malignancies.

Methods: We developed a QPCR assay for DNA, using the human β-actin gene. Plasma samples were analyzed from 58 patients with esophageal cancer (EC; 20 banked samples and 38 prospectively collected samples) and 25 patients with lung cancer (LC; all prospectively collected). Control groups consisting of 51 patients with gastroesophageal reflux disease (GERD; 23 banked samples and 28 prospectively collected) and 11 healthy volunteers were also analyzed.

Results: The assay had an experimental variability <4%. In our banked samples, the mean concentration of plasma DNA in EC was 819.0 (range, 46.2–4738.0) μg/L vs 432.0 (6.0–2888.0) μg/L in GERD (P = 0.02). However, the prospectively collected samples had lower DNA concentrations, and there was no difference between cancer patients and controls. The mean DNA concentration was 10.6 μg/L (range, 7.0–14.0 μg/L) in healthy volunteers and 10.5 μg/L (range, 4.0–23.5 μg/L) in GERD controls vs 13.0 μg/L (range, 4.5–46.5 μg/L) in EC and 14.6 μg/L (range, 3.0–30.0 μg/L) in LC.

Conclusions: Our data indicate that plasma DNA concentrations are of limited diagnostic value when samples are prospectively collected and uniformly handled. This is in contrast to previously published results.

Qualitative analysis of DNA may be needed if plasma nucleic acids are to be used as a diagnostic tool in cancer screening.

Diagnostic assays based on blood sample analysis are attractive because of the simplicity of sample collection. Accurate analysis of tumor markers in blood from cancer patients could have significant impact in facilitating the screening, diagnosis, and monitoring for disease recurrence after initial therapy. The development of a simple standardized assay for cancer detection in thoracic malignancies is appealing because of the complexity and low sensitivity of current screening procedures and the fact that many cases of lung and esophageal cancer present at late stages of the disease. More efficient and noninvasive screening methods could possibly impact on the outcomes of thoracic malignancies by improving patient compliance with screening procedures and by allowing earlier detection of the disease.

The presence of cell-free DNA circulating in plasma has been described in patients with malignant and inflammatory processes, and active release of tumor DNA into the circulation has been reported (1). Circulating DNA is in the form of oligonucleosomes and appears to be a general marker of the cell death process (2–6). Furthermore, several studies have identified DNA alterations in circulating plasma DNA of cancer patients that match genetic changes present in primary tumors (7–13). The low sensitivities reported for detection of DNA alterations in plasma, however, make these labor-intensive approaches less attractive for clinical applications.

A simpler approach is the measurement of total DNA in plasma. Leon and coworkers were the first to describe increased DNA concentrations in plasma as a marker of neoplasia (14, 15). Several studies have since described an increase in circulating DNA in several malignancies, and an association between increased plasma DNA and disease recurrence has been suggested (1, 10, 13, 16, 17). In patients with lung cancer, increased total DNA was found in patients with untreated cancer and in those with...
The supernatant (plasma) was separated into Eppendorf tubes without disturbing the cellular layer and centrifuged for an additional 10 min. Plasma was aspirated carefully, leaving behind any pelleted material in the tube. Plasma was then stored at −80 °C until used for analysis. DNA was isolated from 200-μL aliquots of plasma by use of the Blood DNA Mini Kit (Qiagen, Inc.), according to the manufacturer’s instructions for blood and body fluids, with one minor modification. We added 400 μL of H2O and 400 μL of Buffer AL1 to the sample because we found that this increased our DNA yields.

It has been reported that circulating plasma DNA is highly degraded (3); therefore, to determine whether our DNA isolation procedure would extract small DNA fragments, we added a 100- to 1500-bp human DNA ladder (Invitrogen) to a group of normal plasma samples before extraction. The extracted DNA was then analyzed on agarose gels to evaluate extraction of the different DNA fragments.

**Materials and Methods**

**Patients**

All blood samples were collected after informed consent was obtained for research protocols approved by the University of Pittsburgh Institutional Review Board. Plasma samples from 20 patients with esophageal cancer and 23 patients with gastroesophageal reflux disease (GERD) were obtained from the Division of Thoracic and Foregut Surgery Tissue Bank at the University of Pittsburgh Cancer Institute. In addition, blood samples were collected prospectively from patients undergoing surgical treatment for lung cancer (25 patients), esophageal cancer (38 patients), and GERD (28 patients) as well as from 11 healthy volunteers. The stage representation for the patients with non-small-cell lung cancer was 10 with stage I, 4 with stage II, 3 with stage III, 1 with stage IV, and 7 with unknown stage. The prospectively accrued esophageal cancer patients had predominantly adenocarcinomas (32 with adenocarcinoma and 6 with squamous cell carcinoma): 1 with stage 0, 6 with stage I, 12 with stage II, 15 with stage III, 1 with stage IV, and 3 with unknown stage. With the exception of the healthy volunteers, all samples were drawn from patients at the time of surgery but before any surgical incisions. Cancer patients had not received neoadjuvant radiotherapy or chemotherapy before surgery and blood sample collection.

**DNA Isolation from Plasma**

Banked plasma samples were originally collected in 10-mL EDTA tubes and processed within 1 h of collection by centrifugation at 1600g for 5 min. Plasma was separated and frozen at −80 °C in 1-mL aliquots. All prospective blood samples were collected in 10-mL EDTA tubes and processed in our laboratory within 1 h of collection. Fresh blood samples were centrifuged at 1600g for 10 min.

The objectives of this study were (a) to develop a controlled, reproducible assay for quantification of plasma DNA by real-time, quantitative PCR (QPCR). The objectives of this study were (a) to develop and validate a controlled, simple assay to quantify plasma DNA, and (b) to investigate the plasma DNA concentrations in esophageal and lung cancer as a possible marker for the presence of malignancy when compared with appropriate controls.

**Quantification of Plasma Total DNA by Real-time PCR**

PCR primers and fluorescent probes were designed by use of Primer Express Software (Applied Biosystems), and oligonucleotides were purchased from Integrated DNA Technologies. A 99-bp PCR amplicon was designed with the human β-actin genomic DNA sequence, and a second 72-bp amplicon was designed for the yeast *Saccharomyces cerevisiae* actin gene. The yeast amplicon was used to monitor DNA extraction efficiency as described below. For human β-actin, the primer and probe sequences were as follows: forward primer, 5’-CCA CAC TGT GCC CAT CTA CG-3’; reverse primer, 5’-AGG TTC AGG TAG TCA GTC AG-3’; probe, 5’TET-ATG CCC TCC CCC ATG CCA TCC TGC GT-TAMRA-3’ (where TET is ???, and TAMRA is 6-carboxyfluorescein) for *S. cerevisiae*, the sequences were as follows: forward primer, 5’TGG ATT CCG GTG ATG TT-3’; reverse primer, 5’TCA AAA TGG CTG GAG GTA GAG A-3’; probe, 5’FAM-CTC AGC TCG TTC CAA TIT ACG CTT GTT T-TAMRA-3’ (where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). QPCR was performed with an ABI 7700 Sequence Detection Instrument (Applied Biosystems), and DNA amounts were quantified from genomic DNA calibration curves.

PCR reactions were performed separately for human β-actin and yeast actin DNA quantification. QPCR reaction components were as follows: 1× PCR buffer A (Applied Biosystems), 300 nM each deoxynucleotide triphosphate, 3.5 mM MgCl2, 0.06 U/μL AmpliTaq Gold (Applied Biosystems), 500 nM each PCR primer, and 200 nM probe. DNA-free H2O was used to bring the reaction volume to 45 μL, and 5 μL of extracted DNA was then added. PCR cycling conditions for both amplicons were as follows: 95 °C for 12 min, followed by 95 °C for 15 s and 64 °C for 1 min, repeated for 40 cycles. All samples were processed in duplicate, and the mean value was used for...
quantification. The slope of the calibration curve was used to calculate PCR efficiency, and all correlation coefficients were >0.99.

ASSAY VALIDATION AND CONTROL FOR DNA EXTRACTION
One of the difficulties in quantifying DNA in plasma samples is the uncertainty of DNA extraction yield from each sample. To evaluate the DNA extraction efficiency from our samples, we added 25 ng of genomic DNA from the yeast S. cerevisiae (Invitrogen) to each plasma sample and quantified the DNA in a parallel QPCR reaction. The yeast DNA was used to monitor the efficiency of the extraction procedure for each sample and to identify any problems with DNA isolation on a sample-to-sample basis. There was no cross-species amplification observed between the yeast and human PCR amplicons.

To determine sources of variability in our DNA quantification assay, we performed a series of experiments using several concentrations (0–1200 g/L) of human genomic DNA added to plasma from healthy volunteers. In each case, 25 ng of yeast DNA was again added to determine the DNA extraction yield and the correlation with extraction yield of the human DNA (which can be calculated in these cases but is unknown in the actual patient samples). A total of eight human DNA concentrations were added to plasma samples and extracted in one experiment. This was repeated twice more on the same day (total of 24 extractions per day), and the whole process was performed on 3 separate days (total of 72 DNA extractions, 9 from each DNA concentration). All QPCR reactions were run in triplicate for each DNA extraction. Thus, these experiments were designed to test the interassay variability of DNA extraction performed on the same day as well as the interday extraction variability over a range of DNA concentrations expected to span that seen in clinical samples.

STATISTICAL ANALYSIS
Assay validation. Variance components of total variation in DNA concentrations were estimated with a restricted maximum likelihood from mixed linear models. Both human and yeast DNA concentrations were log-transformed to initially stabilize variances. The random effects estimated in the model were extraction number and day of extraction. Sources of variation were then partitioned into fixed concentrations of plasma DNA, day, extraction number, and random measurement error. Paired DNA concentrations from plasma and yeast were then standardized and plotted to examine trends and correlation.

Plasma sample analysis. The effects of sample preparation (fresh vs banked) and disease (esophageal cancer vs GERD) on plasma DNA concentrations were compared simultaneously by two-way ANOVA with interaction. Plasma DNA concentrations were compared between patients with benign and malignant processes by a one-way ANOVA test for fresh tissue samples and a t-test for banked plasma samples. These group comparisons were also conducted using adjusting plasma DNA concentrations in which the plasma DNA was standardized to the yeast DNA yield. Analyses for correlation of serum DNA concentrations with tumor stage were performed using a one-way ANOVA with log transformation of the DNA concentration data.

ASSAY VALIDATION
With our modified DNA extraction protocol, the yield of yeast DNA added to samples typically ranged from 40% to 60% (mean, 44%). The results of the assay validation demonstrated that the assay variability was <4.1% of the total variability, and the highest contributor to the variability was the plasma sample itself. The measurement of yeast DNA was useful to detect abnormal DNA extraction when the yeast DNA yield was <35% or >60% (4 occurrences in 96 assays; see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue1/). As a result, samples with DNA yields <35% or >60% were excluded and categorized as extraction or assay failure. Gel electrophoresis after extraction of samples with the added DNA ladder demonstrated efficient extraction of fragments as low as 100 bp (Fig. 2 in the online Data Supplement). PCR efficiencies for the human and yeast actin genes were 98% and 97%, respectively, based on linear correlation of the calibration curves.

PLASMA DNA EVALUATION IN BANKED SPECIMENS
A total of 20 banked plasma samples from patients with esophageal cancer were analyzed along with 23 banked samples from patients with GERD that were used as controls. The mean plasma DNA concentrations were 432.0 µg/L (range, 6.0–2888.0 µg/L) for patients with GERD and 819.0 µg/L (range, 46.2–4738.0 µg/L) for patients with esophageal cancer (Fig. 1). The DNA concentrations from esophageal cancer patients were significantly higher than the DNA concentrations from GERD patients (P = 0.0287, t-test).

PLASMA DNA EVALUATION IN PROSPECTIVELY COLLECTED SAMPLES
Eleven volunteers provided plasma for DNA analysis; the mean DNA concentrations in these plasma samples was 10.6 µg/L (range, 7.0–14.0 µg/L), which was substantially lower than the plasma DNA concentration observed with banked samples from GERD patients, and the range of values was unexpectedly small. We therefore collected and analyzed a series of prospective samples to explain this discrepancy and to explore the potential for plasma processing to alter DNA concentration.

Blood samples from 28 patients with GERD, 38 patients with esophageal cancer, and 25 patients with lung cancer were prospectively collected at the time of surgery but
before incision. The assay failure rate (extraction yield of yeast DNA >60% or <35%) was 4%. The mean plasma DNA concentration in patients with GERD was 10.5 μg/L (range, 4.0–23.5 μg/L) vs 13.0 (4.5–46.5) μg/L in esophageal cancer and 14.6 (3.0–30.0) μg/L in lung cancer (Fig. 2). There were no significant differences among healthy controls, GERD patients, or esophageal cancer patients (P = 0.2833, one-way ANOVA) or between healthy controls and lung cancer patients (P = 0.1754, t-test with Welch correction). The results were similar when we adjusted DNA concentrations for yeast yield (healthy vs GERD vs esophageal cancer, P = 0.4099; healthy vs lung cancer, P = 0.7897). When we used the highest detected DNA concentration in normal plasma as a cutoff (14.0 μg/L), the frequency of lung cancer patients with DNA concentrations above this cutoff was 48%. For esophageal cancer, 23.7% of patients had DNA concentrations above this cutoff, but so did 18% of GERD patients, the high-risk group for esophageal cancer screening. When the cutoff was increased above the highest concentration observed in GERD patients (23.0 μg/L; 100% specificity), only 10.5% of esophageal cancer patients had higher plasma DNA concentrations. Shown in Fig. 3 are ROC curves for comparisons of healthy volunteers vs esophageal cancer patients, GERD vs esophageal cancer patients, and healthy volunteers vs lung cancer patients. In each case, the lower bound of the 95% confidence interval for the area under the curve was <0.5, indicating that plasma DNA concentrations were unable to consistently differentiate between the two populations being compared. Comparison of plasma DNA concentrations with tumor stage revealed statistically significant differences (esophageal cancer, P = 0.0049; lung cancer, P = 0.0129) between stages. However, in esophageal cancer this was attributable to unusually high concentrations in stage I patients, and in lung cancer, it was attributable to lower concentrations in stage II patients. Given the small numbers of patients in each stage group, the inconsistency among cancer types, and the lack of differentiation between cases and controls, it is possible that this finding represents a type I statistical error.

EFFECT OF SAMPLE PREPARATION ON REPORTED RESULTS
To ascertain the impact of using banked plasma samples to measure DNA concentration, we analyzed the simultaneous effects of sample preparation and disease status on plasma DNA concentrations with a two-way ANOVA with interaction. This analysis used patients with esophageal cancer and GERD because these patients had both fresh and banked plasma samples for analysis. The results showed that the interaction between sample preparation and disease status was significant (P = 0.0400). This significant interaction supported our earlier findings of differential results for banked samples compared with
fresh samples and that sample preparation can affect the plasma DNA concentration.

Discussion
The ability to use molecular analysis of blood samples for cancer screening and surveillance is appealing because of the technical simplicity of sample collection and the potential for assay automation and large-scale population analysis. Several studies have reported increased concentrations of circulating DNA, up to 20-fold higher, in the plasma of cancer patients compared with controls, but these studies did not evaluate the impact and variability of their blood processing and DNA extraction procedures on the total DNA concentrations (13, 16, 18). This is an important limitation of previous studies because significant differences exist in observed DNA concentrations when the methods of sample processing and DNA quantification vary. For example, in one study evaluating DNA concentrations in lung cancer patients, the mean DNA concentration in cancer patients was 8-fold higher than in controls but was 13-fold lower than concentrations reported previously by the same group using a different DNA analysis method (19). It should be noted, however, that this difference may be accounted for by the fact that the PCR methods used in the second report measured only amplifiable DNA, whereas the methods used in the original report could detect all DNA fragments. With these issues in mind, we decided to control and monitor our methods for blood sample processing, plasma DNA isolation, and DNA quantification and to ascertain whether carefully controlling sample preparation and quantification altered the results and affected our conclusions.

We developed a simple and internally controlled assay for plasma DNA quantification using β-actin as the target sequence for quantitative PCR. The β-actin primers used in this study share high homology with actin pseudo-genes and with other actin family members. Given the genomic instability observed in lung and esophageal tumors (and in most other solid tumors), we believe that amplification from multiple genomic loci (vs use of a single copy gene as the target) is beneficial in this assay because loss or gain of any single locus is less likely to impact the plasma DNA quantification results. In addition, we monitored plasma processing and DNA isolation procedures by adding and quantifying the yield of yeast genomic DNA. Reproducibility experiments with our assay demonstrated low variability and consistent plasma DNA measurements. Our initial experiments analyzing banked plasma samples from patients with esophageal cancer and GERD demonstrated marked differences compared with results obtained from fresh samples processed in our laboratory. This led us to hypothesize that blood sample processing could artificially increase detected concentrations of DNA and alter results. One potential source of increased plasma DNA concentrations could be cell lysis between the time of blood draw and plasma separation. However, because all blood samples in this study were processed within 1 h of collection, and by the same technicians, we do not believe this was the source of the differences. Instead, we believe that the difference was caused by incomplete cell separation in the banked samples because only a single, short centrifugation was used compared with two centrifugation steps for the prospectively collected samples. Prospective collection of blood samples from patients with benign and malignant diseases, using identical plasma processing protocols, demonstrated no difference in total plasma DNA concentrations, a finding that contradicts recent reports in the literature. Our results suggest that once sample preparation is carefully controlled, measured plasma DNA concentrations are neither sensitive nor specific as a marker to differentiate between patients with GERD and those with esophageal cancer. Similarly, plasma DNA concentrations

Fig. 3. ROC curves for comparisons of healthy controls (Normal) and esophageal cancer (left panel), GERD and esophageal cancer (center panel), and healthy controls and lung cancer patients (right panel).

Areas under the curves: healthy controls vs esophageal cancer patients (left), 0.49 (95% confidence interval, 0.29–0.68); GERD patients vs esophageal cancer patients (center), 0.58 (95% confidence interval, 0.44–0.72); healthy controls vs lung cancer patients (right), 0.63 (95% confidence interval, 0.44–0.82).
did not distinguish between healthy controls and patients with lung cancer in our study, although the number of samples is relatively small. Therefore, despite the simplicity of sample collection and analysis of plasma DNA concentrations, we believe that a significant amount of variability can be introduced during sample processing, DNA isolation, and quantification, depending on the methods used. Future studies should be designed with these issues in mind.

In summary, we used a rigorously validated, reliable quantitative assay to measure DNA concentrations in plasma from patients with thoracic malignancies and those without cancer. Our data indicate that there is little diagnostic value of plasma DNA concentrations when samples are prospectively collected and uniformly handled. This is in direct contrast to previously published results. A well-controlled and standardized approach, along with a larger patient series and more appropriate control group for lung cancer, are needed to definitively determine the potential usefulness of DNA concentrations in plasma of cancer patients as a diagnostic tool. If our data are shown to be correct, qualitative analysis of DNA alterations specific to the tumor may be needed to enable use of plasma DNA as a diagnostic tool in cancer screening.

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