Long-term Stability of Circulating Nucleosomes in Serum

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Abstract. Background: Circulating nucleosomes, complexes of DNA and histones, are recognized as a potential new diagnostic tool for therapy monitoring of cancer disease and as prognostic marker in other acute diseases. Because many studies are carried out retrospectively on serum samples collected earlier, we tested the stability of nucleosomes during long-term storage at −70°C. Patients and Methods: Never-thawed aliquots of 154 stabilized serum samples from cancer patients which had been stored between 11/1999 and 07/2001 at −70°C were analyzed by ELISA six to nine months after collection and for a second time in 11/2006, with a median interval of 64.8 months (±5.5 months) between the measurements. Results: Nucleosome levels of the second measurement (median 220.0 ng/ml) were significantly lower than those of the first measurement (354.0 ng/ml; p<0.0001) exhibiting a median decrease of 32.0% (mean 29.7%±39.7%). Single concentrations of both measurements correlated well (R=0.92; p<0.0001). Individual courses of eight cancer patients showed a parallel downshifting of the nucleosome signal. The concordance rate of parallel increases and decreases was 89%. Conclusion: Retrospective studies on nucleosomes in serum can be performed if samples derive from homogeneous time intervals and preanalytical protocols are respected.

Several observations indicate that circulating nucleosomes constitute a major proportion of cell-free DNA, such as the findings that DNA fragments in circulation are mainly sized in multiples of the nucleosomal DNA (1, 2) and that DNA fragments were not associated with greater particles in filtration experiments, whereas RNA was (3). This might be due to its property of being organized in nucleosomal complexes consisting of the double represented histones core proteins H2A, H2B, H3, and H4 and 147 base pairs of DNA on the outside, which seems to be conserved from further plasmatic digestion by this conformation (4).

Qualitative and also quantitative changes of circulating DNA have been reported in various pathological conditions: Elevated DNA concentrations have been found in patients with various types of cancer (5-9), but also in those with acute non-malignant diseases, such as infections, sepsis, trauma and stroke, as well as in autoimmune diseases (5, 10-13). In consequence, several studies have described diagnostic and prognostic power of circulating DNA in cancer and non-malignant diseases, as well as a relevance of DNA kinetics during cancer treatment and tumor recurrence (5-13). Recently, initial changes of nucleosomal DNA during chemotherapy were shown to indicate the efficacy of chemotherapy in lung cancer patients (14).

Because many studies were carried out retrospectively on serum and plasma samples collected earlier, Sozzi et al. investigated the loss of DNA quantity during long-term storage (15). DNA concentrations in 179 plasma samples of lung cancer patients and healthy controls which were stored at −80°C or isolated DNA which was stored at −20°C were determined at the beginning of storage, as well as after a median storage time of 41 months, by quantitative PCR measurement. The authors reported a dramatic decrease of DNA concentration of about 30% per year for plasma DNA and isolated DNA, respectively, while the diagnostic relevance of DNA quantity was maintained (15).

In order to analyze the effect of long-term storage on serum concentrations of circulating nucleosomes, which were determined by an immunological ELISA method (16), we performed the present study.

Patients and Methods

A total of 154 serum samples from patients with lung, breast and gastrointestinal cancer had been collected in cooperation with the Asklepios Lung Cancer Hospital in Gauting, Germany, from 11/1999 to 07/2001. Among them were also samples from eight patients with metastasized breast and colorectal cancer during the course of chemotherapy. The samples were centrifuged at 3000 xg for 15 minutes within one to two hours after blood drawing; sera were subsequently stabilized with 10 mM EDTA (pH 8), aliquoted into two microtubes of Greiner, Germany, and stored at −70°C. First
measurements were conducted within six to nine months after sample collection using the Cell Death Detection ELISA plus (Roche Diagnostics, Penzberg, Germany), as described elsewhere (16); second measurements of nucleosomes in the second, never-thawed serum aliquots were carried out in 11/2006. The median time between both measurements was 64.8 months (standard deviation 5.5 months). Differences in nucleosome concentrations between both determinations were calculated by Wilcoxon test, correlations were tested by the Spearman rank test. A \( p \)-value <0.05 was considered statistically significant. Furthermore, concordance rates of parallel increases and decreases were calculated.

**Results**

Concerning single value comparisons, levels of the second measurement (mean 329.6 ng/ml; median 220.0 ng/ml) were significantly lower than of the first measurement (mean 540.4 ng/ml; median 354.0 ng/ml; \( p<0.0001 \)). Median decrease was 32.0%, with a mean of 29.7% and a standard deviation of 39.7%. Using Passing-Bablok equation, the slope was calculated at 0.67 (95% confidence interval 0.61-0.72) and intercept was +1.7 (95% confidence interval −9.6-13.3). Single concentrations of both measurements correlated well as shown by correlation coefficients of 0.92 for all results (N=154; \( p<0.0001 \)), 0.89 for nucleosome values below 1000 ng/ml (N=133; \( p<0.0001 \)), and 0.83 for nucleosome values in the clinically relevant range below 500 ng/ml (N=100; \( p<0.0001 \); Figure 1A-C).

Courses of nucleosome values in most cancer patients during chemotherapy showed parallel downshifting over the complete follow-up period, indicating a homogeneous loss of the nucleosome signal within individual patients after five years’ storage time. When the occurrence of parallel increases and decreases were tested, both measurements showed a concordance rate of 89% (Figure 1D-K).

**Discussion**

Our results indicate that long-term storage of stabilized serum samples over 5 years at −70°C led only to minor decreases of nucleosome concentrations of about 7% per year, which is considerably lower than the 30% annual loss of plasma DNA reported by the group of Sozzi et al. (15). This remarkable difference cannot be explained solely by the different quantification techniques, as earlier studies have
shown a good correlation between the nucleosome assay and DNA quantification by real-time PCR (17). An important difference between the studies constitutes the use of plasma and serum as source of circulating DNA and nucleosomes and their potentially different behaviour during long-term storage at –70˚C. Although this discrepancy cannot be clarified here, it should be pointed out that in our study, the influence of interfering factors was minimized by following a strict preanalytical protocol for the handling of the serum samples (16) and nucleosome concentrations after stabilization of the serum were found to be constant when exposed to stressful situations such as shaking, rolling and vortexing (18).

In conclusion, serum storage time should be taken into account when studies on circulating nucleosomes with stored serum samples are performed although the annual loss is only about 7%. Particularly, the comparison of two patient groups whose serum samples were collected during different time periods remains critical. However, the high correlations in the single value and follow-up comparisons indicate that retrospective studies can be performed if samples derive from homogeneous time intervals and preanalytical protocols are respected.

References


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