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Plasma DNA as a Prognostic Marker in Trauma Patients

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Background: **Recently, much interest has developed in the potential use of plasma DNA as a diagnostic and monitoring tool. We hypothesized that plasma DNA is increased in patients with trauma and may be prognostic in such patients.**

Methods: **We studied 84 patients who had sustained an acute blunt traumatic injury. We measured plasma DNA by a real-time quantitative PCR assay for the *β-globin* gene. Blood samples were collected at a median time of 60 min following injury. Blood samples were also obtained from 27 control subjects.**

Results: **The median plasma DNA concentrations in the control, minor/moderate trauma (Injury Severity Score <16; n = 47), and major trauma (Injury Severity Score ≥16; n = 37) groups were 3154 kilogenome-equivalents/L, 13 818 kilogenome-equivalents/L, and 181 303 kilogenome-equivalents/L, respectively. Plasma DNA concentrations in patients with adverse outcomes, including acute lung injury, acute respiratory distress syndrome, and death, had 11.6- to 12-fold higher plasma DNA concentrations than those who did not develop these complications. At a cutoff of 232 719 kilogenome-equivalents/L, the sensitivities of plasma DNA analysis for the prediction of acute lung injury, acute respiratory distress syndrome, and death were 100% (95% confidence interval, 100–100%), 100% (95% confidence interval, 100–100%), and 78% (95% confidence interval, 40–97%), respectively. The respective specificities were 81% (95% confidence interval, 71–89%), 80% (95% confidence interval, 70–88%), and 82% (95% confidence interval, 71–90%).**

Conclusions: **Plasma DNA is increased after trauma and may be a potentially valuable prognostic marker for these patients.**

Posttraumatic organ failure is common after severe injury and is an important cause of mortality *(1)*. The current hypothesis is that a systemic inflammatory response syndrome follows severe trauma and that the processes eventually lead to organ failure, including acute lung injury (ALI)[3] and acute respiratory distress syndrome (ARDS) *(1)*. Recently, models have been proposed for the prediction of multiple organ failure as early as 12 h after injury *(1)*. It would be useful to develop new assays that may allow risk stratification to be made even earlier.

Recently, much interest has developed in the use of circulating cell-free DNA in the plasma for clinical diagnosis *(2–4)*. In particular, circulating tumor-, fetal-, and graft-derived DNA has been detected in the plasma of cancer patients, pregnant women, and organ transplantation recipients, respectively *(2–5)*. Although the mechanisms by which cell-free DNA is liberated into the circulation of human subjects are unknown, one possibility is that DNA is released following cell death *(6, 7)*. Along this line of reasoning, we hypothesized that DNA may be liberated from body tissues into the plasma after trauma and that plasma DNA may be a potentially useful prognostic tool.

Materials and Methods

PATIENTS

The study was a secondary analysis of archival plasma samples obtained from 84 patients who had sustained an acute blunt traumatic injury requiring admission to the Emergency Resuscitation Room at the Prince of Wales Hospital. These subjects were recruited between April 1996 and September 1999, with informed consent from either the patient or a relative. The project was approved by the Research Ethics Committee of the Chinese University of Hong Kong. Patients who were less than 12 years of age, pregnant, or were admitted because of drowning,

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[3] Nonstandard abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; AIS, Abbreviated Injury Score; ISS, Injury Severity Score; and CI, confidence interval.

thermal injury, hypothermia, and acute drug overdose were excluded. Two of the 84 patients had a systolic blood pressure <90 mmHg on admission. The Abbreviated Injury Score (AIS) for individual bodily regions was determined as described *(8)*. The total extent of the injury was calculated using an objective Injury Severity Score (ISS) at the time of discharge or death, or at 28 days if the patient was still hospitalized *(9)*. The definitions of ALI and ARDS were as described previously *(10)*. Peripheral venous blood (3 mL) was collected from each patient into heparin-containing tubes after patients were admitted to the resuscitation room. The median time between injury and blood sampling was 60 min (interquartile range, 50–100 min). At the time of blood sampling, 39 patients had received between 500 and 1000 mL of intravenous crystalloids; the rest had received <500 mL. None of the patients had colloids administered before the time of blood sampling. The hematocrit was measured on admission as part of the diagnostic workup for each patient. Plasma creatine kinase and lactate dehydrogenase concentrations were determined using a Dade Behring Dimension® clinical chemistry system. The ISS values, AIS values, outcomes, and other clinical parameters were "blinded" to the researchers carrying out the subsequent plasma DNA analysis. Control blood samples were also obtained from 27 healthy volunteers.

PROCESSING OF BLOOD SAMPLES

Blood samples were centrifuged at 3000*g*, and plasma samples were carefully removed from blood collection tubes and transferred into plain polypropylene tubes. Great care was taken to ensure that the cell pellet was undisturbed when plasma samples were removed. The samples were stored at −80 or −20 °C until further processing.

DNA EXTRACTION FROM PLASMA SAMPLES

DNA from plasma samples was extracted using a QIAamp Blood Kit (Qiagen) using the "blood and body fluid protocol" as recommended by the manufacturer *(2)*. A 400- to 800-μL plasma sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentration *(11)*.

REAL-TIME QUANTITATIVE PCR

The theoretical and practical aspects of real-time quantitative PCR have been described in detail elsewhere *(11–13)*. Real-time quantitative PCR analysis was performed using a PE Applied Biosystems 7700 Sequence Detector. The amplification and product reporting system used was based on the 5′ nuclease assay *(14)* (the TaqMan assay as marketed by Perkin-Elmer), in which the liberation of a fluorescent reporter is coupled to the amplification reaction. A typical analysis (including blood centrifugation and DNA extraction, followed by real-time PCR) took ~3 h.

Plasma DNA was measured using a real-time quantitative PCR assay for the *β-globin* gene, which is present in all nucleated cells of the body *(11)*. The *β-globin* TaqMan system consisted of the amplification primers beta-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′), beta-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′), and a dual-labeled fluorescent TaqMan probe, beta-globin-402T [5′-(FAM)AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3′] *(11)*. The TaqMan probe contained a 3′-blocking phosphate group to prevent probe extension during PCR.

When applied to serial dilutions of human genomic DNA, this real-time *β-globin* quantitative PCR assay was able to detect the DNA equivalent from a single cell. The imprecision of this system has been reported previously, with a CV of the threshold cycle of 1.1% *(11)*.

The expression of quantitative results was as described previously *(11)*. The unit "kilogenome-equivalents/L" was preferred to "genome-equivalents/mL" in accordance with SI unit convention. One genome-equivalent was defined as the amount of a particular target sequence contained in a single diploid human cell.

STATISTICAL ANALYSIS

Descriptive statistics and nonparametric data comparison tests were carried out using the SigmaStat 2.0 software. ROC curve analysis was carried out using the MedCalc 5.0 software. The data files are available as a supplement from the *Clinical Chemistry* Web site. The file can be accessed by a link from the on-line Table of Contents (http://www.clinchem.org/content/vol46/issue3).

Results

PLASMA DNA AND TRAUMA SEVERITY

The median plasma DNA concentrations in the control, minor/moderate trauma (ISS <16; n = 47), and major trauma (ISS ≥16; n = 37) groups were 3154 kilogenome-equivalents/L, 13 818 kilogenome-equivalents/L, and 181 303 kilogenome-equivalents/L, respectively (Fig. 1). The differences between these groups were highly significant (Kruskal–Wallis test, $P < 0.001$). Pairwise comparisons using the Dunn method revealed significant difference between each constituent pair within these three groups ($P < 0.05$). The direct comparison of individual ISS values with the corresponding plasma DNA concentration revealed a positive correlation, both including (Spearman rank-order correlation, $P < 0.0005$; $r = 0.756$) or excluding (Spearman rank-order correlation, $P < 0.0005$; $r = 0.617$) the control group (ISS = 0). Significant correlations were observed between plasma DNA concentrations and the AIS values for the head and neck region (Spearman rank-order correlation, $P < 0.0001$; $r = 0.440$), the thorax (Spearman rank-order correlation, $P < 0.001$; $r = 0.520$), and the abdomen (Spearman rank-order correlation, $P = 0.0002$; $r = 0.418$). No significant correlation was observed between plasma DNA concentrations and

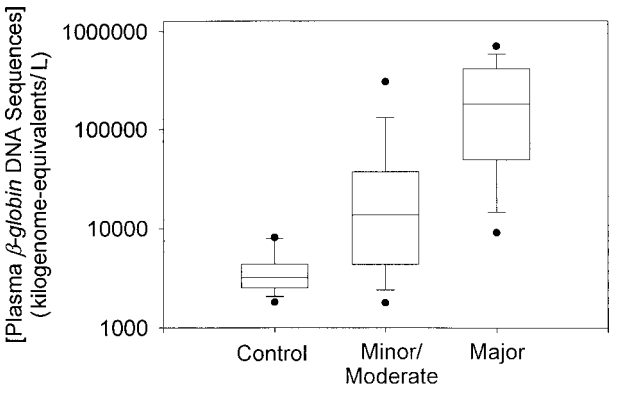

Fig. 1. Plasma DNA concentrations in control subjects and trauma patients.

The subject categories are shown on the *x-axis*. Plasma DNA concentrations (kilogenome-equivalents/L) as determined by real-time quantitative PCR for the *β-globin* gene are plotted on the *y-axis* (common logarithmic scale). The *lines* inside the boxes denote the medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicates the 5th and 95th percentiles.

the AIS values for the extremities (Spearman rank-order correlation, $P = 0.136$; $r = 0.165$).

No significant correlation was observed between the plasma DNA concentrations and the admission hematocrit (Spearman rank-order correlation, $P = 0.311$; $r = -0.111$). The stratification of the patients into those who had received <500 mL of intravenous crystalloids and those who had received between 500 and 1000 mL by the time of blood sampling revealed no significant difference (Mann–Whitney rank-sum test, $P = 0.25$).

With regard to other biochemical markers of tissue injury, positive correlations were observed between plasma DNA and plasma creatine kinase (Spearman rank-order correlation, $P < 0.0005$; $r = 0.492$) and lactate dehydrogenase (Spearman rank-order correlation, $P < 0.0005$; $r = 0.584$).

PLASMA DNA AND CLINICAL OUTCOME

To determine whether plasma DNA analysis may be used as a prognostic indicator, plasma DNA concentrations among groups with different outcomes were compared. Outcomes that were studied included the development of ALI (n = 6), ARDS (n = 5), and death (n = 9). The plasma DNA concentrations in patients stratified according to each of these outcomes are shown in Table 1, which indicates that patients with adverse outcomes had significantly higher plasma DNA concentrations (11.6- to 12-fold) than those who did not develop these complications.

Table 1. Plasma DNA concentrations in trauma patients stratified according to outcome, including ALI, ARDS, and death.

	Median plasma DNA concentration, kilogenome-equivalents/L		
Complication	With complication	Without complication	Mann-Whitney rank-sum test
ALI	398 225	33 176	$P = 0.002$
ARDS	403 999	34 682	$P = 0.005$
Death	315 122	26 244	$P = 0.002$

ROC CURVE ANALYSIS

The ROC curve analysis for the use of plasma DNA measurement for predicting ALI, ARDS, and death is shown in Fig. 2. The areas under the ROC curves for ALI, ARDS, and death are, respectively, 0.882 [SE = 0.091; 95% confidence interval (CI), 0.794–0.942], 0.877 (SE = 0.102; 95% CI, 0.788–0.939), and 0.822 (SE = 0.088; 95% CI, 0.724–0.896).

For each of these adverse outcomes, a plasma DNA of 232 719 kilogenome-equivalents/L corresponded to the highest value for the sum of sensitivities and specificities. Using this cutoff, the sensitivities of plasma DNA analysis for the prediction of ALI, ARDS, and death were 100% (95% CI, 100–100%), 100% (95% CI, 100–100%), and 78% (95% CI, 40–97%), respectively. The respective specificities were 81% (95% CI, 71–89%), 80% (95% CI, 70–88%), and 82% (95% CI, 71–90%).

Discussion

This study shows that circulating plasma DNA in the peripheral blood of trauma patients increases early after injury and that these increases are related to the development of posttraumatic complications. We have designed the study such that analysis was performed on a single sample obtained at a median time of 1 h after trauma, when the physiologic status of the patient was relatively uncomplicated by multiple therapeutic maneuvers, apart from a modest amount (up to 1 L) of intravenous crystalloids.

The mechanisms by which circulating DNA is increased after trauma are unclear at present. Theoretically, such an increase may be the result of increased liberation after cell death, or decreased efficiency of DNA clearance mechanisms after injury. In the former case, the cell types (e.g., myocytes and endothelial cells) primarily responsible for DNA liberation remain to be elucidated. It is likely that cell death as a direct result of trauma, or secondarily via hemodynamic compromise as a result of blood loss may lead to DNA liberation into the circulation. This possibility is supported by the positive correlation between plasma DNA concentrations and other biochemical markers for tissue injury, namely, creatine kinase and lactate dehydrogenase. The clearance mechanisms for circulating DNA are poorly understood at present, but it is possible that direct damage or hemodynamic compromise of the organ systems responsible for circulating DNA clearance may also lead to increased plasma DNA.

The comparison between plasma DNA concentrations and the AIS values obtained for the head and neck, thoracic, and abdominal regions revealed a positive and significant correlation between these parameters. However, there was no significant correlation between plasma DNA concentrations and the AIS values for the extremi-

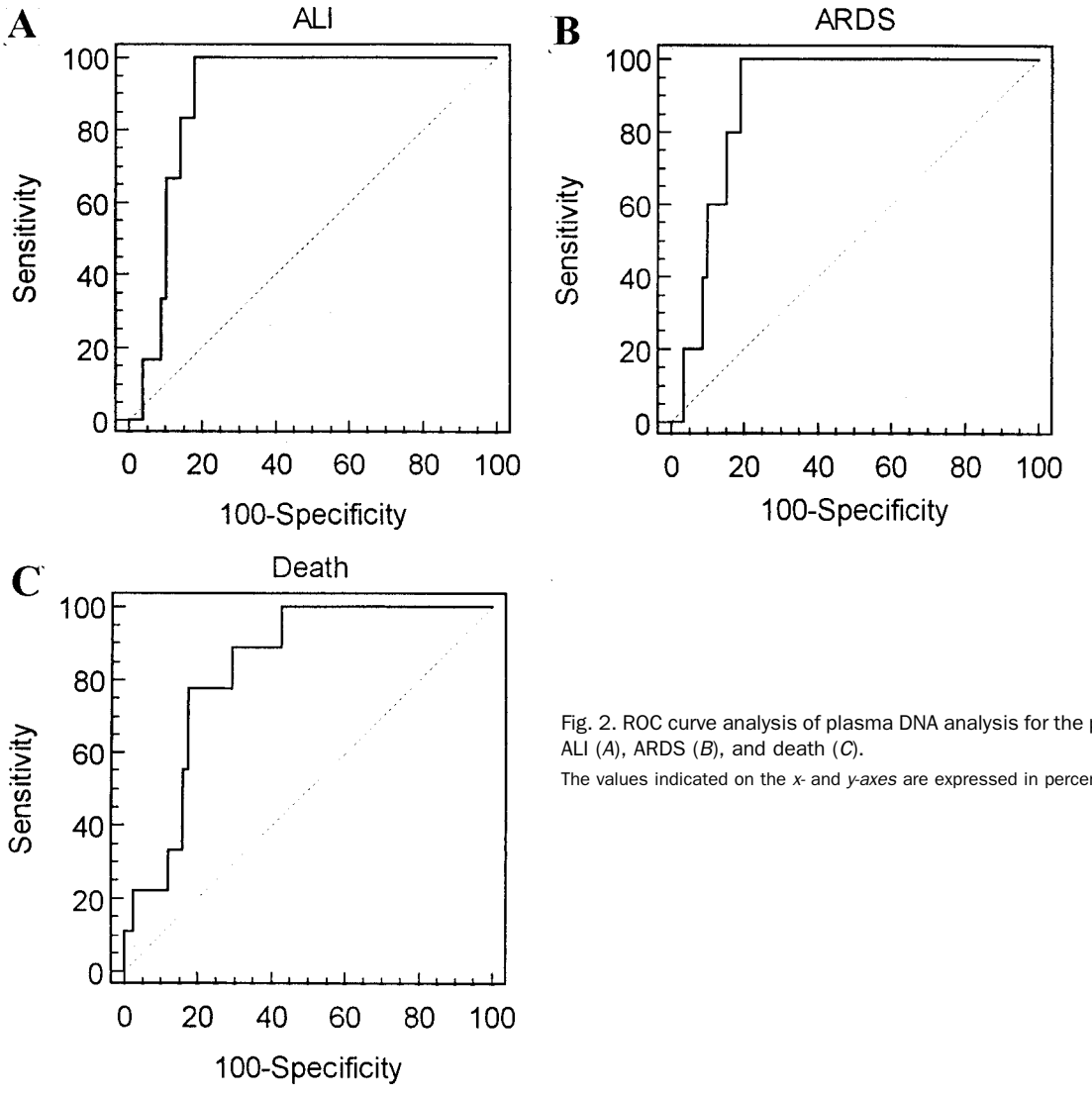

Fig. 2. ROC curve analysis of plasma DNA analysis for the prediction of ALI (*A*), ARDS (*B*), and death (*C*).
The values indicated on the *x*- and *y-axes* are expressed in percentages.

ties. One interpretation of these results is that the extremities are not the predominant organ systems responsible for the increase in circulating DNA in trauma patients. On the other hand, the positive correlation between plasma DNA concentrations and the AIS values for the head and neck, thoracic, and abdominal regions support the hypothesis that organ systems in these anatomic regions are responsible for the increased plasma DNA after trauma. Candidate organ systems include the liver, spleen, and kidneys, which may have a role in both liberating and clearing circulating DNA. For example, evidence of the roles of these organs in circulating DNA clearance has already been demonstrated in animal experiments *(15, 16)*.

The current study was focused on blunt trauma; therefore, it would be interesting to investigate whether other types of tissue insults, e.g., ischemic, infective, toxic, thermal, or radiation injuries, may be associated with cell-free DNA liberation into the circulation. These future studies may open up the possibilities that plasma DNA may be used as a general marker for monitoring diverse types of tissue damage. Further work would be required to elucidate the physicochemical characteristics of trauma-associated circulating DNA and to determine whether this type of plasma DNA differs in any fundamental aspects from other circulating DNA species, e.g., fetal DNA *(4)* and tumor-derived DNA *(2, 3)*.

Clinically, our results suggest that plasma DNA may be a potentially useful marker for monitoring patients after trauma. The plasma concentrations of DNA were correlated with the severity of injury and with outcome. The diagnostic cutoff value of 232 719 kilogenome-equivalents/L established in this pilot study for prediction of outcome may need to be refined when results from larger scale clinical trials become available. Because these data were obtained using a single blood sample taken from the patients at a median time of 60 min after injury, plasma DNA analysis represents an advance over current predic-

tion rules that are applicable from 12 h onward *(1)*. The ability for rapid risk stratification may allow clinicians to make a more rational decision with regard to the type of therapy that is most appropriate for a particular patient.

Recent data indicate that human plasma DNA possesses a short half-life in the circulation *(17)*. The rapid kinetics of plasma DNA suggest that circulating DNA analysis may be useful in monitoring the clinical progress of trauma patients. It is possible that evaluation of the patterns of plasma DNA variation may further enhance the diagnostic accuracy of this type of analysis for predicting adverse clinical outcomes in these patients. There thus is a necessity for future studies to focus on obtaining sequential data from trauma patients. Plasma DNA analysis may also be useful in studying the patients' response to treatment, especially in trials aimed at testing new therapeutic modalities for these patients.

Our current protocol allows the provision of plasma DNA results within 3 h of blood sampling. This rapidity is achieved by the use of a simple column-based DNA extraction method and the utilization of real-time PCR analysis that does not require any postamplification manipulation. With the recent development of rapid capillary-based instrumentation for quantitative PCR analysis *(18)*, this time could be further reduced to 90 min, thus further enhancing the potential clinical usefulness of this assay in accident and emergency departments.

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