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RESEARCH ARTICLE

IDENTIFICATION AND QUANTIFICATION OF APURINIC/APYRIMIDINIC ENDONUCLEASE 1 IN HUMAN PERIPHERAL BLOOD LEUKOCYTES BY LIQUID CHROMATOGRAPHY/ISOTOPE-DILUTION HIGH RESOLUTION MASS SPECTROMETRY

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ABSTRACT

Purpose: Increasing evidence in recent years highlights the predictive and prognostic importance of the expression of DNA repair proteins in cancer treatment. Generally, western-blotting or immunohistochemical staining methods are often used to determine the expression of DNA repair proteins. These methods might cause misleading results such as binding to nonspecific molecules by cross-reaction or false negativity as a result of the inability of antibodies to bind; absolute quantitation of proteins can not be performed. In this study, an analytical measurement technique was developed for human apurinic/apyrimidinic endonuclease 1 (hAPE1) protein for identification and absolute quantification in human leukocyte sample using high resolution mass spectrometry (HR-MS) with the targeted proteomics-based approach.

Methods: Sample preparation was performed by using density gradient centrifugation and total protein extraction cartridges. hAPE1 was analyzed by liquid chromatography isotope-dilution-HR-MS (LC-HR-MS). A fully ^{15}N-labeled analogue of hAPE1 was used for the quantitative measurements.

Results: Six peptides were identified, which matched to a subset of the theoretically predicted tryptic peptides of hAPE1. Mass accuracy was calculated as <1.8 ppm. The amount of hAPE1 protein was calculated as 0.07 ng hAPE1/µg protein in the leukocyte sample.

Conclusion: The absolute quantification of APE1 protein performed within the scope of this study is expected to be used for the follow-up of the prognosis, response to treatment and survival rates of various cancer patients.

Keywords: DNA repair protein, hAPE1, high resolution mass spectrometry, leukocyte, protein quantification, targeted proteomics

INTRODUCTION

DNA damage occurs endogenously under the effect of reactive oxygen species formed as a result of cellular metabolism or as a result of exogenous factors such as ionizing radiation, carcinogenic compounds and environmental toxins. DNA damage is defined as the hallmark of cancers because it causes genetic instability, mutagenize, proliferation,

cell death and apoptosis, as well as being associated with the carcinogenesis process (1,2). On the other hand, DNA repair is carried out by complex mechanisms as a result of the participation of many proteins and DNA repair capacity is important in cancer therapy. Increased DNA repair capacity in malignant tumors and its positive correlation with drug resistance have been demonstrated (1,3). In this context, DNA repair proteins are evaluated as predictive and prognostic markers in most cancer types and inhibitors against DNA repair proteins are being developed in order to increase the effectiveness of cancer treatment (4). Therefore, absolute quantification of DNA repair proteins are very important in the development and selection of treatment strategies including the use of DNA repair protein inhibitors in malignant tumors.

Human apurinic/apyrimidinic endonuclease 1 (hAPE1) is a multifunctional protein that plays a central role in the cellular response to free radicals and oxidative stress. The two major activities of hAPE1 are DNA repair and redox function of transcriptional factors. Functions as a apurinic/apyrimidinic (AP) endonuclease in the DNA base excision repair (BER) pathway of DNA lesions induced by oxidative agents. hAPE1 also reduces certain transcription factors, causing increased transcription activity and upregulation of genes involved in cell growth, inflammation, angiogenesis, and other cellular functions. Expression of APE1 has been observed in most types of cancer. Therefore, hAPE1 inhibitors are thought to play an important role in cancer treatments (5,6).

Previously the measurement of APE1 proteins has been performed by semi-quantitative immunohistochemical techniques or by indirect and relative techniques such as measurement of mRNA level, and there is no study of a methodology that provides absolute quantification with high resolution mass spectrometry at protein levels in the leukocyte samples (7,8). Our previous studies, we have performed the identification of APE1 in tissues with tandem mass spectrometry, a technique with lower resolution than high resolution mass spectrometry (HR-MS) (9). However, in leukocyte samples, which can be obtained relatively less invasively than tissue samples, the measurement was not performed with high-resolution MS technique. With the method optimized within the scope of this study, it is aimed to identify and to perform the absolute quantification of the hAPE1 in human leukocyte samples for the first time with liquid chromatography-high resolution mass spectrometry (LC-HR-MS) technique.

MATERIAL AND METHODS

Sample Collection

This study was conducted in the Department of Molecular Medicine at Dokuz Eylul University (DEU). Six milliliters of venous blood was collected from the author's own blood into EDTA tubes and collected by Dokuz Eylul University Hospital. Ethical approval for this project was obtained from the Dokuz Eylul University Faculty of Medicine Non-Interventional Research Ethics Committee (Protocol number: 2022/20-17, Approval date: 01.06.2022).

Chemicals and Standards

Histopaque 1119 (Cat. No. 1119-1 Sigma, St. Louis, MO, Polysucrose, 6.0 g/dl and sodium diatrizoate 16.7 g/dl) and phosphate buffered saline (1X, Sigma, St. Louis, MO.) were used for leukocytes isolation. Trypsin (Proteomics Grade, Sigma, St. Louis, MO), acetonitrile (UHPLC-MS grade, MERCK & Co., USA), water (UHPLC-MS grade, Sigma, St. Louis, MO), formic acid (MERCK & Co., USA) were used for selective measurement of APE1 using Orbitrap LC-MS system. The stable isotope-labelled internal standard fully ^{15}N-hAPE1 was received from National Institute of Standards and Technology (NIST) (Gaithersburg, Maryland, USA).

Leukocytes Isolation

Leukocytes were isolated from EDTA blood using density gradients. Six milliliters of EDTA blood was carefully layered on six mL of 1119 g/L density polysucrose solution in a conical centrifuge tube and centrifuged at 750g for 30 min at room temperature. Plasma and upper gradient were discarded. The opaque interface of gradients containing the leukocytes was transferred to another tube and washed three times by the addition of 10 mL 1X-isotonic phosphate-buffered saline (PBS). The pellet was obtained by centrifugation at 500g for 10 min and suspended in PBS.

Protein Extraction From Leukocytes

Minute total protein extraction kit for cells and tissues kit from Invent Biotechnologies, Inc. (Plymouth, MN) were used to extract proteins from leukocytes. Prior to extraction, protein extraction filter cartridges with collection tubes were pre-chilled on ice. 200 µL of the

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         10          20          30          40          50
MPKRGKKGAV AEDGDELRTE PEAKKSKTAA KKNDKEAAGE GPALYEDPPD
         60          70          80          90         100
QKTSPSGKPA TLKICSWNVD GLRAWIKKKG LDWVKEEAPD ILCLQETKCS
        110         120         130         140         150
ENKLPAELQE LPGLSHQYWS APSDKEGYSG VGLLSRQCPL KVSYGIGDEE
        160         170         180         190         200
HDQEGRVIVA EFDSFVLVTA YVPNAGRGLV RLEYRQRWDE AFRKFLKGLA
        210         220         230         240         250
SRKPLVLCGD LNVAHEEIDL RNPKGNKKNA GFTPQERQGF GELLQAVPLA
        260         270         280         290         300
DSFRHLYPNT PYAYTFWTYM MNARSKNVGW RLDYFLLSHS LLPALCDSKI
        310
RSKALGSDHC PITLYLAL
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Figure 1. Amino acid sequence of human APE1. **https://www.uniprot.org/uniprot/P27695*

native lysis buffer was added and vortexed briefly to lyse the cells. Then the samples were vortexed for 15 s and incubated on ice for 5 min. The cell lysates were then transferred to filter cartridges in collection tubes and centrifuged in a microcentrifuge at 14000g for 30 s. After centrifuging, the filter cartridges were discarded. The protein concentration of each protein extract was measured using the BCA Protein Assay Kit (BioVision, Inc.) according to the manufacturers' instructions. The optical density of each well was measured at 562 nm using a microplate reader (Biotek, ELX 800, USA). Quantifications were achieved by the construction of six-point standard curve (10, 25, 75, 150, 300 and 600 µg/mL) using known concentrations of albumin for each run. Each standard was measured in duplicate. The results were expressed in microgram per milliliter (µg/mL)(9).

Enzymatic Hydrolysis

For absolute quantification, ^{15}N-hAPE1 was used as an internal standard. An aliquot of 1 µg of ^{15}N-hAPE1 was incubated with trypsin (10 µg trypsin in 200 µL Tris−HCl buffer, 30 mM, pH 8.0) at 37 °C overnight. For the analysis of protein extract (150 µg) from leukocytes, 1 µg of the ^{15}N-APE1 was added to the extract before trypsin hydrolysis. The hydrolysis with trypsin was performed as described above. To remove trypsin, the hydrolysates were filtered by using a Nanosep 3 K Omega tube with molecular-weight-cutoff of 3000 (Pall Life Sciences, Ann Arbor, MI) for 60 min at 14000g. The filtered samples were concentrated in a SpeedVac (Thermo) under vacuum to 100 µL prior to LC-HR-MS analysis (9,10).

Mass Spectrometric Analysis

High resolution LC-MS analyses were performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC consisting of an DGP-3600RS binary pump, WPS-3000 autosampler, and a TCC-3400 column compartment coupled to an orbitrap mass spectrometer (Thermo Scientific™ Exactive Plus™ Orbitrap™ MS) equipped with a heated electrospray-ionization (HESI) ion source in the positive ionization mode. Separation was performed by injecting 100 µL extracts onto a Zorbax Extend-C18, Rapid Resolution HT column (2.1 mm × 100 mm, 1.8 µm particle size) with an attached C8 guard column (2.1 x 12.5 mm, 5 µm particle size). Mobile phase consists of 2% acetonitrile and 0.1% formic acid (v/v) in water (A) and 0.1% formic acid (v/v) in acetonitrile (B). The flow rate was 0.3 mL/min. The total analysis time was 36 min. The system was calibrated daily before analysis for both positive and negative modes. The XCalibur Software (Thermo Scientific™) was used for data analyses. The concentration of each analyte was calculated according to these curves.

RESULTS

In the present work, the absolute quantification of the DNA repair protein APE1 in human leukocyte cells have been performed for the first time with the Orbitrap LC-HR-MS system. APE1 has a monoisotopic molecular mass of 35.5 kDa and contains 318 amino acids (Figure 1). Trypsin cleaves 46 peptide bonds in APE1, resulting in 37 fully tryptic peptides as shown by using the website http://web.expasy.org/peptide_cutter (Figure 2).

Six peptides were identified, which matched to a subset of the theoretically predicted tryptic peptides of APE1. The theoretical masses of peptides were calculated by NIST mass and fragment calculator v.2 program (NIST, USA). Figure 3 shows the mass spectrum ion profiles of the trypsin hydrolysate of APE1. Mass identification and accuracy of peptides were calculated with both full scan and all ion fragmentation spectra. Data for retention times,

Position of cleavage site	Name of cleaving enzyme(s)	Resulting peptide sequence	Peptide length [aa]	Peptide mass [Da]	Cleavage probability
3	Trypsin	MPK	3	374.499	90.7 %
4	Trypsin	R	1	174.203	95.3 %
6	Trypsin	GK	2	203.241	92.9 %
7	Trypsin	K	1	146.189	83.7 %
18	Trypsin	GAVAEDGDELR	11	1131.164	100%
24	Trypsin	TEPEAK	6	673.721	90.5 %
25	Trypsin	K	1	146.189	90.9 %
27	Trypsin	SK	2	233.268	100%
31	Trypsin	TAAK	4	389.452	90.5 %
32	Trypsin	K	1	146.189	100%
35	Trypsin	NDK	3	375.382	83.8 %
52	Trypsin	EAAGEGPALYEDPPDQK	17	1786.869	100%
58	Trypsin	TSPSGK	6	575.619	51.9 %
63	Trypsin	PATLK	5	528.649	89.1 %
73	Trypsin	ICSWNVDGLR	10	1162.329	95.7 %
77	Trypsin	AWIK	4	516.641	85.5 %
78	Trypsin	K	1	146.189	78%
79	Trypsin	K	1	146.189	83.7 %
85	Trypsin	GLDWVK	6	716.835	90.8 %
98	Trypsin	EEAPDILCLQETK	13	1488.673	100%
103	Trypsin	CSENK	5	579.626	95.3 %
125	Trypsin	LPAELQELPGLSHQYWSA	22	2466.732	83.8 %
136	Trypsin	EGYSGVGLLSR	11	1137.258	100%
141	Trypsin	QCPLK	5	587.735	92.4 %
156	Trypsin	VSYGIGDEEHDQEGR	15	1690.700	100%
177	Trypsin	VIVAEFDSFVLVTAYVPNA	21	2267.610	100%
181	Trypsin	GLVR	4	443.547	100%
185	Trypsin	LEYR	4	579.654	100%
187	Trypsin	QR	2	302.333	100%
193	Trypsin	WDEAFR	6	822.875	90.2 %
194	Trypsin	K	1	146.189	100%
197	Trypsin	FLK	3	406.525	100%
202	Trypsin	GLASR	5	502.571	100%
203	Trypsin	K	1	146.189	21%
221	Trypsin	PLVLCGDLNVAHEEIDLR	18	2006.304	79.7 %
224	Trypsin	NPK	3	357.410	94%
227	Trypsin	GNK	3	317.345	84.1 %
228	Trypsin	K	1	146.189	100%
237	Trypsin	NAGFTPQER	9	1019.082	100%
254	Trypsin	QGFGELLQAVPLADSFR	17	1848.089	100%
274	Trypsin	HLYPNTPYAYTFWTYMM	20	2540.896	100%
276	Trypsin	SK	2	233.268	100%
281	Trypsin	NVGWR	5	630.704	100%
299	Trypsin	LDYFLLSHSLLPALCDSK	18	2035.386	100%
301	Trypsin	IR	2	287.362	100%
303	Trypsin	SK	2	233.268	100%
318	**end of sequence**	ALGSDHCPITLYLAL	15	1586.866	-

Figure 2. Tryptic peptides of human APE1. * *https://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl*

experimentally measured and exact protonated ion masses and mass accuracy for each peptides are shown in Table 1. The monoisotopic masses of the protonated molecular ions (MH^+) and doubly-charged molecular ions [$(M+2H)^{2+}$] are given in Table 1.

Note that mass accuracy is expressed in parts per million (ppm) being 10^6 × [(exact mass − measured mass)/exact mass]. Confirmation of each peptide and internal standard relied upon constancy of retention time, and according to the accurate mass agreement to the exact mass; a narrow mass tolerance window (<1.8 ppm) indicated good selectivity of the method towards each compound. Generally good peak shapes for each peptide was attained by the Orbitrap MS, operated with a mass resolution of 140,000 FWHM and a mass tolerance window of 2 ppm.

A combination of any four identified peptides with the use of the SwissProt.2015.3.5 data base with MS-Fit (https://prospector.ucsf.edu/prospector/mshome.htm) resulted in a 100% match with hAPE1. These results mean that the use of any four identified tryptic peptides would be sufficient to positively identify and quantify human APE1. For the quantitative measurement of hAPE1, a fully ^{15}N-labeled analogue of this protein was used. Table 1 shows the monoisotopic masse to charge (*m/z*) of their MH^+ and $(M+2H)^{2+}$ ions of these ^{15}N-labeled peptides. As an example, the mass spectrum of GLVR and ^{15}N-GLVR

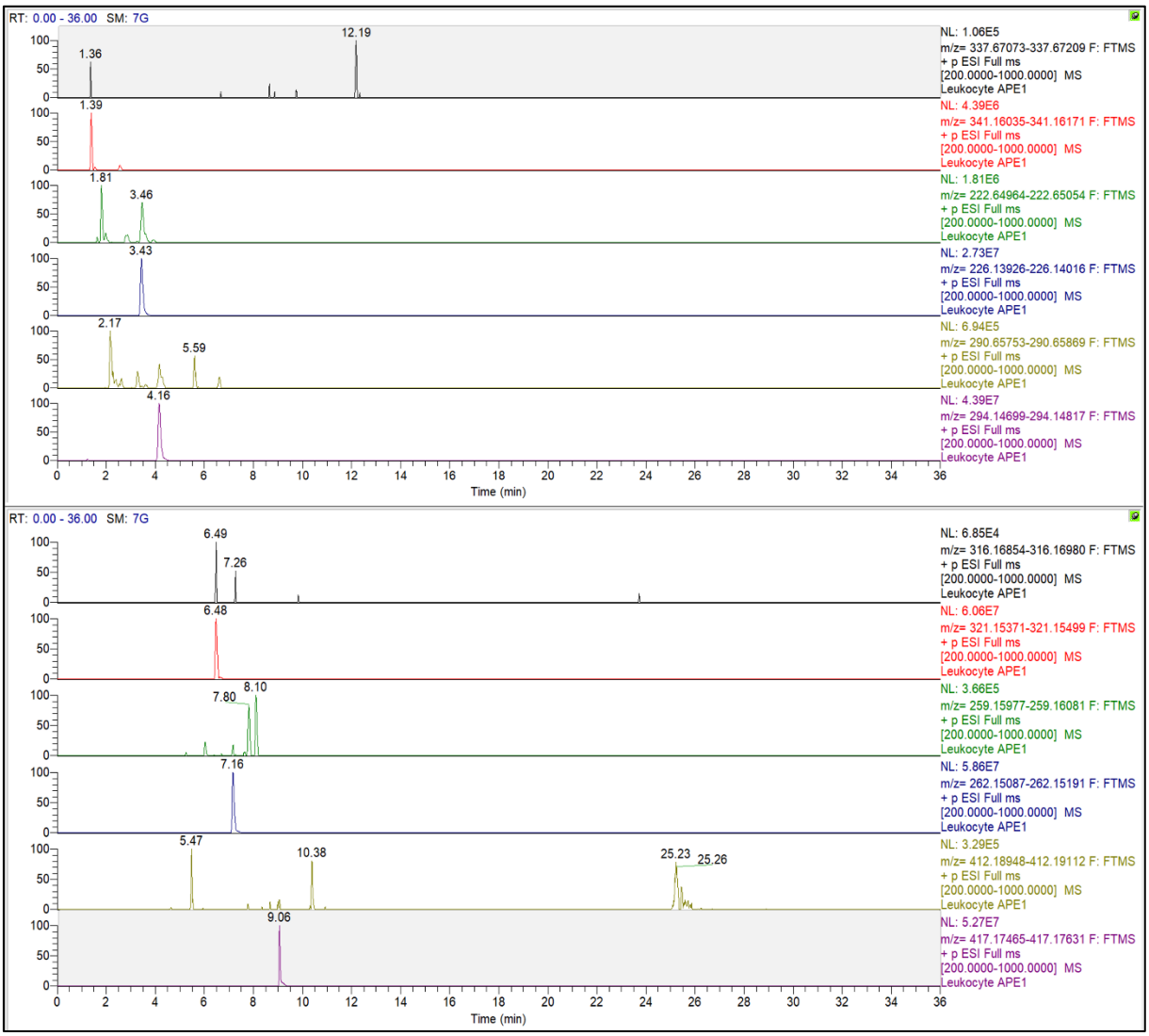

Figure 3. Ion current profiles of precursor ions masses of six tryptic peptides of APE1 and ^{15}N-APE1.

with the $(M+2H)^{2+}$ ions at *m/z* 222.65009 and *m/z* 226.13971, respectively. The precursor ion masses were used for absolute quantification. The results were given in ng/µg protein. The amount of hAPE1 was calculated as 0.07 ng APE1/µg protein in the leukocyte sample.

DISCUSSION

Measurement of DNA repair proteins in biological samples obtained less invasively is important for the evaluation of the clinical course, response to treatment and follow-up of the patients. With this study, a targeted bottom-up proteomics approach was developed for hAPE1 in human leukocyte samples. In this context, as a marker of BER status, the hAPE1 measurement was carried out using different techniques, mainly including ELISA, western-blotting or RT-PCR for messenger RNA (mRNA) expression (11,12). However, these methods may cause misleading results such as cross-reaction binding to nonspecific molecules or false negativity as a result of the inability of antibodies to bind. Also, mRNA expression analyses of proteins can also be performed with various methods. But mRNA analysis is not a direct reflection of protein content in the cell. Many studies have shown a weak correlation between mRNA and protein expression levels. The formation of mRNA is only the first step in a long series of events that results in the synthesis of a protein. The proteins formed are subject to posttranslational modifications. Coşkun et al. measured hAPE1 with isotope dilution tandem MS method in healthy and tumorous tissues breast cancer. However, the method studied has a lower resolution than the method in this study. For this reason, multiple reaction monitoring mode was used

Table 1: Identification of the tryptic peptides of APE1 and the *m/z* values of the monoisotopic masses of their MH^+ and $(M+2H)^{2+}$ ions.

		Unlabeled peptide						^{15}N-labeled peptide			
Peak	**Peptide**	**Retention time (min)**	**MH^+**	**$(M+2H)^{2+}$ (Exact)**	**$(M+2H)^{2+}$ (Measured)**	**Calculated mass accuracy (ppm)**	**MH^+**	**Retention time (min)**	**$(M+2H)^{2+}$ (Exact)**	**$(M+2H)^{2+}$ (Measured)**	**Calculated mass accuracy (ppm)**
1	TEPEAK	1.36	674.33555	337.67141	337.67096	1.33	681.31479	1.39	341.16103	341.16043	1,76
2	GLVR	3.46	444.29289	222.65009	222.65031	0.99	451.27214	3.43	226.13971	226.13969	0,09
3	LEYR	4.16	580.30894	290.65811	290.65845	1.17	587.28818	4.16	294.14773	294.14758	0,51
4	NVGWR	6.49	631.33107	316.16917	316.16910	0.22	641.30142	6.48	321.15435	321.15421	0,44
5	AWIK	7.16	517.31329	259.16029	259.16068	1.50	523.29550	7.16	262.15139	262.15143	0,15
6	WDEAFR	9.06	823.37333	412.19030	412.19092	1.50	833.34368	9.06	417.17548	417.17599	1,22

(13). In addition, no studies have been carried out on leukocyte samples. Demonstrating that hAPE1 is measurable in a sample of leukocytes instead of tissue could help to understand not only cancer but also the role of this molecule in other diseases.

CONCLUSION

Application of LC-Exactive Plus Orbitrap MS for analysis of APE1 protein with a high- resolution MS analysis was demonstrated. High resolution MS analysis produces high selectivity and sensitivity based on the exact mass measurement of protonated ions using a narrow mass extraction window. Thus, in the next stage, the way to perform simultaneous measurement of other proteins in the BER pathway in leukocyte samples with LC-HR-MS has been opened. It is thought that the accurate measurement of DNA repair proteins in various diseases, especially in leukocyte samples, is important for the evaluation of a predictive and prognostic biomarker, the development and use of inhibitors in cancer therapy, and the monitoring of treatment processes.

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