ORIGINAL ARTICLE

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Combining Histone ELISA and Quantitative PCR Assays to Extract and Quantify Histone Methylation-related Circulating DNA

Histon ELISA ve Nicel PCR Testlerinin Birleştirilerek Histon Metilasyonu ile İlişkili Dolaşımdaki DNA'nın İzolasyonu ve Ölçülmesi

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ABSTRACT

Aim: Liquid biopsy-based measurement of post-translational histone modifications (PTHMs) in bodily fluids of patients with cancer represents a novel area of biomarker research. Here, we tested the applicability of an approach combining enzyme-linked immunosorbent assays (ELISA)-like measurement of histone methylation and quantitative polymerase chain reaction to extract and quantify pericentric histone 4 lysine 20 trimethylation (H4K20me3)-related circulating DNA.

Materials and Methods: After adding plasma into ELISA wells and letting H4K20me3-related nucleosomes bind to the antibody, nucleosomal DNA was detached from immune complexes using a buffer including $NaHCO_3$, SDS, and proteinase K and purified. A single copy- and a multiple copy-sequence from pericentric regions of chromosomes 16 and 1 were amplified to quantify H4K20me3-related DNA from patients with colon cancer or colonic polyps.

Results: Relative plasma level of H4K20me3-related nucleosomal DNA was lower in the patients with colon cancer than in controls for both target sequences, with a statistically significant difference at the chr16-specific target (relative values were 0.037 vs. 0.073, respectively; p=0.008).

Conclusion: That H4K20me3-related nucleosomal DNA is present in decreased levels in colon cancer confirms our previous findings attained using other techniques. In conclusion, our findings indicate the applicability of direct extraction of protein-bound DNA from ELISA immune complexes and it could provide a surrogate means in the assessment of PTHMs or other protein-DNA interactions.

Keywords: Colon cancer, histone methylation, circulating nucleosomal DNA, ELISA

ÖZ

Amaç: Kanserli hastaların vücut sıvılarında post-translasyonel histon modifikasyonlarının (PTHM) likit biyopsi bazlı ölçümü, biyobelirteç araştırmalarının yeni bir alanını temsil etmektedir. Bu çalışmada, perisentrik histone 4 lizin 20 trimetilasyonu (H4K20me3) ile ilgili dolaşımdaki DNA'yı çıkarmak ve ölçmek için enzime bağlı immünosorbent testini (ELISA) ve nicel polimeraz zincir reaksiyonu birleştiren bir yaklaşımın uygulanabilirliğini test ettik.

Gereç ve Yöntem: ELISA kuyularına plazma eklendikten ve H4K20me3 ile ilgili nükleozomların antikora bağlanmasına izin verildikten sonra, $NaHCO_3$, SDS ve proteinaz K içeren bir tampon kullanılarak nükleozomal DNA bağışıklık komplekslerinden ayrıldı ve saflaştırıldı. Kolon kanseri ve kolon polipleri olan hastalardan H4K20me3 ile ilgili DNA'nın miktarını ölçmek için kromozom 16 ve 1'in perisentrik bölgelerinden tek kopya ve çoklu kopya dizisi çoğaltıldı.

Bulgular: H4K20me3 ile ilişkili nükleozomal DNA'nın göreceli plazma seviyesi, kolon kanserli hastalarda kontrollere göre daha düşük görüldü ve chr16'ya özgü hedefte istatistiksel olarak anlamlı bir fark saptandı (göreceli değerler sırasıyla 0,037'ye karşı 0,073 idi; p=0,008). H4K20me3 ile ilişkili nükleozomal DNA'nın kolon kanserinde düşük seviyelerde bulunması, diğer teknikler kullanılarak elde ettiğimiz önceki bulgularımızı doğrulamaktadır.

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Sonuç: Sonuç olarak, bulgularımız ELISA bağışıklık komplekslerinden proteine bağlı DNA'nın doğrudan çıkarılmasının uygulanabilirliğini göstermektedir ve PTHM'lerin veya diğer protein-DNA etkileşimlerinin değerlendirilmesinde farklı bir yöntem sağlayabilir.

Anahtar Kelimeler: Kolon kanseri, histon metilasyonu, dolaşımdaki nükleozomal DNA, ELISA

INTRODUCTION

In eukaryotic cells, nucleosomes are basic functional units of DNA packaging in chromatin. Each nucleosome is composed of an octamer of four core histones (H3, H4, H2A, and H2B), with wrapped DNA around them[1]. The genetic information stored in the context of chromatin is subjected to two main types of epigenetic modifications, i.e. DNA methylation and various histone modifications. Post-translational modifications of N-terminal tails of histone proteins protruding from the nucleosomes affect the chromatin structure, thus their dysregulation impacts gene expression and represents a common mechanism across human cancers contributing to oncogenesis through the induction of epigenetic, transcriptomic, and phenotypic alterations[2].

Histone methylation, which is catalyzed by histone methyltransferases on three levels (mono-, di-, or trimethylation), mainly takes place on lysine residues of histone H3 and H4 and is a dynamic process with key roles in development and differentiation. The outcomes of this modification are highly context-dependent and can be associated with transcriptional repression or activation. Methylation of H4K20 is associated with both transcriptional activation and repression depending on methylation states. H4K20me1 catalyzed by PR-Set7 is associated with transcriptional activation, whereas H4K20me2/3 catalyzed by SUV4-20H1/2 is associated with the repression of transcription by maintaining pericentric and telomeric heterochromatin[3]. Loss of H4K20me3 has been described as a hallmark of cancer[4].

Liquid biopsy-based detection of cancer-related alterations in PTHMs has been reported by our group and others[5-9]. In the present study, our goal was to demonstrate the practicability of the extraction of circulating nucleosomal DNA from antigen-antibody complexes in enzyme-linked immunosorbent assays (ELISA) wells. This resembles chromatin immunoprecipitation (ChIP) assays where, however, there are relevant differences between the two approaches. In ChIP assays, protein A/G agarose beads are usually employed to bind antibodies of interest, and this is often associated with significant non-specific binding of DNA or non-target proteins to beads[10-12]. In the approach used in the present study, we extracted H4K20me3-associated circulating DNA directly from H4K20me3-containing nucleosomes that bound to anti-H4K20me3 antibodies immobilized on assay strips. In this way, many background signals could be excluded. Following extraction, a single copy and a multiple copy sequence from pericentric regions of chromosomes 16 and 1 were amplified to quantify H4K20me3-related circulating DNA from patients with colon cancer or colonic polyps.

MATERIALS AND METHODS

Study Participants

The study cohort included individuals who underwent colonoscopy procedures at the Surgical Department of İstanbul University, İstanbul Faculty of Medicine Hospital. Individuals with pathologically confirmed polyps in the colon (control group, n=15) and those with pathologically confirmed colon cancer (cancer group, n=15) were enrolled in the study. The characteristics of the patients with colon cancer are shown in Table 1. The study was approved by the İstanbul University, İstanbul Faculty of Medicine Ethics Committee (approval no: 2018/1095) and informed consent was obtained from each participant. Written informed consent forms were read by each patient and signed approvals were obtained before their operation.

Quantification of Circulating Nucleosomes

Blood samples were taken into EDTA tubes before the colonoscopy procedure and immediately centrifuged (10 min at 717 × g) to obtain the plasma fraction. After re-centrifugation, plasma was stored in aliquots at -80 °C until assayed. Plasma levels of circulating nucleosomes were determined using the Cell-Death Detection ELISA kit (Roche Diagnostics, Mannheim, Germany), as previously reported[6]. We applied 20 µL of plasma twice and the mean signal values, measured in optical density, were considered to be relative plasma concentrations.

Extraction of H4k20me3-related Nucleosomal DNA from Antibody-bound Nuclesosomes in ELISA

We used the commercially available EpiQuik Global Tri-Methyl Histone Quantification Kit (Epigentek, Farmingdale, NY, USA), which enables ELISA-like measurements of H4K20me3. Nuclesomal DNA was, however, extracted from antibody-bound nucleosomes before the H4K20me3 measurement was completed. For this, 50 µL plasma was added into ELISA wells and H4K20me3-related nucleosomes were allowed to bind to the antibody for 1 hour. After several washing steps, bound DNA was extracted as follows: The extraction buffer [100 mM $NaHCO_3$, 1% SDS and proteinase K (250 µg/mL)] was added on H4K20me3-antibody complexes and incubated at 65 °C for 15 min leading to the disassociation of nucleosomal DNA from

immune complexes. Detached DNA was transferred into fresh tubes, diluted to 100 µL, and purified using spin columns. DNA was eluted in 50 µL elution buffer and stored at -20 °C after checking the purity.

Quantitation of Nucleosomal DNA by Quantitative Polymerase Chain Reaction (PCR)

We amplified two genomic sequences from the pericentric regions of chromosomes 1 and 16 because H4K20me3 is enriched in pericentric heterochromatin. The primers for chr16 allow the amplification of a single copy sequence and had the following sequences: 5'AATCCAATGGAATCATCGAGT-3' and 5'-GGTGATTTCATTCAAGTCCATTC-3'.

The primers for chr1 amplify at least 40-copies and were 5'-GGTTCCATTTGATGATGATTCC-3' and 5'-ATCGAGTGGAATCGAATGG-3'. LINE1 element, which is found in all genomic regions of the human genome and enriched near centromeres, was used as the reference sequence to normalize the target sequences. Primer sequences of LINE1 resulting in 148-bp amplicon were 5'-AAAGCCGCTCAACTACATGG-3' and 5'-TGCTTTGAATGCGTCCCAGAG-3'. Real-time PCR was conducted in a light-cycler 480 with SYBR green as the fluorescence molecule, according to the instructions. Reactions were run in double and relative concentrations of pericentric sequences were calculated using the ΔΔCt method.

Statistical Analysis

The study findings are presented as median, quartiles, and total ranges. Discrimination between the study groups was performed using the Mann-Whitney U test. P-values of <0.05 were considered statistically significant. Calculations were performed using the SPSS 21.0 statistical software (IBM Corporation, Armonk, NY, USA).

RESULTS AND DISCUSSION

All plasma samples taken from patients with colon cancer and controls had detectable levels of circulating nucleosomes with slightly higher levels in the cancer group (Figure 1A). Following the extraction of nucleosomal DNA from H4K20me3-ELISA immune complexes, DNA was present in all samples and used as a template for the amplification of pericentric sequences from Chr 1 and 16. The relative plasma level of H4K20me3-related nucleosomal DNA was lower in the patients with colon cancer than in the controls for both target sequences (Figure 1B and 1C). At the chr1-specific target, relative values of H4K20me3-related nucleosomal DNA were 2.74 and 3.2 (p=0.16) in the patients with colon cancer and those with polyps, respectively. At the chr16-specific pericentric region, the difference between the patients with colon cancer and the controls was statistically significant (relative values: 0.037 vs. 0.073, respectively; p=0.008). These findings show that H4K20me3-related circulating nucleosomal DNA is present in decreased levels in colon cancer compared to the premalignant state and confirm our previous findings when ChIP- or ELISA-like approaches were used[5,6]. Even if the number of patients in the study is very small, the patients with more advanced disease (n=7) had lower levels of both target sequences than those with localized disease (n=8) (2.26 vs. 3.4 for Chr1-specific target and 0.036 vs. 0.049 for Chr16-specific target, respectively). Overall, decreased plasma levels of H4K20me3-related nucleosomal DNA are likely to be a reflection of

Table 1. Patient characteristics

	Gender	Age	Tumor localization	T stage	Lymph node metastasis	CEA (ng/mL)	CA19-9 (u/mL)
Patient 1	M	50	Sigmoid colon	2	No	2.81	38.22
Patient 2	F	57	Sigmoid colon	3	No	2.49	8.1
Patient 3	M	60	Right colon	3	No	13.96	12.92
Patient 4	F	54	Right colon	3	Yes	3.25	0.84
Patient 5	F	72	Sigmoid colon	4A	No	23.4	24.42
Patient 6	M	55	Right colon	4A	Yes	11.5	13.4
Patient 7	F	58	Right colon	2	No	1.23	2.45
Patient 8	M	85	Right colon	4A	Yes	6.7	3.5
Patient 9	F	58	Right colon	4A	Yes	41.1	0.68
Patient 10	M	60	Right colon	4A	Yes	1.42	16.1
Patient 11	F	56	Right colon	4A	Yes	3.29	22.2
Patient 12	M	70	right colon	3	Yes	3.79	3
Patient 13	M	22	Right colon	3	Yes	3.11	17.1
Patient 14	M	58	Sigmoid colon	4A	Yes	1.98	8.91
Patient 15	M	56	Right colon	2	No	3.17	57.19

M: Male, F: Female

H4K20me3 loss in colonic tumors, which has been described as a hallmark of cancer[4,13].

To our knowledge, this is the first report to describe the applicability of extracting circulating nucleosomal DNA from ELISA immune complexes. Even though our study does not include a direct comparison of this approach with ChIP, this method may be superior to ChIP in preventing background noise signals because protein A/G agarose beads used in ChIP assays non-specifically bind DNA and non-target proteins[10-12].

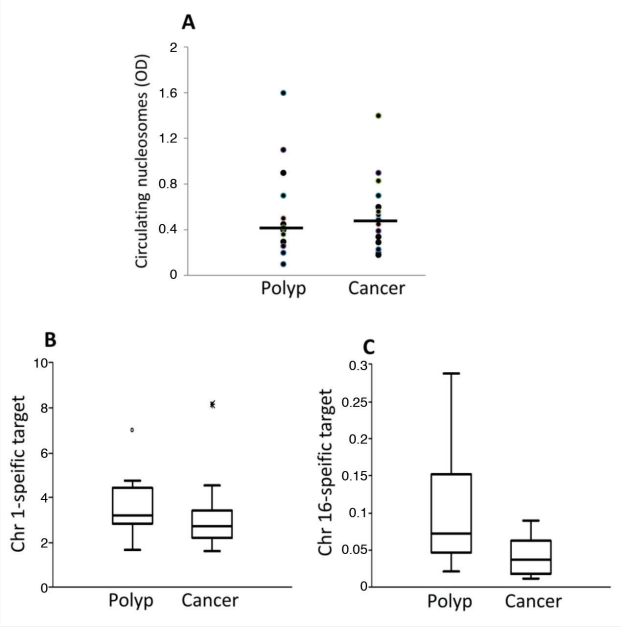

Figure 1. Plasma levels of circulating nucleosomes and nucleosomal DNA extracted from ELISA immune complexes in the study cohort. (A) Circulating nucleosomes were measured using a commercial kit according to the instructions. Mean signal values, measured in optical density, were considered to be relative plasma concentrations. The line mark (-) illustrates median values. (B) Relative plasma levels of extracted H4K20me3-related DNA in the pericentric region of Chr 1. Plasma was added into ELISA wells and H4K20me3-related nucleosomes were allowed to bind to the antibody for 1 hour. After several washing steps, bound DNA was detached from immune complexes using an extraction buffer (100 mM $NaHCO_3$, 1% SDS and proteinase K) and purified. A multiple-copy sequence from the pericentric region of Chr 1 was amplified using qPCR, which was normalized by the LINE1. (C) Relative plasma levels of extracted H4K20me3-related DNA in the pericentric region of Chr 16. After extraction of H4K20me3-related DNA from immune complexes, a single-copy sequence from Chr 16 was amplified. Shown are the 25th and 75th percentiles along with median values

ELISA: Enzyme-linked immunosorbent assays, qPCR: Quantitative polymerase chain reaction

The study of PTHM alterations in bodily fluids of individuals with disease represents a relatively new research field. Different techniques such as ChIP, ELISA-like measurement or more recently mass spectrometry have been used for the measurement of PTHMs in circulating nucleosomes[5-9], where cancer has been the main topic. The research conducted up to now has revealed the potential of liquid biopsy-based detection of PTHMs as surrogate cancer biomarkers. However, given the complexity in the number and the regulation of PTHMs, the lack of standardization of pre-analytics, and the method of their measurement, considerable research is required before validated markers become available for clinical use.

Study Limitations

The limitation of our study is the small number of patients. Further studies with higher number of patients are needed.

CONCLUSION

The detection of biomarkers in blood or other biologic fluids offers many advantages, including being minimally invasive and easily accessible. Here, we demonstrate the applicability of direct extraction of nucleosomal DNA from ELISA immune complexes, which represents a simplified method for the analysis of protein-DNA interactions and could provide an additional means in the assessment of PTHMs in liquid biopsy measurements.

Ethics

Ethics Committee Approval: The study was approved by the İstanbul University, İstanbul Faculty of Medicine Ethics Committee (approval no: 1095, date: 14.08.2018).

Informed Consent: Informed consent was obtained from each participant.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.K., Concept: U.G., Design: U.G., Data Collection or Processing: E.Ö., E.E.Y., Analysis or Interpretation: E.Ö., E.E.Y., Literature Search: U.G., Writing: E.Ö., E.E.Y., M.K., U.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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