Assessment of Deoxyribonuclease Activity in Biological Samples by a Fluorescence Detection-Based Method

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ABSTRACT

Objective: To develop a speedy, sensitive, low-cost fluorescence detection assay for assessing deoxyribonuclease activity in biological samples.

Methods: Deoxyribonuclease activity was measured in serum samples of 15 patients with lung cancer and 15 healthy individuals. The assay is based on hydrolysis of fluorescently labeled DNA into fragments that are subsequently measured by capillary electrophoresis.

Results: Mean (SD) reduction in signal intensity compared with the negative control reaction was 0.04 (0.09) for the group of patients with lung cancer and 0.01 (0.07) for the control group. The difference in signal intensity between the 2 groups was not statistically significant.

Conclusion: The main advantages of the fluorescence detection assay are its high sensitivity, the short time required for it to obtain results, and its low cost. The method provides a useful alternative to the conventional enzyme-linked immunosorbent assay (ELISA) and can potentially be applied to a variety of studies, especially when assessment of deoxyribonuclease activity in large sample series is required.

Keywords: deoxyribonuclease, fluorescence, capillary electrophoresis, serum

Exogenous and endogenous DNA is degraded in blood plasma due to the presence of several enzymes that display deoxyribonuclease-like activity. The major DNA hydrolyzing enzyme in the blood, responsible for almost 90% of its hydrolytic activity, is deoxyribonuclease I (DNase I).[1] Acid DNase II, phosphodiesterase I, DNA-hydrolyzing autoantibodies, and lactoferin also digest DNA.[1-4] The inhibitors of DNases, mainly actin, are also found in the bloodstream.[5] Therefore, DNase activity in the serum is dependent on the concentrations and composition of DNA-degrading enzymes and factors influencing the activity of those enzymes.

Plasma DNase activity protects host cells from exogenous DNA and degrades endogenous DNA released into the blood after cellular disruption.[6] Alterations in DNase activity have been observed in disease. High serum DNase activity has been reported in patients with breast cancer and oral cancer, whereas patients with malignant lymphomas, stomach and colon cancers, systematic lupus erythematosus, and pancreatic cancer had lower DNase activity compared to healthy individuals.[7-12] These results suggest that variations in DNase activity could be a useful marker for monitoring different human malignant neoplasms. Thus, methods enabling measurement of DNase activity in biological fluids are important for investigating these associations.

Methods for measuring DNase activity include enzyme-linked immunosorbent assay (ELISA), colorimetric assay, radial immunodifusion (RID) assay, and radial enzyme diffusion (RED) assay.[1,11,13,14] All these assays are relatively expensive, and results are time-consuming to obtain; therefore, they are not suitable

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Abbreviations

DNase, deoxyribonuclease; ELISA, enzyme-linked immunosorbent assay; RID, radio immunodifusion; RED, radial enzyme diffusion; PCR, polymerase chain reaction; bp, base pair; dNTP, deoxynucleoside triphosphate

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for processing a large number of samples. The aim of this study was to develop a novel method for DNase activity assessment in serum samples that would be characterized by high reproducibility, high sensitivity, and relatively low cost, which could provide an alternative to, or supplement, currently available methods.

Materials and Methods

Samples

This study was performed on 30 serum samples collected from subjects at University Clinical Center Zvezdara in Belgrade, Serbia; 15 of these samples were from patients diagnosed with primary lung cancer and 15 were from healthy individuals used as the control group. The patients with lung cancer were composed of 12 men and 3 women, with a mean (SD) age of 59.5 (7.6) years. Most of the patients were heavy smokers (93%), with a mean of 40 pack/years smoking history. More than half of all patients (60%) with primary lung cancer had extrathoracic metastases (in the liver, spleen, pancreas, bone, and brain). The control individuals were composed of 13 men and 2 women, with a mean (SD) age of 46.5 (8.2) years; all were heavy smokers diagnosed with mild lung disease (ie, bronchitis) and had been confirmed via spirometric testing to have normal lung function. The study protocol was approved by the hospital ethic committee; informed written consent was obtained from all participants in the study. Blood samples were centrifuged after collection and the sera were stored at -80°C before analysis.

Polymerase Chain Reaction (PCR) Preparation of Fluorescently Labeled Fragment

A fluorescently labeled probe as part of an assay for DNase activity was prepared by amplifying a 214-base pair (bp) fragment of ANKRD2 promotor cloned into pGL4.10 vector and previously used in DNase footprint assays. The fragment was amplified using the following primers: 5'-VIC-GGGTTTCCAGGCATCCAGCAGGTGGCACT-3' and 5'- AGCAGAGCCAGTTGCCCCCCCAACTCCTG-3'. Amplification was performed in a reaction mixture containing, in a total volume of 100 µL, approximately 50 ng of plasmid DNA, 2 U of FIREPol DNA Polymerase (Solis BioDyne, Tartu, Estonia), 1× Buffer B (Solis BioDyne), 2.5 mM MgCl2, 0.2 mM of each type of deoxynucleoside triphosphate (dNTP), and 20 pmol of each primer. The amplification was performed under the following conditions: initial denaturation at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with the final elongation step at 72°C for 10 minutes. The obtained PCR product was purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific, Waltham, MA).

DNase Activity Assay

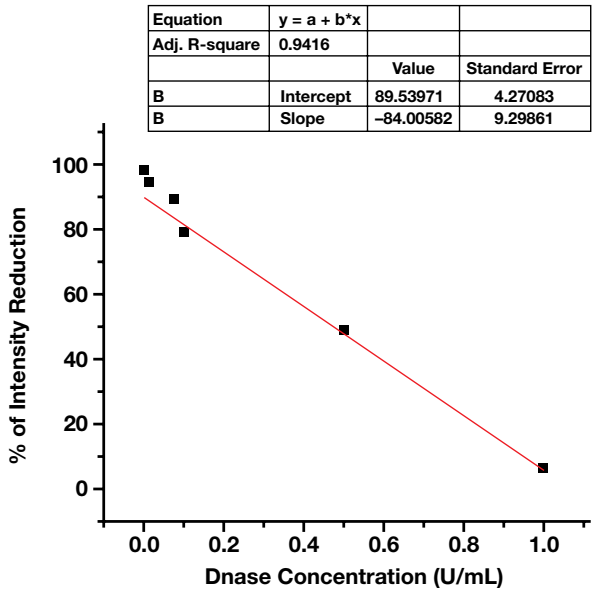
Activity of DNase in serum was determined by incubation of the serum samples with fluorescently labeled PCR fragments, followed by detection of fluorescently labeled products by capillary electrophoresis on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems Corporation, Carlsbad, CA). The assay was performed in a reaction mixture containing, in a total volume of 10 µL, 2.5 ng of PCR product and 2 µL of serum sample 10× diluted in saline. The positive control contained 0.05 U of DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden) instead of serum, whereas the negative control contained only the labeled fragment, with no source of DNase activity. The reaction mixture was incubated for 2 minutes at room temperature, after which the reaction was stopped by incubation at 75°C for 10 minutes. The reaction mixtures were purified using the GeneJET PCR Purification Kit (ThermoFisher Scientific). Each sample subjected to fragment analysis contained 15 µL of the purified reaction mixture, 0.5 µL of GeneScan-500 LIZ Size Standard (Applied Biosystems Corporation) and 15 µL of HiDi Formamide (Applied Biosystems Corporation). Capillary electrophoresis was performed with POP-7 Polymer (Applied Biosystems Corporation), using the default genotyping module for the G5 dye set. The results were analyzed using the GeneMapper Software, version 4.0 (Applied Biosystems Corporation). DNase activities of serum samples are expressed as percentage differences of signal intensity compared with control material containing no DNase, which was assigned the value of 1 (100%).

Statistical Analysis

Statistical analysis was performed with Microsoft Excel, version 2007 (Microsoft Corporation, Redmond, WA). The student *t* test (2-sample, assuming equal variances)

Figure 1

The calibration curve.



was used to assess whether there was a significant difference in serum DNase activity between patients with lung cancer and healthy individuals. $P < .05$ was considered statistically significant.

Results

The activity of serum DNase was determined in 15 samples from patients diagnosed with primary lung cancer and 15 samples from healthy individuals. Fragments of ANKRD2 promoter (GenBank accession number AJ304804) fluorescently labeled with VIC dye was used as a substrate for serum DNase. The purified product of this reaction was analyzed by capillary electrophoresis; signal intensity of 214-bp fluorescently labeled fragment was registered. DNase activity was assessed based on reduction in signal intensity compared with the control reaction that contained no DNase. Reduction in signal intensity was mean (SD) 0.04 (0.09) for the group of patients with primary lung cancer and 0.01 (0.07) for the control group. The calibration curve was linear ($r^2 > 0.77$) from 0.005 to 0.05 U/mL of DNase I, with 1% of reduction in signal intensity corresponding to 0.02 U/mL (**Figure 1**). The sensitivity of the assay was evaluated by using serial dilutions of a commercially available DNase; the reproducibility was assessed by repeating the measurements for each dilution 3 times. Based on the obtained calibration curve, the sensitivity of the assay is 0.001 U/ml.

DNase activity was slightly higher in patients with lung cancer compared with healthy individuals; however, no statistically significant difference was observed between the 2 groups ($P < .05$).

Discussion

This study describes development of a molecular-based method that can be applied to the measurement of DNase activity in serum. Published studies suggest that the DNase activity in human serum may be altered in several malignant diseases, such as lymphomas and breast, oral, stomach, colon, and pancreatic cancers.[7-10,12] Further, measurement of DNase activity is helpful for adjusting the therapeutic dose of recombinant human DNase I used in the treatment of patients with systemic lupus erythematosus and cystic fibrosis.[13,14]

The described method for determining DNase activity in serum samples uses a fluorescently labeled DNA fragment as a substrate for DNase and subsequent analysis of degradation products via capillary electrophoresis. In this study, DNase activity in patients

with lung cancer did not differ from that in healthy individuals. Fifteen samples from each group were analyzed; further analysis on larger number of samples is required to confirm this finding.

The main advantage of fluorescence detection compared with absorbance detection is sensitivity; this assay for DNase activity assessment is more sensitive than methods based on spectrophotometric analysis.[15] The key improvement of the assay is its use of fluorescently labeled fragments, which enable highly sensitive measurements. Substrates used for other assays, namely DNA-methyl green for the colorimetric assay, ethidium bromide or SYBR green (Life Technologies Corporation, Carlsbad, CA) for RED assay, and biotinylated DNA bound to avidin-coated wells for ELISA, provide significantly lower sensitivity.[11,13,14] The RED assay has relatively high sensitivity of 10 × 4 U/mL; however, this assay is susceptible to interference by DNase I inhibitor, which can affect the accuracy of the measurement.[11] All of the established methods are time consuming, taking at least 6 hours through as long as 16 hours; by contrast, the fluorescently labeled DNA method requires no more than an hour to perform.[11,13,14] Like ELISA, the method is based on measuring the nonhydrolyzed DNA fragment; whereas colorimetric, RID, and RAD assays measure the amount of hydrolyzed DNA. The measured activity represents the activity of the DNase and the factors that influence its activity, such as actin, which acts as its inhibitor.[5] Therefore, different concentrations of actin in some physiological states may lead to variations in detected DNase activity, regardless of the method used.

The DNase activity assay we developed is based on measurement of a fluorescently labeled DNA fragment serving as a DNase substrate, and is potentially applicable for research purposes when assessment of DNase activity in many samples is required. This method provides an alternative to the conventional methods that are more expensive and time-consuming, such as ELISA. The method we describe requires a small volume of sample, is simple to perform, and the results are reproducible. Although the assay described here was designed for the measurement of DNase activity in serum samples, it may be adaptable to measurement of DNase activity in a variety of biological specimens. LM

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