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Profiling of Cross-Functional Peptidases Regulated Circulating Peptides in BRCA1 Mutant Breast Cancer

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Abstract

Women with inherited *BRCA1* mutations are more likely to develop breast cancer (BC); however, not every carrier will progress to BC. The aim of this study was to identify and characterize circulating peptides that correlate with BC patients carrying *BRCA1* mutations. Circulating

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Author Contributions

J. F. and M.-K.M.T. contributed equally to this work. J.F., M.-K.M.T., T.Y.H., and M.F. designed the research plan. J.F. performed the experiments. M.-K.M.T. and C.F.S. collected the clinical samples. J.F. and M.-K.M.T. performed data analysis. J.F., M.-K.M.T., T.Y.H., and M.F. wrote the manuscript, and all authors contributed to the revision of the manuscript. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00010. Peptide profiles obtained by MALDI-TOF MS after Nanotrap enrichment and fractionation from clinical serum samples; MS/MS spectra of the two peptides biomarkers; expression levels of daughter peptides from KNG1K438-R457 at 2209 *m/z* (KHNLGHGKHHERDQGHGHQ) and 2080 *m/z* (HNLGHGKHHERDQGHGHQ); mass spectra of peptide products of KNG1E434-L461 after cleavage by purified KLK2; phase contrast and GFP expression under a fluorescence microscope; mass spectra of KLK2 related peptide products in MCF-7 cell extraction before and after knockdown of MCF-7; immunoblotting image of CPN1 in serum samples (PDF)

Demographic information for cancer patients; individual patient information in SBC; individual information on healthy women and healthy BRCA1 carriers; individual patient information on BBC; identification of peptides by LC-MS/MS; identified peptides *t* test analysis; area under the curve; actual numbers divided by optimal cutoff value; enzymes predicted by MEROPS to generate kininogen-1 fragments; matching peptides for unspecific cleavage (PDF)

Supplementary Table S-5. Identification of peptides by LC-MS/MS (XLSX)

Supplementary Table S-6. Identified peptides *t*-test analysis (XLSX)

peptides were enriched using our well-designed nanoporous silica thin films (NanoTraps) and profiled by mass spectrometry to identify among four clinical groups. To determine the corresponding proteolytic processes and their sites of activity, purified candidate peptidases and synthesized substrates were assayed to verify the processes predicted by the MERPOS database. Proteolytic processes were validated using patient serum samples. The peptides, KNG1_{K438-R457} and C3f_{S1304-R1320}, were identified as putative peptide candidates to differentiate *BRCA1* mutant BC from sporadic BC and cancer-free *BRCA1* mutant carriers. Kallikrein-2 (KLK2) is the major peptidase that cleaves KNG1_{K438-R457} from kininogen-1, and its expressions and activities were also found to be dependent on *BRCA1* status. We further determined that KNG1_{K438-R457} is cleaved at its C-terminal arginine by carboxypeptidase N1 (CPN1). Increased KLK2 activity, with decreased CPN1 activity, results in the accumulation of KNG1_{K438-R457} in *BRCA1*-associated BC. Our work outlined a useful strategy for determining the peptide–peptidase relationship and thus establishing a biological mechanism for changes in the peptidome in *BRCA1*-associated BC.

Keywords

breast cancer; *BRCA1* mutation; circulating peptides biomarker; kallikrein-2; Kininogen-1

INTRODUCTION

Breast cancer (BC) ranks first among cancer-related deaths in women aged 20–60 years, and it is expected to account for about 29% of all new cancer cases among women in 2016.¹ Hereditary factors dictate about 10% of BC cases, chief among them are mutations of the tumor suppressor gene *BRCA1* identified in 1994.² Genetic alterations in *BRCA1* are responsible for approximately 50% of these hereditary malignancies.^{2,3} *BRCA1* functions via a homologous recombination-mediated, double-stranded DNA-repair mechanism, which serves to maintain genome stability. DNA damage due to a malfunctioning repair system increases the risk and incidence of tumorigenesis.^{4,5}

The probability of developing BC is significantly increased in women with highly penetrant germ-line mutation(s) in *BRCA1*. About 57% of female carriers of *BRCA1* mutations develop BC by 70 years of age.^{6–11} These women also tend to develop BC at a younger age compared to their peers with sporadic BC. A notable portion (30–50%) of women carrying *BRCA1* mutations never develop BC,^{6,12,13} but there are no clear features associated with BC development in this population, and only a few studies have attempted to determine the

protein profile associated with *BRCA1* mutant BC.^{14–16} Becker et al. detected 35 proteins that were overexpressed in *BRCA1* cancer patients using surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) mass spectrometry¹⁶ but reported only protein molecular weights without identifying specific proteins. Warmoes et al. identified several markers associated with *BRCA1* deficiency in a proteomics study of mouse *BRCA1*-deficient mammary tumors, but these results have yet to be replicated with *BRCA1* BC patients.¹⁵ Finally, a recent study profiled the plasma proteomes of four patients with *BRCA1* mutant BC, four healthy carriers, and four healthy relatives and found an association with gelsolin although this was a small study.¹⁴ Better understanding of what drives *BRCA1* mutant BC may lead to new biomarkers and new treatments. PARP inhibitors are currently under evaluation as targeted therapy for metastatic *BRCA1* mutant BC in a phase III clinical trial;¹⁷ however, there are remaining questions in ongoing targeted therapy research, where better understanding may identify resistance mechanisms and potential therapy targets.

Currently, genetic tests for *BRCA1* and other BC-related gene mutations are used in the clinic to estimate risk and formulate prevention strategies. Although genetic testing identifies mutation status, it does not provide information about additional factors that influence disease development. Recent studies indicate the important role of proteases and peptidases during tumor angiogenesis, invasion, and metastasis.¹⁸ Biopsies are usually needed to evaluate tumor-resident proteases/peptidases for their disease biomarker potential; however, protease/peptidase cleavage products, due to size, likely enter the blood circulation where they may serve as more accessible information conduits than the enzymes themselves.¹⁹ Previous studies have illustrated the use of circulating peptides as potential biomarkers for cancer diagnostics and therapeutic evaluations;^{20–23} however, only a few studies have shown a direct correlation between those circulating peptides and their associated proteases/peptidases.^{24–26}

Our group has developed nanoporous silica thin films (NanoTraps) for peptide enrichment prior to mass spectrometry (MS) analysis as a robust technology platform for accurate and reproducible biomarkers detection.^{27–30} We have used NanoTrap-MS to monitor peptides secretion at different stages of melanoma with lung metastases and to identify peptide markers for early detection in breast cancer.^{20,23} In this study, we applied NanoTraps to identify and profile circulating peptides that could distinguish *BRCA1* carriers with breast cancer from the healthy carriers and sporadic BC. We further demonstrated a direct link between these peptides and their corresponding tumor-resident peptidases.

MATERIALS AND METHODS

Clinical Samples Collection

The human specimens (132 serum samples) used in this study were collected at the Medical University of Vienna from patients who gave informed consent in a study approved by the Institutional Review Board. All specimens were collected as nonfasting samples in an outpatient setting. Specimens were allowed to clot at room temperature for 60 min before centrifugation. The serum was then collected and aliquoted immediately and stored at -80°C . Serum samples from cancer patients were collected at the time of diagnosis or a few

days after diagnosis. Retrospective samples were given coded labels, and ages were assigned at the time of collection for healthy controls, and at the time of cancer diagnosis for BC patients. Each sample was tested for the *BRCA1* mutation(s). Patient characteristics are listed in Table 1, Supplementary Table S1, and the individual patient information, including age, histologic types, grading, TNM staging, and hormonal factors, is shown in Supplementary Tables S2–S4.

Cell Lines

MDA-MB-231, MCF-7, and MCF-10A cell lines were obtained from the ATCC. The HCC1937 cell line was kindly donated by Dr. Haifa Shen (Houston Methodist Research Institute). Two human BC cell lines, MDA-MB-231 and MCF-7, were maintained separately as adherent monolayers in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum (FBS) for the studies described below. The human BC cell line HCC1937, harboring *BRCA1* mutations, was cultured in RPMI-1640 medium with 10% FBS. The human breast epithelial cell line MCF-10A was cultured in DMEM/F12 (1:1 vol/vol) medium supplemented with 5% horse serum, hydrocortisone (0.5 $\mu\text{g/mL}$), insulin (10 $\mu\text{g/mL}$), and epidermal growth factor (20 ng/mL). All cell cultures were also supplemented with penicillin (100 U) and streptomycin (100 $\mu\text{g/mL}$). Authentication of cell lines was conducted utilizing short tandem repeat (STR) DNA fingerprinting at MD Anderson Cancer Center's "Characterized Cell Line Core". The *BRCA1* mutation in HCC1937 (a C insertion at nucleotide 5382) was determined by DNA sequencing.

Peptide Expression Profiling

Sample buffer consisting of 50% acetonitrile (ACN; Sigma-Aldrich, St. Louis, MO, USA) and 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, St. Louis, MO, USA) was prepared in deionized water, and 2.2 μL was added to a 20 μL sample of serum that was thawed on ice. NanoTraps were fabricated as described previously.²⁹ Five microliters of each serum was pipetted into the sample chamber. Samples were incubated at 25 °C in a humidified chamber for 30 min, after which the wells were washed four times with water. Five microliters of sample buffer were added to release peptides from the nanopores, and the peptides fractions were transferred into a tube for further analysis.

MALDI-TOF MS Detection

To prepare the samples for matrix-assisted laser desorption/ionization (MALDI)-TOF MS analysis, 0.5 μL of each sample was spotted on the MS target plate and allowed to dry completely. Once dry, 0.5 μL of matrix solution [5 g/L of acyano-4-hydroxycinnamic acid (CHCA) in 50% ACN and 0.1% TFA] was spotted on the target plate and left to air-dry. All of the samples were analyzed on an Applied Biosystems 4700 MALDI-TOF Analyzer (Applied Biosystems, Inc., Framingham, MA, USA), operated in positive ion mode with reflector (set laser intensity at 4300 and 5000 shots/sample and mass range 800–5000 Da, with target mass of 3000 Da).

Peptide Identification by LC–MS/MS

Reversed-phase chromatography was performed on an Agilent 1200 series HPLC autosampler. As gradient solvents for liquid chromatography (LC) analysis, 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used. Samples were dried in a vacuum centrifuge and resuspended in solution (1% formic acid and 5 mM NH₄OAc) prior to loading into the HPLC sample port. Analysis of the peptides was conducted on an Orbitrap-XL mass spectrometer (Thermo Scientific, Waltham, MA). The peptides were eluted using a linear gradient of 5–40% ACN over 75 min with flow rate at 0.3 μ L/min. The electrospray source maintained at 2.1 kV. Acquisition parameters included: 1 FTMS scan at 60 000 resolution followed by three MS/MS product ion scans (in the ion trap) of two microscans each, 400–2000 Da mass range for MS1, 2000 ion counts as the threshold for triggering MS2, 0.5 Da for mass window of precursor ion selection, relative collision energy at 30%; +2, +3, +4, and +5 charge state for screening, 15 s as dynamic exclusion. The MS data obtained were processed using Proteome Discoverer (Version 1.4.1.14, Thermo Fisher Scientific, Germany) and screened against the SwissProt (SwissProt 010913 (538 849 sequences; 191 337 357 residues)) protein database using the Mascot search engine (Matrix Science, Boston, MA). The precursor and fragment mass tolerances were set to 15 ppm and 0.5 Da, respectively, with a 1.5 signal-to-noise ratio allowance. False discovery rates (FDRs) were determined by searching against a decoy database (0.01 FDR strict –0.05 FDR relaxed). Parameters for the searches were no enzyme, and allowance of nine missed cleavages, the oxidation of methionine and pyro-glutamate formation as the dynamic modification.

BRCA1 shRNA Knockdown in MCF-7 and MDA-MB-231 Cells

MCF-7 or MDA-MB-231 cells were transduced with lentiviral vectors carrying BRCA1_shRNA_1, BRCA1_shRNA_2, or a control shRNA (Ctrl_shRNA) (Dharmacon, Chicago, IL). The sequences of shRNA 1 and 2 were 5'-TAAGGGACCCTTGCATAGC-3' and 5'-TTCAGTACAATTAGGTGGG-3', respectively. The transduced cells were selected using 2 μ g/mL puromycin (Invitrogen, Carlsbad, CA) for 48 h. Transduced cells were analyzed using immunoblotting to determine the level of BRCA1 expression.

Preparing Conditioned Medium

Once grown to approximately 80% confluence, the cells were washed three times with phosphate-buffered saline (PBS) and maintained in serum-free medium for an additional 24 h. Cells were removed by a two-step centrifugation process (300g, 5 min, 4 °C, and then 2000g, 10 min. Four °C) and lysed in Mammalian Protein Extraction Reagent (M-PER) (Pierce, Rockford, IL) containing protease inhibitors (Pierce). Clarified supernatant was collected and concentrated using 10K Millipore centrifugal devices (Amicon Ultra 10K, Millipore, Bedford, MA).

Peptidase Expression Assays

To examine KLK2 expression in serum samples, high-abundance proteins were first removed using Seppro IgY14 according to the manufacturer's instructions. The pass-through fraction containing KLK2 was measured using an in-house enzyme-linked immunosorbent

assay (ELISA) according to the direct ELISA using primary antibody protocol provided by Abcam. The primary anti-KLK2 antibody was obtained from Abcam (Cambridge, MA). Expression levels of CFI were measured using ELISA assay according to the manufacturer's instructions (USCN Life Science Inc., Wuhan, PR China). Western blotting analysis was performed as follows: proteins were separated by gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (precast gels from Bio-Rad); separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA); the membranes were probed with peptidase-specific primary antibodies and peroxidase conjugated secondary antibodies. Signals were visualized by chemiluminescence. The ImageJ program was used to analyze the density profiles for the protein bands and to normalize samples to the loading control. The primary antibodies used include anti-CPN1 (rabbit polyclonal antibody, Pierce) and anti-CFI (mouse monoclonal, Abcam).

RNA Isolation and Quantitative PCR

Total RNA was isolated from cells using TRIzol reagent (Life Technologies, Gaithersburg, MD) and reverse transcribed for quantitative real-time PCR. Expression of each peptidase was normalized to expression of GAPDH. The KLK2 primer sequences (forward) 5'-TCAGAGCCTGCCAAGATCAC-3' and (reverse) 5'-CACAAGTGTCTTTACCACCTGT-3' yielded a 250 bp PCR product.

Depletion of KLK2 and CPN1 from Patient Serum and Conditioned Media

Anti-KLK2 or anti-CPN1 antibodies were incubated for 2 h at 37 °C with either diluted patient serum or cell culture conditioned media (CM). Each mixture was incubated for 2 h at 37 °C with Protein A/G agarose beads (Pierce) and centrifuged to remove the antibody-peptidase complexes.

Assay for Peptide Degradation in Sera and CM

His-tagged KNG1 fragments (His₃-KNG1_{E434-L461}-His₃ and His₃-KNG1_{K438-R457}-His₃) were synthesized (98% purity) by GL Biochem (Shanghai, PR China). Prior to addition of the peptides, the serum samples were diluted 1/10 in Tris-Cl buffer, pH 7.5, and the CM were concentrated by buffer exchange into Tris-Cl buffer using 10 kDa centrifugation filters. The synthetic KNG1 fragments were then spiked into serum or concentrated CM at a final concentration of 100 μ M, and incubated for 3 h at 37 °C. Cleaved peptide products were fractionated and analyzed via Nanotrap-MS.

Statistical Analysis

The MALDI-TOF MS data were processed using MarkerView software v. 1.2.1 (AB SCIEX, Concord, Canada), and normalized to the internal standard peptide ACTH 18–39 (Sigma-Aldrich, St Louis, MO). Comparisons of MS data sets (i.e., different patient cohorts) were performed using the unpaired *t* test with a *p*-value cutoff of 0.05. Principal components analysis-discriminant analysis (PCA-DA) was carried out with a Pareto Scaling for MS data analysis. Receiver operating characteristic (ROC) curves, used to assess the accuracy of biomarker analysis, were performed with a logistic regression model using SPSS version 22 (Chicago, IL). Sensitivity against 1-specificity was plotted, and the area under the curve

(AUC) values were computed. Mann–Whitney U analysis of the target peptides was performed. Youden Index (sensitivity-specificity +1) was calculated, and the optimal cutoff values were determined by the maximal Youden index. Kendall's tau-b analysis was used to test the correlations between patient characteristics and each peptide markers. One-way or two-way ANOVA was performed for each comparison of ELISA, Western blotting data, and age differences among the four clinical groups using GraphPad Prism v.6 (GraphPad Software, La Jolla, CA). Quantitative image analysis of immunoblots was conducted using the software ImageJ (Bethesda, MD). All numerical data are presented as mean $\pm$ standard deviation, mean $\pm$ standard error, or 95% confidence intervals. All statistical tests were considered statistically significant if P was less than 0.05.

RESULTS

Serum Peptide Profiling by Nanotrap-MS

Clinical serum specimens (132 total) used in this study were collected from female patients who were tested for *BRCA1* mutations (Table 1). Of these 132 patients, 55 were carriers of hereditary *BRCA1* mutations, of whom 28 (median age = 48 years) were diagnosed with BC (BBC), and 27 (median age = 43 years) remained cancer-free (BH). Of the remaining patients, 39 (median age = 50 years) were diagnosed with sporadic breast cancer (SBC), and 38 (median age = 46) were healthy volunteers (WT). Samples in the four groups were age-matched, yielding a p -value of 0.124 when one-way ANOVA was performed to evaluate statistical differences.

All patient serum samples were processed on NanoTraps as previously described,^{20,28} and the enriched peptide fractions were subsequently analyzed by MALDI-TOF MS in the mass range of 800–5000 m/z . Approximately 500 monoisotopic peaks were observed in each MS spectrum (Supplementary Figure S1). Of those, 62 peaks were confirmed by LC-MS/MS (Supplementary Table S5) and imported into MarkerView software for standard t test analysis (Supplementary Table S6). We performed pairwise comparisons of the MS spectra generated from the four sample cohorts. A comparison of the BBC and WT spectra showed a significant increase in the expression of seven peptides in the BBC samples (fold change >1.5 , $p < 0.01$). The expression of two peptides was significantly increased in the BBC spectra when compared to that of SBC (fold change >1.5 , $p < 0.01$). When we aligned these two pair-comparisons, the peak at 2365 m/z appeared as a common factor. Thus, we considered it a potential biomarker for BBC (Figure 1A). When we compared the BBC spectrum against that of BH, one peak at 2021 m/z appeared elevated in the BBC sample (Figure 1B). The median relative intensity and 95% confidence interval (CI) values of the two peptides' relative intensity were shown in Table 1. There was no statistically significant correlation between the two circulating peptides and clinical variables (Supplementary Table S1). Taken together, these results implicate at least two putative circulating peptides were associated with BBC.

As shown in Supplementary Figure S2, LC-MS/MS analysis identified the 2365 m/z peptide peak (KHNLGHGKHERDQGHGHQR, KNG1_{K438-R457}) as part of high-molecular weight KNG1 and the 2021 m/z peak (SSKITHRIHWESASLLR, C 3f_{S1304-R1320}) as part of complement C3. To assess the diagnostic value of KNG1_{K438-R457} and C 3f_{S1304-R1320}, we

calculated the receiver operating characteristic (ROC) curves. AUC values were determined to be 0.794 (95% CI = 0.683–0.905) for KNG1_{K438-R457} for distinguishing BBC from SBC (Figure 1C and Supplementary Table S7), and 0.758 (95% CI = 0.638–0.877) for distinguishing BBC from WT (Figure 1D and Supplementary Table S7). The AUC values for KNG1_{K438-R457} and C 3f_{S1304-R1320} shown in Figure 1, panel E are 0.640 (95% CI = 0.489–0.791) and 0.685 (95% CI = 0.542–0.828), respectively, when comparing BBC to BH. The ROC curves were improved in a multivariate model using the two peptides, and AUC value was 0.739 (95% CI = 0.601–0.878) for distinguishing BBC from BH (Figure 1E). The optimal cutoff value (BBC vs WT) was obtained by the Youden index: the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy (ACC) were 0.750, 0.769, 0.700, 0.811, and 0.742, respectively, to differentiate patients with BBC from WT by KNG1_{K438-R457} (Figure 1F). By using the two peptides pattern, KNG1_{K438-R457} and C 3f_{S1304-R1320}, to identify BBC from BH, the sensitivity, specificity, PPV, NPV, and ACC are 0.540, 1.000, 1.000, 0.675, and 0.764, respectively. The optimal cutoff values for different comparison between groups, BBC versus WT, BBC versus SBC, or BBC versus BH, are shown in Supplementary Table S8. Therefore, we proposed to investigate the clinical value of KNG1_{K438-R457} and C 3f_{S1304-R1320} for distinguishing BBC from WT, SBC, or BH.

Peptidase Prediction and Validation

We searched the peptidase database, MEROPS, for peptidases that have potential to cleave KNG1 to KNG1_{K438-R457} (Supplementary Table S9). Human kallikrein-2 (KLK2) was identified as being capable of cleaving KNG1 at two sites, R437-K438 and R457-G458. We observed increased amounts of the Des-Arg-KNG1 fragment in BBC versus BH (Supplementary Figure S3). Carboxypeptidase N (CPN) cleaves peptides at the carboxy-terminal arginine, and it was demonstrated to cleave arginine from C 3f to generate Des-Arg-C 3f.³¹ We believe CPN1, the catalytic subunit of CPN, to be the peptidase responsible for generating Des-Arg-KNG1-fragments. For the other peptide marker, C 3f_{S1304-R1320}, a heptadecapeptide, is known to be cleaved from C3b by complement factor I (CFI).³¹

To verify the suggested peptidase–substrate pairing, we synthesized two versions of the KNG1 fragments, His₃-KNG1_{E434-L461}-His₃ and His₆-KNG1_{K438-R457}-His₆, containing KLK2 and CPN1 cleavage sites, respectively. These peptides were added to a solution containing purified recombinant peptidase or clinical samples. The degraded peptide fragments were extracted and analyzed using Nanotrap-MS. Each prominent peak in the MS spectra, representing a cleaved peptide fragment, was matched to a cleavage prediction generated by FindPept (<http://web.expasy.org/findpept/>) (Supplementary Table S10).

The 2365 *m/z* peak of KNG1_{K438-R457} was observed after incubating His₃-KNG1_{E434-L461}-His₃ with purified KLK2 (Supplementary Figure S4 and Table S10). Given the complexity of serum, His₃-KNG1_{E434-L461}-His₃ may also be cleaved by other peptidases. To demonstrate specificity to KLK2, we spiked the fragment into whole serum or KLK2-depleted serum, respectively, and then processed the samples through Nano-trap-MS. In the whole serum sample, a 3140 *m/z* peak (2365 *m/z* fragment with His-tags) appeared with increasing intensity and a corresponding decrease in intensity of the 4093 *m/z* peak (full-

length peptide subtract). This observation suggests that 3140 m/z signifies a cleavage product of KLK2 (Figure 2A,B). After the depletion of KLK2 from whole serum, the signal corresponding to the cleavage product at 3140 m/z was dramatically lower; in contrast, the full-length substrate remained stable in KLK2-depleted serum. We also incubated whole serum with protein A/G agarose beads as a negative control, and the resulting cleavage product at peak 3140 m/z showed a signal comparable with that detected in whole serum. This result indicates the degraded product is KLK2 specific. The slightly lower intensity in the negative control may be due to nonspecific binding between KLK2 and protein A/G. Taken together, these results support the notion that KLK2 is the major peptidase largely responsible for cleaving KNG1 to generate KNG1_{K438-R457} under biological conditions.

A similar experiment to examine CPN1 peptidase activity in serum revealed a 3031 m/z peak (the fragment at 2021 m/z , with His-tags) increased in intensity when the substrate was incubated with whole serum, whereas little to no fragment accumulated in CPN1-depleted sera (Figure 2C,D). The 3740 m/z peak lacks a histidine compared to fragment His₆-KNG1_{K438-R457}, and was likely generated by a peptidase other than CPN1. As expected, the peak intensity at 3740 m/z remained unchanged irrespective of CPN1 depletion (Figure 2D). These observations suggested that KLK2 acts on KNG1 to generate KNG1_{K438-R457}, which in turn serves as a substrate for CPN1, cleaving it to des-Arg-KNG1_{K438-R457}.

Specific Peptidase Activities Correlate with the Appearance of Peptide Fragments in Serum

To demonstrate a direct correlation between the appearance of peaks at 3140 m/z and 3031 m/z to KLK2 and CPN1 cleavage, respectively, we added His-tagged peptides substrates into sera from each of the four sample cohorts and processed them through Nanotrap-MS. We observed a significant increase in the relative peak intensity of 3140 m/z in the BBC cohort, indicating an increase in KNG1 cleavage by KLK2 (Figure 3A), confirming the initial analysis with the endogenous KNG1 fragment. We conducted a similar experiment to evaluate the activity of CPN1, which, unlike KLK2, appeared to be lower in BBC compared to the WT group (Figure 3B). In fact, CPN1 activity appeared diminished in all three groups with BC, regardless of *BRCA1*-mutation status. This observation aligns with the appearance of CPN1-dependent fragments at 2209 and 2080 m/z , both of which were reduced in the BC groups (Supplementary Figure S3). These results indicate two possible regulatory factors that could give the accumulation of fragment KNG1_{K438-R457} in BBC: one is the increased peptidase activity of KLK2 and the other is reduced activity of CPN1, leading enhanced KNG1_{K438-R457} levels in BBC patients.

Intracellular and Extracellular Peptidase Expression of BC Cells

To determine the degree to which the *BRCA1*-associated peptidases are expressed within BC cells or released to the extracellular medium, we examined the expression of KLK2 in four different cell lines: MCF-10A (nontumorigenic), MCF-7 (tumorigenic, triple-positive, *BRCA1* WT), MDB-MB-231 (tumorigenic, triple-negative, *BRCA1* WT), and HCC1937 (tumorigenic, *BRCA1*-mutated). Compared to MCF-10A cells, we detected a 58-fold increase in *KLK2* mRNA expression in HCC1937 cells, a 23-fold increase in MCF-7 cells,

and a 16-fold increase in MDA-MB-231 cells (Figure 3C). To evaluate whether elevated *KLK2* mRNA expression corresponded to increased secretion of the peptidase, we assessed the enzyme levels in CM of these cells. Consistent with *KLK2* mRNA expression, secreted levels of KLK2 also appeared higher in HCC1937 CM, as determined by ELISA for detection of low-abundance KLK2 than immunoblotting (Figure 3D).

CPN1 combined with KLK2 was demonstrated to regulate a peptide at 2365 *m/z*. CFI releases C 3f from C3b to form inactive iC3b (iC3b). We thus analyzed the expression and secretion of CPN1 and CFI, both *BRCA1*-associated peptidases. In all of the cell lines tested, CPN1 was almost entirely secreted (Figure 4A), as CPN1 signal in cell lysates was barely detectable (Figure 4B). Approximately 50% less CPN1 was detected in the CM of all three breast cancer cell lines (Figure 4C) compared to MCF-10A cells. CPN1 levels were higher in CM from *BRCA1* mutant BC cells compared to *BRCA1*-wildtype BC cells. The level of secreted CFI decreased in CM from all of the cancer cell lines tested, and no significant difference was seen between *BRCA1*-wild-type and *BRCA1* mutant cancer cells. These results indicate that tumor-resident factor(s) can regulate the expression of KLK2 and CPN1.

Correlation between KLK2 and BRCA1 Expression

To further determine whether KLK2 expression or activity was associated with *BRCA1* status, we generated stable MCF-7 and MDA-MB-231 cell lines with shRNA-mediated knockdown of *BRCA1* and measured KLK2 expression and activity in these cells. Puromycin was used to select cultures containing >90% stable viral transductants (Supplementary Figure S5). As shown in Figure 5, panels A and B, *BRCA1* expression decreased by ~70% in both MCF-7 and MDA-MB-231 cells transduced with *BRCA1*_shRNA_2 compared to the controls. We therefore focused on MCF-7/MDA-MB-231 cells expressing *BRCA1*_shRNA_2 (MCF-7^{BRCA1-} or MDA-MB-231^{BRCA1-}) for further related experiments. KLK2 levels were elevated in MCF-7^{BRCA1-} cells and tended to increase in MDA-MB-231^{shBRCA1-} cells (Figure 5C). KLK2-dependent peptide products of *BRCA1*-knockdown MCF-7 cells were analyzed by MS (Supplementary Figure S6), and after normalization with the internal standard peptide (ACTH), KLK2-dependent peptide products exhibited significantly higher levels in *BRCA1* knockdown cells (Figure 5D). Thus, the result further demonstrated that the correlations between the activities and expressions of KLK2 and *BRCA1* status.

Peptidase Expression in Serum

We examined the expression of KLK2, CFI, and CPN1 in sera to explore their implications for biological events (i.e., secretion into blood circulation). Because of the low abundance of KLK2 in circulation, high abundance proteins were depleted with an multifactor affinity column prior to ELISA to improve the sensitivity and specificity of KLK2 detection. The amount of KLK2 appeared significantly increased in BBC sera (Figure 6A). CFI levels were significantly reduced in BH sera (Figure 6B). Immunoblots revealed that CPN1 expression remained unchanged across the samples (Supplementary Figure S7). These results indicate a direct correlation of KLK2 and CFI expression with their reference peptides in serum.

DISCUSSION

Many of the efforts on BC prevention and early detection have focused on identifying *BRCA1* mutation carriers,^{16,32} but we are only beginning to elucidate the mechanisms that increase BC risks for *BRCA1* carriers. Only a limited number of studies have attempted to determine protein profiles associated with *BRCA1* mutant BC,^{14–16} and to date no study has focused on peptide–peptidase interactions in *BRCA1*-related BC, largely due to the lack of tools for peptide profiling and limited access to populations with inherited *BRCA1* mutant BC. In this study, we employed our NanoTraps technology coupling with MS to search circulating peptide candidates differentially presented in *BRCA1* mutation carriers and also attempted to decipher the proteolytic mechanisms involved in producing these peptides.

The peptide KNG1_{K438-R457} originates from domain 5 of HMW kininogen a 120-kDa glycoprotein, which is composed of heavy and light chains with domains 1–3 and domains 5 and 6, respectively.^{33,34} Amino acid residues 441–457 are part of a histidine-glycine-rich region of the protein, which was demonstrated to be responsible for binding to negatively charged surfaces.³⁵ This may explain the preference of KNG1_{K438-R457} and its daughter fragments for the negatively charged NanoTraps used for peptide fractionation in this study. Small peptides cleaved from domain 5 have been indicated as biomarkers for bladder and gastric cancer.^{22,36} Although we believe proteases play an important role in generating biologically relevant peptides, little is known about the direct correlation between specific peptidases and their peptide products. This is the first report to our knowledge that shows a direct correlation for KLK2 and KNG1_{K438-R457}.

We report that the appearance of peptide fragments KNG1_{K438-R457} and C3f_{S1304-R1320} in serum depended on the presence of *BRCA1* mutations, as KNG1_{K438-R457} was up-regulated in *BRCA1* mutation carriers with BC. We also provide evidence that identifies KLK2 as the peptidase responsible for generating KNG1_{K438-R457}. We also show that the peptidase CPN1 subsequently acts on KNG1_{K438-R457} at its C-terminal arginine residue (Figure 7A). Interestingly, KLK2 peptidase activity increased, while CPN1 activity decreased in sera from *BRCA1* carriers, which resulted in the elevated level of KNG1_{K438-R457} in *BRCA1*-associated BC.

Although the peptidase activity of CPN1 differed among the sample groups, its expression level remained steady. We speculate that changes in CPN1 peptidase activity may be influenced by other factors that affect its stability in serum (e.g., CPN2). Despite no obvious differences in CPN1 expression in sera among the four groups, we observed less CPN1 secretion by BC cell lines compared to nontumorigenic MCF-10A cells, although it is possible that such differences were obscured by signal saturation since CPN1 is highly abundant in serum. C 3f_{S1304-R1320} is cleaved from C3b as a result of C3 activation by CFI.³¹ The expression of CFI in BH samples was significantly lower than that in BBC samples, and its secretion was lower in BC cell lines compared to MCF-10A cells. It is straightforward to assume that peptidase abundance or activity changes in the tumor resulted in the detectable changes in cleaved peptides in the circulation, although tumor and blood changes were not completely identical. This may be partially due to the different methods used to measure peptidases in tumor cells and serum. In addition, *BRCA1*-associated BC

biology remains only partially understood, we cannot definitively differentiate the peptidase activity of tumor-resident enzymes from that of their circulating counterparts. “Mapping” the peptide biomarker landscape of tumor formation and progression will require more information about other organs and tissue networks.

We further demonstrated that KLK2 expression and activity are associated with *BRCA1* status using shRNA to achieve stable knockdown of BRCA1 in wild type BC cells. We performed an ingenuity pathway analysis, which maps tentative network connections (Figure 7B) to identify possible mechanisms for how the peptidases are activated and how they relate to *BRCA1* in BC development. The link between BRCA1 and KLK2 is better recognized than that between BRCA1 and CPN1 or CFI. Three proteins, E1A-binding protein p300 (EP300), androgen receptor (AR), and β -catenin (CTNNB1), were found to be directly connected to BRCA1 and KLK2. Overexpression of *BRCA1* down-regulates cellular expression of the transcriptional coactivator EP300 in BC lines.³⁷ Recent microarray analysis of prostate cancer cells identified KLK2 as an EP300-dependent gene.³⁸ It was also recently reported that loss of BRCA1 leads to impaired expression of the nuclear protein CTNNB1 in BC, implicating it in connecting BRCA1 and KLK2.³⁹ Another study revealed that CTNNB1 could enhance AR signaling, possibly affecting KLK2 expression.⁴⁰ AR signaling is a third potential pathway connecting BRCA1 to KLK2. Studies with purified protein *in vitro* have shown that AR binds to a protein fragment of BRCA1, and that this interaction can allow activation of AR in prostate cancer cells.⁴¹ AR is also expressed in the ER and PR double negative cell line HCC1937.⁴² AR increases *KLK2* mRNA expression in prostate cancer cells,⁴³ and it differentially modulates KLK2 in different BC cells. KLK2 is strongly associated with BRCA1 through various pathways, and more studies are needed to gain a clearer understanding of their relationship and implications in breast cancer development and progression.

We applied a simple, robust, and relatively noninvasive approach to identify *BRCA1*-associated BC peptide biomarkers KNG1_{K438-R457} and C 3f_{S1304-R1320}. We also presented an analysis of their associated peptidases CFI and KLK2/CPN1. In both the tumor microenvironment and the circulation system, KLK2 cleaves KNG1 to produce KNG1_{K438-R457}, and CPN1 removes the terminal residue to form KNG1_{K438-456}. CFI cleaves C3 to produce C 3f_{S1304-R1320}. Both peptides can be captured using NanoTraps, and their expression levels were associated with cancer status in *BRCA1* carriers. We outline a new approach for profiling circulation peptide and determining their relationship with the activity of the corresponding peptidases. Most published cancer biomarkers fail to enter clinical practice. We believe that our strategy for discovering peptide-peptidase relationships in cancer may prove useful for biomarker discovery, but we acknowledge that our results are still in the early phase of biomarker discovery and that future prospective studies are required to validate our findings. We are currently conducting a prospective study to address this issue. Women carrying *BRCA1* mutations typically present with BC at a younger age; therefore, the average age of the patients, whose samples are used in this study, is around 45 years. Including older women in the sample cohort would broaden the impact of these results. The long-term longitudinal information would also be greatly beneficial, particularly for cancer-free *BRCA1* mutation carriers who maintain their high-risk status. We intend for

this strategy to improve the early examination of cancer in the *BRCA1* carriers based on the suggestions from the blood-based test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Peptide biomarker levels in serum from clinical samples. Comparison of the relative intensities of MS peaks at (A) 2365 m/z (KNG1_{K438-R457}) and (B) 2021 m/z (C3f_{S1304-R1320}), which represent peptide fragments cleaved from KNG1 and complement C3, respectively. Mean $\pm$ standard error is also shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Peptide at 2365 m/z yielded AUC value of 0.794 (95% confidence interval [CI] is 0.683 to 0.905) in distinguishing BBC to SBC. (D) Peptide 2365 m/z yielded an AUC value of 0.758 (95% CI is 0.638 to 0.877) in distinguishing BBC to WT. (E) ROC curve generated by comparing the BBC versus BH sample cohorts. Blue dotted line, 2021 m/z ; green dotted line, 2365 m/z ; red line, multivariate model based on both peptide fragments (2365 m/z and

2021 m/z). The multivariate model AUC is 0.739 (95% CI is 0.601 to 0.878). (F) Sensitivity, specificity, PPV, NPV, ACC, and FDR of the two peptides.

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Figure 2.

MS spectra showing the cleaved peptide fragments after incubation with whole serum or KLK2/CPN1 depleted serum. (A) Degraded products at 3140 m/z derived from His₃-KNG1_{E434-L461}-His₃ in whole sera or KLK2-depleted sera. (B) The stability of full length of His₃-KNG1_{E434-L461}-His₃ in whole sera and KLK2-depleted sera. (C) Degraded products with 3031 m/z generated from His₆-KNG1_{K438-R457}-His₆ in whole sera and CPN1-depleted sera. (D) The stability of Des-His-KNG1_{K438-R457} in whole sera and KLK2-depleted sera. All the assays were performed by spiking synthesized His₃-KNG1_{E434-L461}-His₃ or His₆-KNG1_{K438-R457}-His₆ into biological fluids followed by NanoTraps enrichment.

Figure 3.

KLK2 and CPN1 activities in patient serum samples. (A) The relative intensity of the KLK2-dependent His-tagged cleavage product at 3140 *m/z*. (B) The relative intensity of the CPN1-dependent His-tagged cleavage product at 3031 *m/z*. (C) The fold changes of *KLK2* mRNA expression in normal and breast cancer cells. (D) *KLK2* expression in conditioned medium (CM) collected from normal and BC cells; equivalent amounts of proteins were coated on the ELISA plate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. CPN1 and CFI expression in and secretion from MCF-10A, MDA-MB-231, MCF-7, and H1937 cell lines. (A) CPN1 and CFI expression in cell CM, and a silver stained gel to assess equal sample loading. (B) CPN1 and CFI expression in cell lysates (CL). (C) Histogram of quantitative image analysis of immunoblots of CPN1 and CFI and the silver stained gel performed by software ImageJ. **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. Expressions and activities of KLK2 in BRCA1 knocked down cells. (A) Immunoblotting images of BRCA1 expression and (B) relative BRCA1 levels in MDA-MB-231 and MCF-7 cells transduced with lentiviruses carrying BRCA1_shRNA_1, BRCA1_shRNA_2, or a Ctrl_shRNA. The density of each protein band was normalized to that of corresponding GAPDH. (C) Expressions and (D) activities of KLK2 in BRCA1 knockdown MDA-MB-231 and MCF-7 cells. *, $P < 0.05$; ***, $P < 0.001$.

Figure 6. KLK2 and CFI expression in serum from clinical samples. (A) KLK2 expression levels in serum. Highly abundant serum proteins, such as albumin and IgG, were removed from serum before ELISA. (B) CFI expression levels in serum from four clinical groups. *, $P < 0.05$; **, $P < 0.01$.

Figure 7.

(A) Schematic representation of the peptide generation pathway. KNG1 is first cleaved by KLK2 to form KNG1_{K438-R457}, and subsequently the C-terminal arginine is removed by CPN1. (B) Ingenuity pathway maps between BRCA1 and KLK2. EP300, AR, and CTNNB1 are three key molecules that connect BRCA1 and KLK2.

Table 1Clinical Characteristics and Age Distribution of the Clinical Samples ($n = 132$) Used for Peptidomic Profiling

groups	n	age median (95% CI)	<u>age range</u>		ER(+)	PR (+)	Her2 (+)	<u>peptide biomarker median (95% CI)</u>	
			min	max				KNG1 _{K438-R457}	C 3f _{S1304-R1320}
wild type (WT)	38	46(44–50)	23	64				6.85(4.89–7.76)	38.16(32.23–47.95)
sporadic breast cancer (SBC)	39	50(46–53)	31	63	26	20	2	5.76(5.27–7.20)	24.38(22.44–41.50)
BRCA1 mut healthy (BH)	27	43(40–46)	31	57				8.36(7.63–9.79)	19.30(17.20–24.13)
BRCA1 mut breast cancer (BBC)	28	48(42–50)	31	65	3	2	1	10.40(9.48–13.86)	27.24(26.58–49.63)