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Extreme Expression of DNA Repair Protein Apurinic/Apyrimidinic Endonuclease 1 (APE1) in Human Breast Cancer As Measured by Liquid Chromatography and Isotope Dilution Tandem Mass Spectrometry

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Supporting Information

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 for measuring APE1 levels are indirect and not quantitative. We previously developed an approach using liquid chromatography and tandem mass spectrometry with isotope dilution to accurately measure APE1 levels. 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 for the development of novel treatment strategies and APE1 inhibitors as anticancer 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 >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 because of defects in APE1 activity.[4−13] Other adverse effects that are caused by depletion and inhibition or downregulation 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 upregulated 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 make 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 in the development of inhibitors as anticancer 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 of positive identification of APE1 and accurate quantification of its levels in human tissues. In general, methods such as Western blotting and quantitative real-time polymerase chain reaction (PCR) analysis have been used to estimate APE1 levels (e.g., see refs 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 of positive identification is provided, and no internal standards are used for absolute quantification. We recently developed a novel approach involving liquid chromatography−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 analogue of the target protein with identical chemical and physical properties, is absolutely essential for accurate measurements. Such a standard can be added to the samples at the earliest step of experiments such as prior to the enrichment of target proteins by high-performance liquid chromatography (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. We also demonstrated the identification of APE1 variants found in the human population.

In this work, we attempt, 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

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APE1 can be identified and quantified, and whether differences exist between the levels of APE1 expression in these tissues. We used commercially available protein extracts isolated from the disease-free breast tissues of eight individuals and from the malignant breast tumors of 22 breast cancer patients. Only eight 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 previously.[28] Selected reaction monitoring was used to monitor typical mass transitions of at least eight previously detected tryptic peptides of APE1 (GLVR, NAGFTPQER, NVGWR, GAVAEDGDELR, WDEAFR, GLDWVK, EGYSGVGLLSR, and QGFGELLQAVPLADSFR) and their ^{15}N-labeled analogues resulting from the internal standard ^{15}N-APE1. As examples, panels A and B of Figure 1 illustrate the ion current profiles of the mass transitions of five 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 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 four tryptic peptides.

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 four tryptic peptides. This means that the simultaneous measurement of four tryptic peptides of APE1 suffices for its positive identification and quantification in protein extracts. In general, malignant tumors exhibited levels of APE1 far greater than those of 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 eight lowest APE1 levels in malignant tumors (numbers 1–8 in Figure 1 and Table S1 of the Supporting Information) with those in eight 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 this work (Figure 2 and Table S1) with those in nuclear and

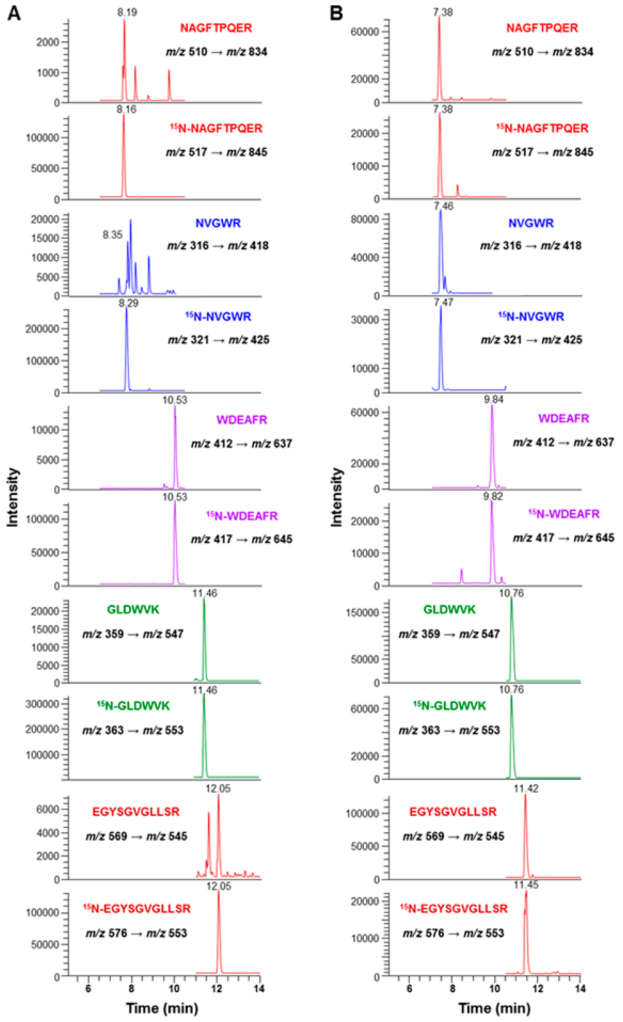

Figure 1. Ion current profiles of mass transitions of five tryptic peptides of APE1 and ^{15}N-APE1 obtained using the tryptic hydrolysate of a protein fraction, which was collected during separation by HPLC of a protein extract from a disease-free breast tissue (A) and a malignant breast tumor (B).

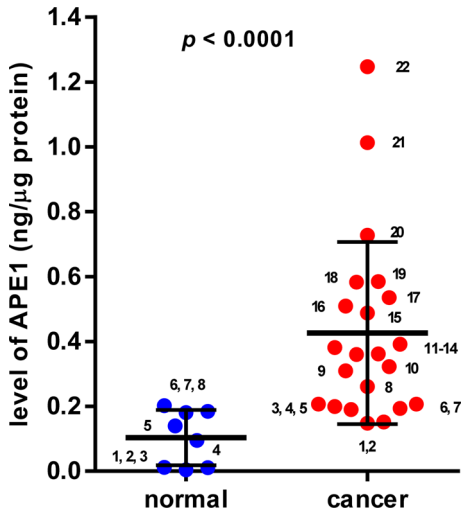

Figure 2. Levels of APE1 in disease-free breast tissues and in malignant breast tumors.

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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 previous work 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−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 aforementioned 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 previous work.[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.

This work shows that the level of APE1 expression is drastically increased in malignant breast tumors when compared to those in 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 analogue of APE1 as an internal standard. Because of 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 the development of treatments, potentially yielding the greatest therapeutic response. This approach may also help in the development of APE1 inhibitors as potential anticancer drugs for personalized therapies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00928.

Table S1, Methods, and Figure S1 (PDF)

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Notes

The authors declare no competing financial interest.

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Certain commercial equipment or materials are identified in this paper 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 is necessarily the best available for the purpose.

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