species is fragmented, cell-free DNA the size of nucleosomal DNA, mostly derived from cells undergoing apoptosis. As discussed earlier, the source of mutated \(k\)-ras DNA in the circulation could be apoptotic cells. Thus, mutated \(k\)-ras DNA should be enriched in the fragmented, nucleosomal DNA species relative to the large species found near the sample wells of the gel. Therefore, the DNA isolation method that favors isolation of fragmented DNA should produce a better ratio of \(k\)-ras-mutated DNA to wild-type DNA. As shown in Table 1, mutated \(k\)-ras was readily detected in the DNA isolated by the G/R method but was detected less frequently in DNA isolated by the Qiagen method from the specimen from the same patient, although more DNA was recovered by the Qiagen method. Another possible contributor to this discrepancy in assay results is the nature of the RE-PCR assay. This is a PCR-based assay, and with more wild-type genomic DNA in the assay, the sensitivity of detection of the mutated DNA is reduced because of interference with the PCR.

This study clearly demonstrates that the method chosen for isolation of DNA can contribute significantly to the outcome of mutation detection (in this case, \(k\)-ras mutations). It has been suggested that malignant, benign, and even preneoplastic cells often proliferate at abnormal rates, accompanied by an increase in apoptotic cell death (9–11), and that this small, fragmented DNA may accumulate in the circulation. These results further suggest that to enhance assay sensitivity for detection of somatic mutations or epigenetic modifications in circulating DNA for cancer detection, monitoring, or prognosis, a method that can preferentially isolate small DNA should be used.

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Plasma Mitochondrial DNA Concentrations after Trauma, Nicole Y.L. Lam, 1 Timothy H. Rainer, 1 Rossa W.K. Chiu, 2 Gavin M. Joynt, 3 and Y.M. Dennis Lo 4 (1 Accident and Emergency Medicine Academic Unit, 2 Department of Chemical Pathology, and 3 Department of Anaesthesia and Intensive Care, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR; 4 address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Room 38023, 1/F Clinical Sciences Bldg., 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong SAR; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

Both qualitative and quantitative studies on circulating DNA (1–3) and RNA (4–6) in plasma and serum have shown their usefulness in clinical diagnosis and prognosis. In addition to DNA and RNA, there is another species of nucleic acids, mitochondrial DNA (mtDNA), that is usually confined to and functions in the mitochondria. Mutations in the mitochondrial genome are associated with various diseases (7). A recent study reported the presence of a known mtDNA mutation in the serum and plasma of patients with type II diabetes mellitus (8). Mutant mtDNA has been detected in plasma of patients with hepatocellular carcinoma (9). However, unlike circulating DNA, there is a paucity of quantitative data on circulating mtDNA. The reason might be partly attributable to the presence of pseudogenes in the nuclear genome that may lead to coamplification of these nuclear copies of mtDNA (10). To overcome this problem, a mtDNA-specific real-time quantitative PCR assay has recently been developed (11).

Significant increases in circulating DNA in the plasma of trauma patients have been reported and found to be useful in posttraumatic diagnosis (12). One hypothesis for the increased plasma DNA concentration after trauma is that cell-free DNA is released from damaged tissues to the nearby bloodstream. We therefore proposed that
mtDNA in plasma may also be increased after trauma. In this study, we aimed to investigate and compare changes in cell-free mtDNA and nuclear DNA concentration in plasma after trauma, their relationships with injury severity, and the potential prognostic value of plasma mtDNA concentrations.

Thirty-eight patients who had sustained an acute blunt traumatic injury and had been admitted to the resuscitation room at the Prince of Wales Hospital between July 2000 and October 2002 were recruited. Inclusion criteria included time from injury to admission of <4 h and age >13 years. Exclusion criteria were pregnancy, drowning, hanging, thermal injury, hypothermia, and acute drug overdose. Injury severity was calculated using an objective Injury Severity Score (ISS) (13) at the time of discharge or death, or at 28 days if the patient was still hospitalized. Twenty-eight patients had minor/moderate injury (ISS <16), and 10 patients were severely injured (ISS >16). Two of the 38 patients died 12 h and 1 day after their injuries, respectively. Peripheral blood was collected into EDTA-containing tubes (Vacuette; Greiner) within minutes of the patients’ arrival at the hospital. Median time from injury to blood sampling was 63 min. Blood samples were centrifuged at 1600g for 10 min (Labofuge400R; Heraeus), and the plasma was removed, placed in clean plain polypropylene tubes, and stored at temperatures below −80 °C pending further processing. Blood samples from 10 healthy controls were also collected and processed in the same way.

mtDNA and nuclear DNA in the plasma were extracted together by use of a QIAamp Blood Kit (Qiagen GmbH). We used 200 μL of plasma per sample for the extraction, and the extracted mtDNA and nuclear DNA mixture was eluted with 50 μL of doubly distilled water. The amounts of mtDNA and DNA in the mixture were then measured in two separate real-time quantitative PCR assays. Real-time quantitative PCR (14) was performed on an Applied Biosystems 7700 Sequence Detector (Applied Biosystems). The theoretical and practical aspects have been described previously (14, 15).

Plasma mtDNA quantification was based on the recently established mtDNA-specific assay, in which serially diluted cloned plasmid DNA was used in the calibration curve (11). The primer sequences Mit 3130F (5'-AGG ACA AGA GAA ATA AGG CC-3') and Mit 3301R (5'-TAA GAA GAG GAA TTG AAC CTC TGA CIG TAA-3') have been reported previously by Parfait et al. (10). The TaqMan probe sequence was Mit 3153T (5'-FAM-TTC ACA AAG CCC CTC CCC CGG TAA ATG A-TAMRA-3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine), designed using Primer Express software, Ver. 2.0 (Applied Biosystems). The assay was linear over five orders of magnitude, and it could detect one copy of mtDNA (11). The assay was also tested to be mtDNA specific by decreases in mtDNA over five successive generations in the 143B cell line; no mtDNA was detectable from the sixth generation onward (11).

mtDNA concentrations are expressed in copies/mL of plasma.

The number of copies of β-globin DNA, which is present in all nucleated cells of the body, was used for the quantification of plasma nuclear DNA in this study. The actual conditions for the measurement have been well established and applied in many studies (12, 16, 17). The expression of quantitative results was genome-equivalents/mL. One genome-equivalent was defined as the amount of a target sequence contained in a single diploid human cell. Both the mtDNA and nuclear DNA concentrations in the plasma samples collected from each individual were assessed. We used 5 μL of extracted plasma DNA for each reaction, and the final reaction volume was 50 μL for both the mtDNA and β-globin DNA real-time quantitative PCR assays. Each sample was run in duplicate, and the mean was used for further calculations.

The median concentrations of mtDNA and β-globin DNA were both significantly higher in trauma patients than in the controls. The median plasma mtDNA concentration in trauma patients was 8 586 300 copies/mL (vs 1 607 000 copies/mL in the controls; P = 0.003, Mann–Whitney test), whereas the median β-globin DNA concentration in trauma patients was 6 100 genome-equivalents/mL (vs 6 80 genome-equivalents/mL in the control group; P <0.0001, Mann–Whitney test). The concentrations of mtDNA were much higher than that of β-globin DNA in both trauma patients and controls because mtDNA exists as multiple copies per cell. In contrast, β-globin DNA is a nuclear gene that exists only in pairs. Nevertheless, plasma mtDNA and β-globin DNA concentrations were positively correlated (Spearman rank correlation, r = 0.35; P = 0.016).

The results shown in Fig. 1A indicate a positive correlation between plasma mtDNA and injury severity (P = 0.0002, Kruskal–Wallis test). The median plasma mtDNA concentrations in the severely injured subgroup and the minor/moderate subgroup were 15 105 000 and 7 115 000 copies/mL, respectively. The difference between these two groups was highly significant (P = 0.002, Mann–Whitney test). The results for β-globin DNA were similar: positive correlation with injury severity (P <0.0001, Kruskal–Wallis test) and a statistically significant difference between the two trauma subgroups (P = 0.005, Mann–Whitney test). Comparison of individual ISS values with corresponding plasma mtDNA and β-globin DNA concentrations revealed positive correlations (Spearman rank correlation, r = 0.49, P = 0.0009; and r = 0.78, P <0.0001, respectively). Moreover, the median plasma mtDNA concentration in trauma patients who later died was higher than that of the survivors (340 000 000 vs 8 325 000 copies/mL) and was statistically significant (P = 0.02, Mann–Whitney test; Fig. 1B). Similarly, the median plasma β-globin DNA concentration was significantly higher in those patients who died than in those who survived (384 000 vs 5700 genome-equivalents/mL; P = 0.03, Mann–Whitney test).

In this study, we assessed the circulating mtDNA concentrations in the plasma of trauma patients. This is
the first quantitative study of plasma mtDNA in a clinical scenario. Plasma mtDNA was increased shortly after trauma, was increased with injury severity, and was the highest in patients who died. Because the pattern of plasma mtDNA was similar to that of \( \beta \)-globin DNA, both may be released from the same tissues of origin and by similar mechanisms. This requires further investigation. Moreover, the number of patients in this preliminary study was limited, especially in the “severe” and “died” groups. Further confirmation with larger and better distributed patient groups is necessary.

Recently, two consecutive centrifugations and filtration have been recommended in studies on circulating mtDNA to rid plasma of residual blood cells \((11, 18)\). Future work should use this regimen to assure maximum purity of the isolated mtDNA.
A previous study indicated that the sequential hourly and daily changes in plasma β-globin DNA concentrations after trauma were different between mildly and severely injured patients and between patients with and without organ failure (19). The differences may also be useful for monitoring the progress of post-trauma complications (19). Further studies will be required to investigate the dynamic changes in plasma mtDNA and may provide additional and valuable information on trauma prognosis. In addition, quantitative plasma mtDNA analysis may also be useful in the prognosis and monitoring of other diseases, such as cancer, and the high concentration of mtDNA in plasma may allow its use for noninvasive study of mtDNA-related diseases.

References


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Testing for Anti-Human Transglutaminase Antibodies in Saliva Is Not Useful for Diagnosis of Celiac Disease, Valentina Baldas,1 Alberto Tommasini,1 Daniela Santon,1 Tarcisio Not,1 Tania Gerarduzzi,1 Gabriella Clarich,1 Daniele Sblattero,2 Roberto Marzari,3 Fiorella Florian,3 Stefano Martellossi,1 and Alessandro Ventura1 (1 Department of Reproductive and Development Science and Service of Dentistry, I.R.C.C.S. “Burlo Garofolo”, and 2 Department of Biology, University of Trieste, Trieste, Italy; 3 address correspondence to this author at: Clinica Pediatrica, Istituto per l’Infanzia “Burlo Garofolo”, Via dell’Istria 65/1, IT-34100 Trieste, Italy; fax 39-040-3785-210, e-mail not@burlo.istriete.it)

Undetected celiac disease (CD) can cause serious complications and excessive mortality (1–3). Noninvasive tests could be useful in physicians’ offices to identify patients for intestinal biopsies (4). Serum IgA anti-endomysium (AEA) and anti-human transglutaminase (anti-htTG) antibody assays are the most widely used laboratory tests for CD (5). Autoantibody determinations have been simplified by the use of rapid testing (6,7), but when saliva specimens were evaluated for AEA testing, they were found to be not suitable (8).

To evaluate the potential utility of saliva as a sample for anti-htTG testing, we compared salivary IgA anti-htTG (measured by ELISA with a dot-blot method and an AEA test) with serum IgA anti-htTG antibodies (measured by ELISA and an AEA test) in patients with CD and in controls. Salivary IgA anti-htTG activity was analyzed for the IgA secretory chain by use of a monoclonal antibody. The recognition patterns of the saliva samples were compared with those for the serum samples and with the pattern for a gut-derived IgA monoclonal antibody to hTg from one CD patient, using two transglutaminase deletion mutants, one recognized and one not by the patients’ sera (9).

We studied 47 untreated CD patients (29 females and 18 males; median age, 19 years; range, 2–52 years) diagnosed in 2002–2003 according to ESPGHAN criteria (10) and 47 patients with celiac disease (26 females and 21 males; median age, 19 years; range, 4–50 years) on a gluten-free diet (GFD) for at least 12 months. We recruited 51 healthy controls with no history of gastrointestinal or autoimmune diseases (25 females and 26 males; median age, 14 years; range, 6–50 years) and identified samples from 49 individuals suffering from various gastrointestinal diseases (18 with Crohn disease, 10 with ulcerative colitis, 10 with milk allergy, 6 with acute diarrhea, 5 with gastroesophageal reflux). Of these 49 individuals, 11 had undergone intestinal biopsies showing nonceliac mucosa.

Serum IgA anti-htTG antibodies were measured by ELISA (11) in plates coated with hTg (1 μg/well). Serum samples diluted 1:100 were incubated for 1 h, and then 1 h more with anti-human IgA. Absorbance was read at 405 nm. The results were expressed as percentages of the positive control serum. The cutoff for IgA was set at <16%, >2 SD above the mean of 400 healthy individuals. Serum IgA AEA concentrations were measured by indi-