Extreme expression of DNA repair protein apurinic/apyrimidinic endonuclease 1 (APE1) in human breast cancer as measured by liquid-chromatography isotope-dilution tandem mass spectrometry

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Supporting Information Placeholder

ABSTRACT: Apurinic/apyrimidinic endonuclease 1 (APE1) is a DNA repair protein and plays other important roles. Increased levels of APE1 in cancer have been reported. However, available methods to measure APE1 levels are indirect and not quantitative. We previously developed an approach using LC-MS/MS with isotope-dilution to accurately measure APE1. Here, we applied this methodology to measure APE1 levels in normal and cancerous human breast tissues. Extreme expression of APE1 in malignant tumors was observed, suggesting that breast cancer cells may require APE1 for survival. Accurate measurement of APE1 may be essential to develop novel treatment strategies and APE1 inhibitors as drugs.

Apurinic/apyrimidinic endonuclease 1 (APE1) is a DNA repair protein in the base excision repair pathway, which cleaves apurinic/apyrimidinic sites generated by removal of modified DNA bases by DNA glycosylases.[1-3] APE1 provides over 95% of the total apurinic/apyrimidinic endonuclease function in mammals, and also possesses a redox regulatory portion with multiple other functions. The critical nature of APE1 functions is evidenced by early embryonic lethality in *ape1*$^{-/-}$ mice, by increased oxidative stress, mutagenesis and cancer incidences in *ape1*$^{+/-}$ mice, and by loss of neuronal cell function due to defects in APE1 activity.[4-13] Other adverse effects that are caused by depletion, and inhibition or down-regulation of APE1 include apoptosis[14,15], and sensitization to DNA-damaging agents.[16] Moreover, APE1 polymorphisms are associated with the disposition to cancer[9,10] APE1 expression is up-regulated or dysregulated in many human cancers. In general, APE1 overexpression is associated with many adverse effects, including resistance to therapy, prolonged therapeutic response and lower survival rates.[3,17-19] DNA repair and redox regulatory activities of APE1 affect various signaling pathways suggesting that cancer cells may be addicted to APE1 functions for survival.[3] Many aspects of APE1 as outlined above makes it a well-justified target in cancer for development of inhibitors as anticancer drugs. Thus, knowledge of APE1 levels in normal and malignant tissues may have prognostic and predictive significance in cancer treatment, potentially yielding the greatest therapeutic response, and helping develop inhibitors as anti-cancer drugs.[3,9,10,18]

Despite the importance of APE1 in cancer development and treatment, and extensive research in this field, there has been a paucity in positive identification of APE1 and accurate quantification of its levels in human tissues. In general, methods such as Western blotting and quantitative real-time PCR analysis have been used to estimate APE1 levels (e.g., see[20-27]). Some of these methods use antibodies, which may potentially exhibit some off-target binding, leading to false identification and quantification of the target protein. Comparison of stained areas, but no absolute quantification is given. No mass spectrometric evidence for positive identification is provided, and no internal standards are used for absolute quantification. We recently developed a novel approach involving liquid-chromatography isotope-dilution tandem mass spectrometry (LC-MS/MS) with isotope-dilution to positively identify and accurately quantify APE1 in human cells and tissues.[28] We produced and purified a completely ^{15}N-labeled full-length human APE1 (^{15}N-APE1) that can be used as an internal standard for quantification. For mass spectrometric measurements of proteins, a stable isotope-labeled internal standard, which is the analog of the target protein with identical chemical and physical properties, is absolutely essential for accurate measurements. Such a standard can be added into samples at the earliest step of experiments such as prior to the enrichment of target proteins by HPLC. Furthermore, it compensates for eventual losses during all stages of sample preparation and analysis, and avoids the measurement bias due to trypsin hydrolysis, which can be inefficient. We applied this approach to the measurement of APE1 in nuclear and cytoplasmic extracts of multiple human cultured cell lines and mouse liver. Identification of APE1 variants found in the human population has also been demonstrated.

In the present work, we attempted, for the first time, to identify and quantify APE1 levels in human tissues using the previously developed approach. Human disease-free breast tissues and malignant breast tumors were chosen to determine whether APE1 can be identified and quantified, and whether differences exist between the expression levels of APE1 in these tissues. We used commercially available protein extracts isolated from the disease-free breast tissues of 8 individuals and from the malignant breast tumors of 22 breast cancer patients. Only 8 protein extracts of disease-free breast tissues were commercially available for purchase. We were not able to obtain human tissue samples from other sources because of the lack of material transfer agreements with other institutions that would provide human tissues.

Table S1 of the Supporting Information shows the list of all female control individuals and breast cancer patients, from whom the protein extracts were obtained. An aliquot of ^{15}N-APE1 as an internal standard was added to 150 µg of each protein extract. The extracts were then separated by HPLC to enrich APE1. Figure S1 of the Supporting Information shows the superimposed elution profiles of protein extracts from samples of a disease-free breast tissue and a malignant breast tumor along with the superimposed elution profile of APE1. Fractions corresponding to the elution period of APE1 in this figure were collected, and then lyophilized, hydrolyzed with trypsin and analyzed by LC-MS/MS as described.[28] Se-

lected-reaction monitoring was used to monitor typical mass transitions of at least 8 previously detected tryptic peptides of APE1 (GLVR, NAGFTPQER, NVGWR, GAVAEDGDELR, WDEAFR, GLDWVK, EGYSGVGLLSR and QGFGELLQAVPLADSFR) and their ^{15}N-labeled analogs resulting from the internal standard ^{15}N-APE1. As examples, Figure 1A and B illustrate the ion-current profiles of the mass transitions of 5 tryptic peptides of APE1 and ^{15}N-APE1 obtained with a disease-free tissue sample (A) and a malignant tumor sample (B). In each case, the signal

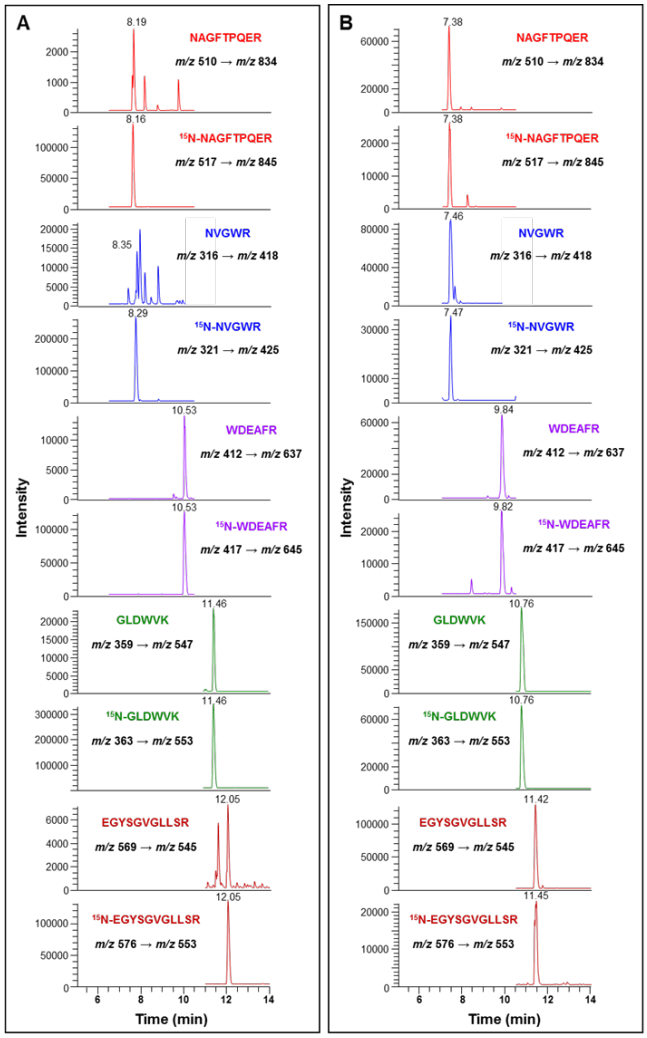

Figure 1. Ion-current profiles of mass transitions of 5 tryptic peptides of APE1 and ^{15}N-APE1 obtained using the tryptic hydrolysate of a protein fraction, which was collected during separation by HPLC of a protein extract from a disease-free breast tissue (A) and a malignant breast tumor (B).

of the mass transition of a tryptic peptide of APE1 was observed at the retention time of the corresponding tryptic peptide of the added internal standard ^{15}N-APE1. These results unequivocally identified the presence of APE1 in protein extracts of all tested tissue samples. The level of APE1 was calculated using the integrated signals of the mass transitions of the tryptic peptides of APE1 and ^{15}N-APE1, and the amount of ^{15}N-APE1 and the protein content. Figure 2 shows the levels of APE1 in disease-free tissues and malignant tumors. The numbers on the scattered data plot correspond to the disease-free individuals and cancer patients shown in Table S1 of the Supporting Information. The exact levels and the associated uncertainties of measurements are also given in this table. The numbers represent the mean of the levels of at least 4 tryptic peptides.

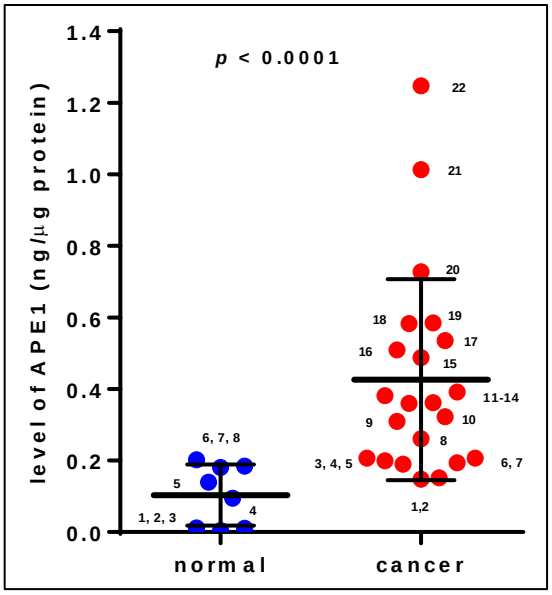

Figure 2. Levels of APE1 in disease-free breast tissues and in malignant breast tumors.

It should be pointed out that the SwissProt database (http://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi) using the taxonomy search *Homo sapiens* and 20203 entries yields a 100 % identification of APE1 with just 4 tryptic peptides. This means that the simultaneous measurement of 4 tryptic peptides of APE1 suffices for its positive identification and quantification in protein extracts. In general, malignant tumors exhibited far greater levels of APE1 than disease-free tissues. The statistical difference was highly significant with $p < 0.0001$ and a confidence level of 99 %. In a few cases, the levels of APE1 in both tissues were similar. However, a comparison of the 8 lowest APE1 levels in malignant tumors (numbers 1-8 in Figure 1 and Table S1 of the Supporting Information) with those in 8 disease-free tissues still yielded a statistical significance with $p = 0.015$ and a confidence level of 98 %.

Table S2 shows a comparison of the levels of APE1 measured in the present work (Figure 1 and Table S1) with those in nuclear and cytoplasmic protein fractions of mouse liver, and human cultured cells, which had been previously reported.[28] It is interesting to note that the APE1 level in mouse liver is quite similar to the average of the levels found in disease-free breast tissues. The APE1 levels found in cultured human cells were greater than those in normal human and mouse tissues. This may indicate the effect of cell culturing on expression levels of proteins and reflect the differences between cell culture and mammalian tissues. Moreover, it is well known that cultured cells are only one cell type, whereas tissues consist of mixtures of cell types. The APE1 levels measured by LC-MS/MS in this work and previously cannot be compared to those measured by other methods mentioned above,[20-27] because of the lack of absolute quantification.

It is well known that APE1 undergoes post-translational modifications such as phosphorylation, S-nitrosation, ubiquitination and acetylation.[29] Acetylation on K6 and K7, and on the K27 to K35 cluster has been linked to modulation of several APE1 activities.[22,29-32] Ubiquitination occurs at several lysines of the N-terminus of APE1 such as K7, K24, K25, K27, K32 and K35.[21,22,29] None of the tryptic peptides of APE1, which were identified by us in this work and previously,[28] contains the afore-mentioned acetylated or ubiquitinated lysines of APE1. On the other hand, APE1 phosphorylation was predicted to occur at 19T, 123T and 233T, affecting its repair activity and its redox regulation.[29,33,34] Only 233T is contained in one of the tryptic peptides (NAGFTPQER) of APE1 reported in this work (see Figure 1) and previously.[28] Taken together,

none of the reported post-translational modifications of APE1 would affect its identification and quantification in human tissues by our method. In other words, the reported levels of APE1 will include post-translationally modified APE1 molecules as well.

The present work shows that APE1 expression is drastically increased in malignant breast tumors when compared to disease-free breast tissues. This observation is on a par with the highly significant expression of APE1 in human mammary gland epithelial adenocarcinoma cells (MCF-7) when compared with MCF-10A normal cells of the same origin.[28] These findings suggest that breast cancer cells may be addicted to APE1 functions for survival. However, all cancers are not identical and APE1 expression differs among many human solid cancers.[3,35,36] This fact points to the importance of the accurate measurement of APE1 expression levels in disease-free tissues and malignant tumors, if APE1 is to be used as a reliable biomarker in cancer treatment. Our work is the first to report on the positive identification and absolute quantification of APE1 in human tissues using mass spectrometry and a stable isotope-labeled analog of APE1 as an internal standard. Due to our present limitation on obtaining human samples, commercially available protein extracts of breast tissues from different individuals were used. However, expression levels of a protein may differ among individuals. Therefore, expression levels of APE1 or any other DNA repair protein should be measured in malignant tumors and surrounding disease-free tissues of the same cancer patient. Such measurements would be ideal for future applications of our methodology to clinical samples, and may help predict and guide development of treatments, potentially yielding the greatest therapeutic response. This approach may also help in the development of APE1 inhibitors as potential anti-cancer drugs for personalized therapies.

ASSOCIATED CONTENT

Supporting Information

Table S1, Methods and Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

Extreme expression of DNA repair protein apurinic/apyrimidinic endonuclease I (APE1) in human breast cancer as measured by liquid-chromatography isotope-dilution tandem mass spectrometry

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Methods

Materials. Trypsin (Proteomics Grade), acetonitrile (HPLC-grade), acetonitrile plus 0.1 % formic acid (HPLC-grade), water (HPLC-grade), water plus 0.1 % formic acid (HPLC-grade) and trifluoroacetic acid (TFA) (HPLC grade, ≥ 99 %) were purchased from Sigma (St. Louis, MO). Water purified through a Milli-Q system (Millipore, Bedford, MA) was used for other applications. Protein extracts from human breast tissues (150 µg each) were purchased from OriGene (Rockville, MD).

Separation and enrichment of APE1 from protein extracts by HPLC. Protein extracts from human breast tissues (150 µg each) with a volume of ≈ 25 µL to 50 µL were spiked with an aliquot of ^{15}N-APE1 (0.15 µg) and used directly. In order to isolate and enrich APE1 prior to LC-MS/MS analysis, protein extracts were separated by HPLC using a liquid chromatograph equipped with an automatic injector and a diode-array detector (Agilent Technologies, Wilmington, DE), and a column specifically designed for protein separations (XBridge Protein BEH C4 column, 4.6 mm x 250 mm, 3.5 µm) with a precolumn insert (Delta-Pak C4, 5 µm) (Waters, Milford, MA). Mobile phases A and B were water with 0.1 % TFA (v/v) and acetonitrile with 0.1 % TFA (v/v), respectively. A gradient starting from 30 % B and linearly increasing to 66 % B over 9 min was used. B was then increased to 90 % in 0.1 min, kept at this level for 5 min and then decreased to 30 % B to equilibrate the column for 6 min. The flow rate was 1 mL/min. The effluents were monitored at 220 nm. Prior to separation of protein extracts, an aliquot of APE1 was injected to determine its elution time. The fractions at the elution time period of APE1 (≈ 1 min) were collected, and then dried in a SpeedVac prior to trypsin digestion.

Hydrolysis with trypsin and analysis by LC-MS/MS. Collected and dried fractions of protein extracts were incubated with 1 µg trypsin at 37 ºC for 2 h. Then, a second aliquot of 2 µg trypsin was added and incubation continued for an additional 22 h. Hydrolyzed samples were filtered by using a Nanosep® 3K Omega tube with molecular weight cutoff of 3000 (Pall Life Sciences, Ann Arbor, MI) for 20 min at 14000 x g. This was done to remove any particles and trypsin. Then, the filtered samples were concentrated in a SpeedVac under vacuum to ≈ 50 µL prior to LC-MS/MS analysis. The samples were then analyzed by LC-MS/MS as described previously.[1,2]

Statistical analysis. The statistical analysis of the data was performed using the GraphPad Prism 6 software (La Jolla, CA, USA) and unpaired, two-tailed nonparametric Mann Whitney test with Gaussian approximation and confidence level of 99 %.

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Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Table S1. Breast tissue samples and their characteristics as listed on OriGene's website (http://www.origene.com/tissue/tissueSearch.aspx), and the corresponding numbers and levels of APE1 in Figure 2. The levels of APE1 represent the mean of the levels of at least 4 tryptic peptides of APE1 in each sample. The uncertainties are standard deviations.

Type	Stage	Age	OriGene's code	Ethnicity (all females)	Number on Fig. 2	Level of APE1 (ng/μg protein)
breast within normal limits	N/A	63	565546	not reported	1	0.004 ± 0.001
breast within normal limits	N/A	61	606249	not reported	2	0.010 ± 0.001
breast within normal limits	N/A	72	565513	white or caucasian	3	0.012 ± 0.003
breast within normal limits	N/A	32	565676	black or African American	4	0.095 ± 0.014
breast within normal limits	N/A	34	565636	black or African American	5	0.139 ± 0.016
breast within normal limits	N/A	22	565648	not reported	6	0.172 ± 0.025
breast within normal limits	N/A	45	565563	black or African American	7	0.180 ± 0.033
breast within normal limits	N/A	30	565652	black or African American	8	0.202 ± 0.010
adenocarcinoma of breast, ductal	IIC	56	607640	not reported	1	0.148 ± 0.017
adenocarcinoma of breast, ductal	I	44	531533	white or caucasian	2	0.152 ± 0.020
adenocarcinoma of breast, ductal	IIC	50	542781	not reported	3	0.190 ± 0.024
adenocarcinoma of breast, ductal	IIB	44	544778	not reported	4	0.194 ± 0.046
adenocarcinoma of breast, ductal	IIIA	53	648319	not reported	5	0.199 ± 0.040
adenocarcinoma of breast, ductal	IIIC	27	629057	not reported	6	0.207 ± 0.056
adenocarcinoma of breast, ductal, metastatic	IIB	71	565628	white or caucasian	7	0.207 ± 0.041
adenocarcinoma of breast, ductal, mucinous	IIIB	86	815065	not reported	8	0.261 ± 0.043
adenocarcinoma of breast, ductal, metastatic	IIIA	61	552484	not reported	9	0.309 ± 0.050
adenocarcinoma of breast, ductal	IIA	57	626552	not reported	10	0.322 ± 0.042
adenocarcinoma of breast, lobular	IIA	77	543487	white or caucasian	11	0.360 ± 0.050
adenocarcinoma of breast, ductal	IIA	46	565605	not reported	12	0.362 ± 0.029
adenocarcinoma of breast, ductal	IIIC	61	603959	not reported	13	0.381 ± 0.092
adenocarcinoma of breast, metastatic	IV	55	624650	not reported	14	0.392 ± 0.067
adenocarcinoma of breast, ductal	IIB	89	531866	not reported	15	0.488 ± 0.095
adenocarcinoma of breast, metastatic	IV	41	518780	white or caucasian	16	0.509 ± 0.054
adenocarcinoma of breast, ductal	IIIA	34	565484	white or caucasian	17	0.535 ± 0.069
adenocarcinoma of breast, ductal	IIB	57	537436	white or caucasian	18	0.583 ± 0.077
adenocarcinoma of breast, ductal	IIB	89	565632	not reported	19	0.585 ± 0.091
adenocarcinoma of breast, lobular	IIIA	49	537799	not reported	20	0.728 ± 0.050
adenocarcinoma of breast, ductal, metastatic	IIIA	40	545030	not reported	21	1.013 ± 0.287
adenocarcinoma of breast, lobular, metastatic	IIIC	57	627491	not reported	22	1.247 ± 0.139

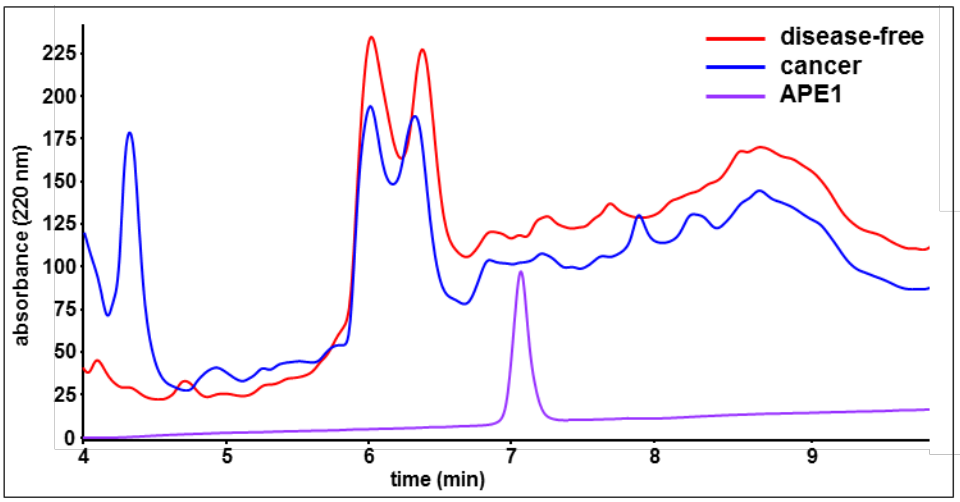

Figure S1. Superimposed elution profiles of protein extracts from a disease-free breast tissue and a malignant breast tumor. The elution profile of APE1 was also superimposed to show the retention time period where collections were made to enrich APE1 for subsequent analysis by LC-MS/MS.

Table S2. Comparison of the levels of APE1 in human tissues (this work) with those in nuclear and cytoplasmic protein fractions of mouse liver, and human cultured cells, which were previously reported.[1] The numbers for the human breast tissues represent the average of the numbers shown in Table S1. The uncertainties are standard deviations.

Breast normal	Breast cancer	Mouse liver nuclear	Mouse liver cytoplasmic	MCF10A nuclear	MCF10A cytoplasmic	MCF7 nuclear	MCF7 cytoplasmic	HepG2 nuclear	HepG2 cytoplasmic
0.104 ± 0.085	0.43 ± 0.28	0.091 ± 0.009	0.029 ± 0.006	1.12 ± 0.21	0.22 ± 0.05	2.86 ± 0.50	0.39 ± 0.06	1.68 ± 0.23	0.35 ± 0.07

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