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Differential Protein Citrullination in Human ER− and ER+ Tumor and Adjacent Healthy Breast Tissue

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ABSTRACT: Post-translational modification of arginine to citrulline is catalyzed by members of the peptidylarginine deiminase (PAD) family. Dysregulation of this catalysis is a significant driver of the pathogenesis of numerous inflammatory diseases, including cancer. However, dysregulation of PAD activity has not been examined in breast cancer with respect to hormone receptor status. In this study, we measured PAD enzyme levels using Western blotting and investigated protein citrullination using a mass spectrometry-based proteomics approach in primary estrogen receptor negative (ER−) or positive (ER+) breast tumor and matched adjacent normal tissue. Our findings reveal 72 and 41 citrullinated proteins in ER− tumor and adjacent healthy tissue, respectively, where 20 of these proteins are common between the two groups. We detected 64 and 49 citrullinated proteins in ER+ tumor and adjacent healthy tissue, respectively, where 32 proteins are common. Interestingly, upon comparison of ER− and ER+ tumor tissue, only 32 citrullinated proteins are shared between the two and the rest are unique to the tumor's receptor status. Using the STRING database for protein−protein interaction network analysis, these proteins are involved in protein-folding events (i.e., heat shock proteins) in ER− samples and blood-clotting events (i.e., fibulin) in ER+ samples. Constituents of the extracellular matrix structure (i.e., collagen and fibrinogen) were found in both. Herein, we establish evidence that supports the role of this unique post-translational modification in breast cancer biology. Finally, to aid drug discovery against citrullination, we developed a liquid chromatography−ultraviolet method to measure PAD enzymatic activity and optimized glucagon-like peptide II to quantitatively measure the ability of PADs to citrullinate its substrate.

Arginine deimination (citrullination) is a type of post-translational protein modification in which arginine residues are converted into the amino acid citrulline.[1] This biological event is catalyzed by a group of calcium-dependent enzymes collectively known as the peptidylarginine deiminases (PADs), of which five isozymes are expressed in humans.[1,2] Citrullination has come into focus in recent years because of its involvement in not only physiological processes such as gene regulation, cell differentiation, and apoptosis but also pathological processes such as rheumatoid arthritis and cancer.[3−6] Previous studies have shown the overexpression of PAD enzymes in breast cancers and their roles in histone citrullination and tumor suppression.[7−9] Despite the many attempts to understand the roles of PAD enzymes in the onset and progression of various cancers such as breast cancer and other solid tumors, citrullination has not been extensively studied in cancer progression, oncologic therapeutic outcomes, or development of oncology drug resistance.[10,11]

Inflammation has been found to promote multiple hallmark features of cancer, in which citrullination has been reported as one of the ongoing processes.[12−14] Yuzhalin et al. reported that the extracellular matrix (ECM) of human liver metastatic tissues had higher levels of citrullinated proteins compared to those of unaffected liver tissue and that genetic and pharmacological intervention of PAD4, one of the isozymes of the PAD family, reduced the level of citrullination of proteins and ultimately liver metastatic growth.[14] Therapeutic estrogen found in hormone replacement therapy (HRT) is a known inducer of citrullinated neoantigens, but the identity and causative link between citrullination and tumorigenesis have not been established.[14−16] Consequently, there is preliminary evidence supporting the study of the entire complement of citrullinated proteins in breast cancer and, more specifically, the differences in the citrullinome between hormone receptor status, as it may contain information necessary for identifying markers of disease progression and to support the development of diagnostics and targeted therapeutics.[15]

The concentrations of PAD enzymes are known to increase during the progression of various cancers compared to those of matched normal tissues, including breast cancer.[5,17] The molecular weights of intact PAD enzymes range from 74 to 76 kDa and have been previously reported within this range using Western blot analysis.[18] Measurement of the concentration of PAD enzymes is considered to be a robust way of interpreting protein citrullination; however, studies comparing PAD levels in tumor versus matched adjacent tissue are lacking in terms of their hormone receptor status. We used Western blot and mass spectrometry-based methods in tumor and adjacent normal tissues to decipher these differences from patient samples. Whole tissue lysates were prepared from tumor and adjacent

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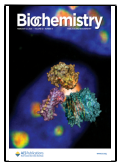

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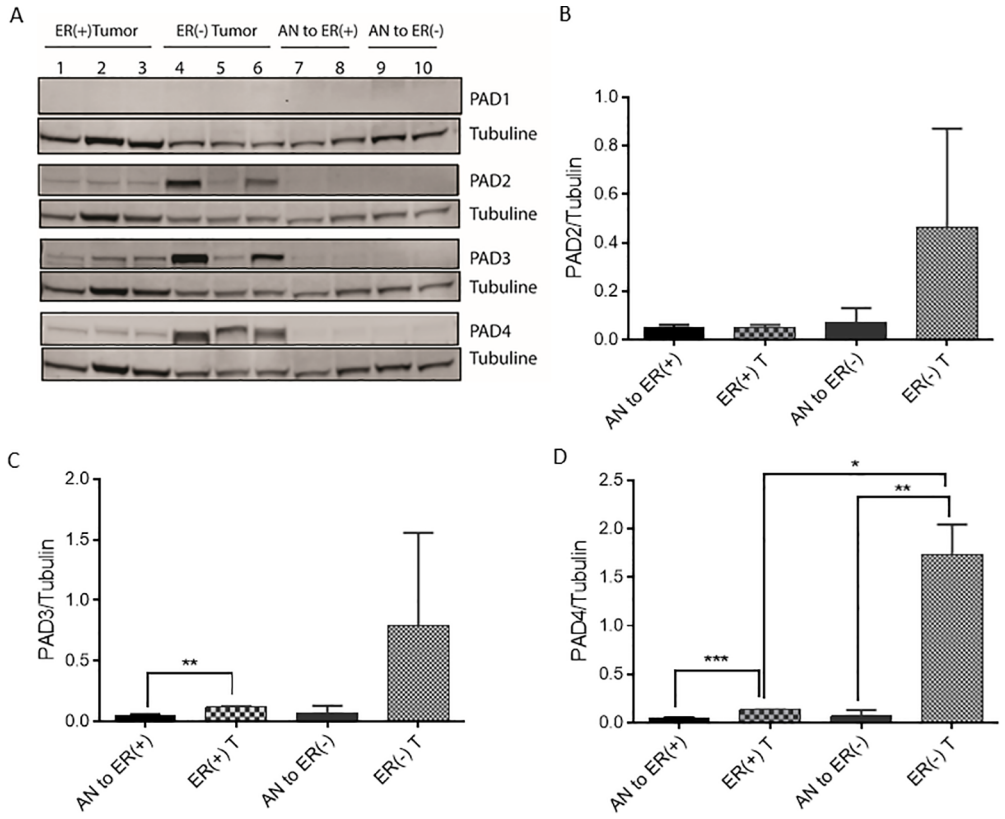

Figure 1. Western blot analysis of PAD enzymes in primary breast tumor and adjacent normal tissue. (A) Representative Western blot images of PAD1−PAD4 and tubulin and semiquantitative analysis of (B) PAD2, (C) PAD3, and (D) PAD4 normalized to tubulin in the corresponding blot. The level of PAD1 was below the detection limit and was not included for statistical evaluation. PAD protein levels in tumor tissue with ER+ or ER− with corresponding matching adjacent normal tissue were analyzed using an unpaired two-tailed Student's *t* test and are expressed as the mean ± standard deviation from three independent biological replicates with at least three technical replicates for each biological replicate. All three adjacent normal samples to ER− and ER+ breast tumor tissue were included in the analysis because a 12-well gel was used, and only two matching normal tissue samples to ER+ and ER− are shown in the representative gel. AN = adjacent normal. T = breast tumor. *$p < 0.05$. **$p < 0.001$. ***$p < 0.0001$.

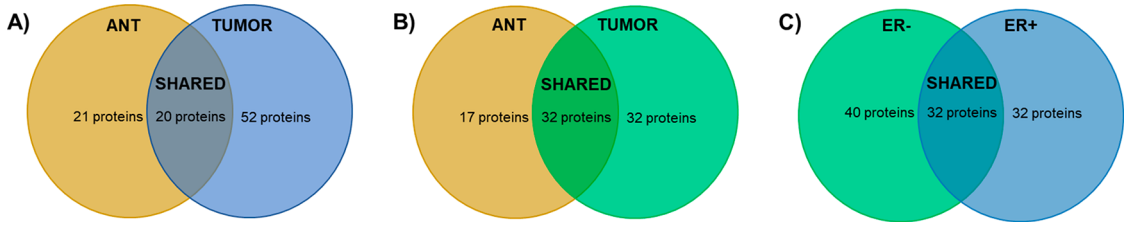

Figure 2. Identification of citrullinated proteins in (A) ER− and (B) ER+ tumor and adjacent normal tissue (ANT) of breast. (C) Comparison of citrullinated proteins between ER− and ER+ breast tumor tissue.

normal tissue and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis for Western blot analysis. We observed greater levels of PAD2−PAD4 in estrogen receptor negative (ER−) breast tumor tissue than in estrogen receptor positive (ER+) tumor tissue or adjacent normal tissue. The level of PAD4 was significantly greater in breast tumor tissue than in adjacent normal tissue, and PAD4 levels were significantly greater in ER− breast tumor tissue than in ER+ breast tumor tissue. In general, breast tumor tissues were found to contain greater levels of all PAD enzymes compared to those of adjacent normal tissue, but because of the high variability between patient samples and the limited number of samples used in this study, the expression level was not statistically significant for all PAD enzymes (Figure 1A−D). PAD1 levels were found to be below the detection limit in all tissue types. Overexpression of PAD1, PAD2, and PAD4 has been reported in a variety of cancers, including breast cancer, although the ER status has not been defined in those studies.[7,10,19,20]

To compare the citrullinome in tumor and matched adjacent normal breast tissue, we used a liquid chromatography−mass spectrometry-based proteomics approach. The tissues were homogenized to extract proteins, and peptides from the trypsin-digested protein extracts were injected into the mass spectrometry instrument as detailed in the Supporting Information. The resulting mass spectrometry data were analyzed using PEAKS software where citrullination was defined as a +0.98 Da shift at arginine residues. Consistent with greater levels of PAD enzymes in ER− breast tumor tissue, we observed a greater number of citrullinated proteins in ER− breast tumor tissue than in ER+ tumor tissue, and overall, more citrullinated proteins were found in tumor tissue than in adjacent healthy tissue from the same patient. A full list of citrullinated proteins

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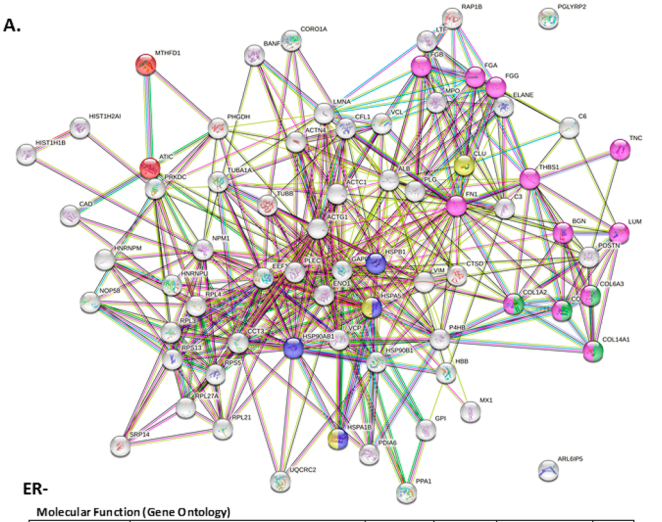
Biochemistry 2023, 62, 893−898

detected and peptides with citrullinated sites and subcellular localization is provided in supplementary file S2. We compared citrullinated peptides identified in our study with full or partial citrullinated peptides and citrullinated sites previously published by other studies. The matching citrullinated peptides with their respective citrullinated sites and relevant references are also provided in supplementary file S2. We identified a total of 72 citrullinated proteins in ER− breast tumor tissue and 41 citrullinated proteins in adjacent normal tissue, where 20 of these proteins are shared between the two (Figure 2A). For ER+ breast tissue, 64 proteins were found to be citrullinated in tumor and 49 in adjacent normal tissue, where 32 proteins are shared (Figure 2B). With this data set, we sought to compare the similarities and differences between the citrullinated proteins found in ER− and ER+ breast tumor tissue and provide hypothesis-generating leads about potential mechanisms for the differences. Interestingly, we found 32 citrullinated proteins shared between the two data sets, and the rest were unique to their respective receptor status (Figure 2C). A full list of citrullinated proteins identified in ER+ tumor, ER− tumor, and corresponding adjacent normal tissues and a list of shared proteins are provided in supplementary file S3.

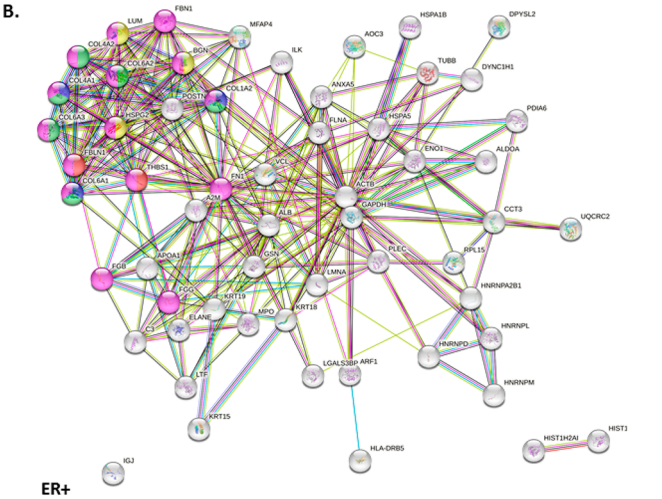
To further investigate the mechanistic drivers of differences between ER− and ER+ tumor tissue regarding the citrullinated proteins identified, we utilized the STRING database (https://string-db.org) to generate a protein−protein interaction (PPI) network. This allowed us to determine the functional interactions, i.e., molecular function, in which these proteins are involved and how these might be linked to the tumor status of the tissue.[21,22] The top five molecular functions were selected on the basis of the strength of the enrichment effect reported. Interestingly, the top molecular functions in ER− involved constituents of the ECM structure and protein-folding events (Figure 3A), while proteins found in ER+ tissue were also found to be associated with constituents of the ECM structure but differed in blood-clotting events (Figure 3B). ECM is a major structural component for the tumor microenvironment, and its remodeling promotes tumorigenesis, metastasis, and drug resistance.[23] The matrix composition plays a key role in the proliferation and progression of both ER+ and ER− cancer cells.[24] Hormone receptor status and selective estrogen modulator treatment of tumors such as tamoxifen impact the occurrence of recurrence or secondary malignancies. While having breast cancer likely increases the risk for recurrent breast cancer, more studies are needed to address the hypothesis that the ER status of tumors may be a risk factor for subsequent cancers in breast cancer.[25,26] Our preliminary data suggest that different levels of protein citrullination exist in ER+ and ER− breast cancers with more citrullinated proteins in ER− tumors than in ER+ tumors; therefore, citrullination status may be a critical factor for recurrence or occurrence of secondary malignancies given the link between citrullination and tumor microenvironment.

To establish differential citrullination in tumor tissue and test mitigation strategies that target increased levels of citrullination, it is critical to analyze PAD activity using a reliable and quantitative method. In the past, several techniques, including colorimetric, spectrophotometric, and fluorometric methods, were developed to measure PAD activity on arginine deimination.[27] Although most of these assays are reasonably easy to perform, they do come with significant technical and translational limitations. Colorimetric and spectrophotometric assays suffer from low sensitivity; fluorometric assays, although highly sensitive, can be costly, and a small amount of impurities may influence fluorescent readouts.[27,28] Recent advances in mass spectrometry (MS) have made it one of the most common



Molecular Function (Gene Ontology)

GO-term	Description	Count in network	Strength	False discovery rate	
GO:0019238	Cyclohydrolase activity	2 of 5	2.04	0.0254	
GO:0044183	Protein folding chaperone	4 of 26	1.62	0.00072	
GO:0030020	Extracellular matrix structural constituent conferring tensile strength	4 of 28	1.59	0.00089	
GO:0051787	Misfolded protein binding	3 of 26	1.5	0.0180	
GO:0005201	Extracellular matrix structural constituent	12 of 119	1.44	8.23e-11	



Molecular Function (Gene Ontology)

GO-term	Description	Count in network	Strength	False discovery rate	
GO:0070051	Fibrinogen binding	2 of 4	2.23	0.0188	
GO:0048407	Platelet-derived growth factor binding	3 of 11	1.96	0.0019	
GO:0030020	Extracellular matrix structural constituent conferring tensile strength	6 of 28	1.86	5.21e-07	
GO:0030021	Extracellular matrix structural constituent conferring compression resistance	3 of 14	1.86	0.0031	
GO:0005201	Extracellular matrix structural constituent	15 of 119	1.63	5.49e-17	

Figure 3. STRING plot showing the molecular function of proteins found to be citrullinated in ER− and ER+ breast tumor tissue. Each node corresponds to a protein, and the thickness of the lines represents the strength of the interaction. The colored nodes (not white) indicate the molecular function in which a protein is involved. (A) Proteins found in ER− tumor have molecular functions associated with cyclohydrolase activity (red), protein folding (blue), extracellular matrix structural constituent (green and fuchsia), and misfolded protein binding (yellow). (B) Proteins found in ER+ tumor have molecular functions associated with fibrinogen binding (red), platelet-derived growth factor binding (blue), and extracellular matrix structural constituent (green, fuchsia, and yellow).

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Biochemistry 2023, 62, 893−898

analytical tools for characterizing and quantifying post-translational modifications (PTMs) of proteins.[29] Unfortunately, a small change in mass (0.98 Da) observed upon citrullination often makes it difficult to differentiate between citrullinated and noncitrullinated peptides. In an effort to develop a sensitive and quantitative method for measuring PAD activity, we first established the kinetic parameters of PAD1−PAD4 and PAD6 using glucagon-like peptide II (GLPII) as their substrate. We then optimized a liquid chromatography−ultraviolet (LC−UV) detection method to distinguish citrullinated and noncitrullinated peptides by leveraging the changes in their retention time.

In pilot experiments, we determined that GLPII was the preferred substrate for this assay due to the clearest separation of the citrullinated versus noncitrullinated peaks in the LC−UV chromatograms (not shown). The assay was therefore first optimized using seven different concentrations of the GLPII substrate in a 50 μL reaction mixture containing 500 ng of PAD1−PAD4 and PAD6 with 10 mM calcium, as these enzymes are dependent on calcium for their activity.[4] After 15 min, a small aliquot was removed from the reaction mixture and the reaction was quenched with ethylenediaminetetraacetic acid (EDTA) before analysis. GLPII was confirmed to be a substrate of PAD1−PAD4, as shown by their kinetic parameters (Table 1 and Figure S1). Due to critical mutations, PAD6 is considered inactive and no protein substrate to PAD6 has been identified; however, its expression is important for citrullination as it may function as an activator of other active PAD enzymes.[30−32] As expected, PAD6 showed no activity toward GLPII. Our method was highly reproducible and is based on changes in retention time due to citrullination. While this method may not be suitable as a high-throughput tool for the measurement of PAD activities in a complex biological environment such as tissue samples, it can be applied for accurate measurement of PAD activities during drug development and screening of therapeutics that inhibit or reduce PAD enzyme activity.

Table 1. Kinetic Parameters for GLPII Using PAD1−PAD4

	K_m (μM)	V_{max} (μM/min)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
PAD1	32.81	1.31	0.17	5181.35
PAD2	44.01	1.58	0.20	4544.42
PAD3	40.21	2.08	0.27	6714.75
PAD4	86.77	4.50	0.57	6569.09

In this exploratory study, we established two novel tools and data sets to allow for expanded research on citrullination in cellular processes and drug development. Western blot analyses and proteomics approaches not only revealed similarities and novel differences between adjacent normal and tumor breast tissue with regard to PAD enzyme concentrations and citrullinated proteins but also distinguished the molecular functions impacted by this PTM (Figure 3). On the basis of our study, PAD1−PAD4 can citrullinate the same peptide but with distinct reaction rates (Table 1). The differences in reaction rates for each of the PADs, coupled with differential expression of the PADs in breast ER− and ER+ tumors, may account for the variability in citrullinated proteins between tumor types and rates of progression or occurrence of secondary malignancies.[25] Proteomics, in general, permits for the discovery of new, clinically relevant, protein markers and an overall better understanding of biological pathways.[33] Given our small number of human samples, four ER+ normal adjacent and tumor tissues and three ER− normal adjacent and tumor tissues, in this pilot study, additional and larger studies are needed to confirm the citrullinated proteins identified and to perform a correlation with clinical outcomes. Citrullination of proteins between ER− and ER+ tissue can also be further explored by improving tools such as the analytical method presented in this report to allow medium- and high-throughput drug screening. The LC−UV approach allows us to reproducibly discriminate between citrullinated and noncitrullinated peptides by their retention times and to determine enzyme kinetic parameters for the physiologically relevant GLPII substrate. We established an analytical tool for measuring the activity of PAD enzymes to aid drug discovery in this context. These advancements could pave the way for better interpretation and integration of citrullination as part of proteomic data sets, and that data could be applied for the development of potential diagnostic and therapeutic applications.

■ ASSOCIATED CONTENT

Data Availability Statement

All data supporting the findings of this study are available within the manuscript and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00551.

Experimental procedure and Figure S1 (PDF)

A list of citrullinated peptides and a list of the corresponding proteins and their subcellular localization (XLSX)

A list of citrullinated proteins shared among ER+ tumor, ER− tumor, and corresponding adjacent normal tissues as used in the Venn diagram in Figure 2 (XLSX)

Accession Codes

Access numbers to the data sets generated and or analyzed during this study are available in the UniPort repository: Q9ULC6 for PAD1, Q9Y2J8 for PAD2, Q9ULW8 for PAD3, and Q9UM07 for PAD4.

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Notes

The authors declare the following competing financial interest(s): J.T. is currently employed by Pfizer Inc., and L.L. is currently employed by BTIG, LLC.

This study was approved by FDA Research Involving Human Subjects Committee, an institutional review board (IRB) of FDA.

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Biochemistry 2023, 62, 893−898