Circulating Proteolytic Products of Carboxypeptidase N for Early Detection of Breast Cancer

Yaojun Li,† Yueguo Li,† Tao Chen,† Anna S. Kuklina, Paul Bernard, Francisco J. Esteva, Haifa Shen, Mauro Ferrari, and Ye Hu*

BACKGROUND: Carboxypeptidase N (CPN) is important in regulating vasoactive peptide hormones, growth factors, and cytokines by specifically cleaving their C-terminal basic residues. We investigated whether circulating peptides specifically cleaved by CPN in the tumor microenvironment can be stage-specific indicators of breast cancer.

METHODS: CPN activity was measured using an ex vivo peptide cleavage assay by incubating synthesized C3f peptide (His6-C3f_S1304-R1320-His6) in interstitial fluids of breast tumors and adjacent normal breast tissues in mice with orthotopic implantation of the human cell line MDA-MB-231. The nature and extent of peptide cleavage by CPN was investigated by fragment profiling using nanopore fractionation and mass spectrometry. The fragment profiles in interstitial fluid correlated with concentrations of CPN-catalyzed peptides in blood samples taken from the tumor-bearing mice, healthy women, and breast cancer patients. CPN expression in the same set of samples was further examined by immunohistochemistry and immunoblotting.

RESULTS: We showed that generation of C3f_R1310-L1319 specifically correlated with the CPN expression level. In both the mouse and clinical patient samples, CPN was clearly increased in tumor tissues compared with normal breast tissue, whereas corresponding CPN abundance in blood remained constant. Concentrations of 6 CPN-catalyzed peptides predominantly increased in sera taken from the mice (n = 8) at 2 weeks after orthotopic implantation. Six homologous peptides displayed significantly higher expression in the patients’ plasma as early as the first pathologic stage of breast cancer.

CONCLUSIONS: Circulating CPN-catalyzed peptide concentrations reflect the CPN activity in tumors. These biomarkers show strong potential for the noninvasive and early diagnosis of breast cancer.

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Perhaps in preparation for or as a result of disease progression, cells in the tumor microenvironment typically secrete several kinds of proteins, such as cytokines, growth factors, proteases, and peptidases, to name a few (1, 2). In theory, any or all of these secreted proteins can serve as the biomarkers of tumor progression (3, 4). However, the reality is much more challenging to monitor because of the large degree of fluctuation in abundance and localization of these tumor-secreted proteins, especially in the early stage of tumor development and/or metastasis (5, 6). As such, it seems feasible that we might take advantage of the fact that secreted proteases/peptidases in the tumor microenvironment generate proteolytic products, also referred to as circulating peptides, and these are continuously released into interstitial fluid, lymph, eventually making their way into the bloodstream (7). Consequently, blood samples can provide ample information about the body, “coded” in the patterns and quantity of these peptides (8). An additional advantage of seeking this type of information from blood samples is that these samples are readily accessible and less prone to selection bias than tissue samples. Several studies have presented comprehensive qualitative and quantitative profiling of circulating peptides, demonstrating their enormous potential for use as a rapid, cost-effective, and accurate platform for cancer diagnosis and therapeutic evaluation despite the complexity of the cancer pathophysiology landscape (9–11). However, only a few studies have shown a direct correlation between the presence (and/or quantity) of these circulating pep-
tides, the proteases/peptidases that generate them, and the roles these play in cancer progression (12–15).

Carboxypeptidase N (CPN), also known as an arginine/lysine carboxypeptidase, kininase I, or anaphyla-toxin inactivator, is a member of a larger family of zinc metallopeptidases (16). Many metallopeptidases cleave the carboxy (C)-terminal arginine or lysine of endogenous peptides, thus changing the peptide substrates’ activity and receptor-binding capability (17, 18). CPN is known to act on peptides, bradykinin (BK₉), anaphylatoxins (C3a, C4a, and C5a), and fibrinopeptides A and B (19). The nonapeptide BK₉, whose amino acid sequence RPPGFSPFR remains identical in the human and mouse genomes, enlarges blood vessels and causes the blood pressure to decrease as a result. Removal of its C-terminal arginine by CPN, and thus loss of the function of binding with its receptor, is an important regulatory step for the inactivation of BK₉ (18). C3f (C3f_S₁₃₀₄-R₁₃₂₀) is a heptadecapeptide (amino acid sequence SSKITHRIHWESASLLR in human and SSATTFRLWENGNLLR in mouse) that is generated from the proteolysis of C3b by complement factor I in blood (20). C3f_S₁₃₀₄-R₁₃₂₀ has also been reported as a substrate of CPN, as determined in an in vitro study (21). Profumo et al. and Villanuva et al. have both attributed several daughter fragments of C3f_S₁₃₀₄-R₁₃₂₀ and BK₉ to human bladder cancer, prostate cancer, and breast cancer (10, 11). Given these results, the only study that we could identify that attempts to draw a relationship between CPN and cancer was a study conducted decades ago in 1983, in which the investigators observed increased CPN activity in the sera of lung cancer patients (22). In our present study we sought to link the catalytic activity of CPN to the patterns of its proteolytic products during tumor progression in a breast cancer mouse model and in clinical samples from breast cancer patients. Our results strongly indicate that circulating peptides generated by CPN, as a representative member of the metallopeptidases, can serve as clear signatures of early disease onset and progression.

Materials and Methods

BREAST CANCER MOUSE MODEL AND BLOOD SAMPLE COLLECTION

All animal study protocols were approved by the Institutional Animal Care and Use Committee of the Methodist Hospital Research Institute. Female mice (age-matched between 6 and 8 weeks, homozygous for severe combined immune deficiency) were purchased from Charles River Laboratories. Eight mice were injected subcutaneously into the right flank with 1 × 10⁷ MDA-MB-231 cells suspended in 100 μL of PBS. Tumor volume was calculated every 2 weeks using the formula: $\pi \times \frac{\text{length} \times \text{width}^2}{2}$. Blood samples were collected from 8 mice by retroorbital bleeding at 5 different time points (before injection and 2, 4, 6, and 8 weeks after injection). At each time point, 100-μL blood samples were collected from each mouse. The blood samples were kept at 25 °C for 1 h and then centrifuged at 4000g for 15 min at 25 °C. The sera were collected and stored at −80 °C until use. After the mice were killed at the eighth week, adjacent normal breast tissue and tumor tissue from mice were collected and then stored at −80 °C until use.

EXTRACTION OF INTERSTITIAL FLUID

Five milligrams of tissue (normal or tumor) were homogenized in 300 μL of PBS on ice with an OMNIFlex homogenizer. The interstitial fluid was obtained by centrifugation of homogenized tissue samples at 3000g for 30 min at 4 °C. Protein concentration was determined by Bradford assay (Bio-Rad). Aliquots of the interstitial fluid were stored at −80 °C until use.

CPN DEPLETION FROM INTERSTITIAL FLUID

The anti-CPN antibody (Abcam) was immobilized on Dynal magnetic beads (Life Technologies) according to the manufacturer’s instructions. Aliquots (1 g/L) of the interstitial fluid or conditioned cell-culture medium (CM) were incubated with 0.4 mg of antibody-coated beads for 1 h at 25 °C. After 5 washes with 0.5 mL of PBS, CPN was eluted from the beads with 100 μL of 0.1 mol/L glycine (pH 3). The flow-through and eluate were analyzed by immunoblotting to evaluate the depletion efficiency.

EX VIVO PEPTIDE CLEAVAGE ASSAY

Aliquots (1 g/L) of the interstitial fluid were preincubated at 37 °C for 15 min with 10 mmol/L EDTA for an inhibition test. His-tagged C3f peptide (His₅-C3f_S₁₃₀₄-R₁₃₂₀-His₅), synthesized with 95% purity by GL Biochem, was used at a final concentration of 100 μmol/L for this assay. Cleaved peptides were extracted from the reaction solution by nanopore fractionation and detected by MALDI-TOF mass spectrometry (MS) according to protocols described in our previous publication (9).

NANOPORE FRACTIONATION

Five microliters of each serum/plasma sample, collected from either mice or clinical breast cancer patients at 5 different stages (see details in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/
issue1), were spiked-in with 100 nmol/L of internal standard peptide corticotropin (ACTH) 18–39 (Sigma-Aldrich) for subsequent quantification. Then the samples were pipetted into individual wells arrayed on a 4-inch nanoporous silica (NPS) chip and incubated at 25 °C in a humidified chamber for 30 min. After the residual samples were removed, the wells were washed with 10 mL of deionized water 4 times. For elution, 5 mL of a solution containing 50% acetonitrile and 0.1% trifluoroacetic acid was applied to each well for 90 s, allowing the peptides to be extracted from the NPS chip. The eluates were then sent to profile and peptide sequencing by MALDI-TOF-MS (see protocols in the online Data Supplement).

STATISTICAL ANALYSIS
MALDI-TOF MS data were processed by MarkerView software v. 1.2.1 (AB SCIEX). The data were normalized by the internal standard peptide ACTH 18–39 (Sigma-Aldrich). Student t-tests were performed using MarkerView for comparisons of control and case samples.

Results
WORK FLOW OF PEP TIDOMIC STUDY ON CIRCULATING PEPTIDE BIOMARKERS
To verify our hypothesis that CPN-catalyzed peptide products found in circulation can indeed be strong indicators of disease, we designed and implemented a systematic work flow to culminate in a set of signatures that could inform disease diagnosis (Fig. 1) as follows. Some of the foundational experiments involved inducing a breast cancer mouse model by injecting MDA-MB-231 human breast cancer cells into the animals. It was important to first evaluate the activity of CPN in the microenvironment by an ex vivo cleavage assay. Here, interstitial fluids extracted from the animals were incubated with synthesized C3f peptides (His6-C3f_S1304-R1320-His6), as the substrate of CPN, with the cleavage sites predicted by bioinformatics tools [e.g., the protease substrate specificity webserver (PROSPER) (23), http://lightning.med.monash.edu/PROSPER/webserver.html], and marked in Fig. 2A.
The peptides were isolated using a nanopore fractionation method previously developed by our group (9, 24–28). Briefly, low molecular weight peptides are captured and preserved on NPS chips, separating them from often overwhelming, high molecular weight proteins before MS analysis. Analysis by MS can not only provide the quantitative information for multiple peptides species in a single sample, it can also help to identify their sequence information (9, 24, 25, 27–29). The in vivo expression of CPN in tissues [evaluated by immunohistochemistry (IHC)] or blood (by immunoblotting) is correlated to the presence or quantity (see protocols in the online Data Supplement). Taking these results, we then perform clinical validation, in which the peptide biomarker candidates are examined in plasma samples collected from patient cohorts at different stages of cancer. Expression of CPN in human tumor tissue and blood is also evaluated and correlated to that of its peptide substrates.

**Fig. 2. Ex vivo peptide cleavage assay on the synthetic C3f peptide.**

(A). Specific cleavage sites are indicated on the C3f peptide, a substrate of the enzymes factor I, chymotrypsin and CPN. (B). Detecting CPN in different assay conditions. CPN-depletion in normal [NIF (CPN⁻)] or tumor [TIF (CPN⁻)] interstitial fluid; CPN protein pull-down from normal [CPN (NIF)] or tumor [CPN (TIF)] interstitial fluid. (C, D), NIF and TIF (20 μL of 1 g/L) from a breast cancer mouse model or CM of MB-MDA 231 cells were incubated with synthetic C3f peptides (final concentration is 100 μmol/L) at 37 °C for 1 h, followed by nanopore fractionation and MALDI-TOF MS. C3f, synthetic peptide His6-C3f_S1304-R1320-His6 only; NIF or TIF, normal or tumor interstitial fluid only; EDTA, an inhibitor of CPN; NIF (CPN⁻) and TIF (CPN⁻), CPN-depleted normal and tumor tissue interstitial fluid; CM (CPN⁻), CPN-depleted conditioned medium.
sin, a serine protease that cannot be inhibited by EDTA. If cleaved by chymotrypsin, a peptide peak can be detected in an MS spectrum at an m/z of 2206.13.

After factor I removes the His6-tag on both termini of the synthetic peptide, CPN cleaves the C-terminal “R” of C3f_R1310-L1320 to produce C3f_R1310-L1319 with an m/z of 1227.68. The addition of EDTA inactivates CPN by chelating the zinc ion in its catalytic domain. For further ex vivo validation of these cleavage events, we conducted the peptide cleavage assay by incubating synthetic peptides with tumor interstitial fluid (TIF) and CM of MDA-MB-231 cells. Results indicated that production of C3f_R1310-L1319 in TIF was 6-fold higher than in normal interstitial fluid (NIF). As shown in Fig. 2, C and D, the addition of EDTA clearly inhibited cleavage of C3f_R1310-L1319; however, it could not prevent the generation of the peak at m/z = 2206.13 in samples containing TIF, NIF, and CM, suggesting the likelihood that C3f_R1310-L1319 was finally generated by a metallopeptidase, which was proven to be CPN in this study. Most notably, the generation of C3f_R1310-L1319 with an m/z of 1227.68, but not the peptide with an m/z of 2206.13, was inhibited if CPN was removed by immunodepletion (confirmed by immunoblotting, Fig. 2B) from the interstitial fluids or CM. Expression of CPN, validated by anti-CPN immunoblotting and IHC staining, was increased in tumor tissue compared to that in normal breast tissue (Fig. 3, A and B). In fact, the adipocytes and glandular cells in normal mouse breast tissue expressed CPN weakly or not at all, whereas the myoepithelial cells exhibited an increased concen-
As shown in Fig. 3C, the concentration of CPN in mouse sera remained constant during tumor growth.

**SERUM PEPTIDOMIC PROFILING IN MOUSE MODEL**

We introduced MDA-MB-231 cells into nude mice by orthotopic implantation to induce tumor growth. Tumor volume was measured at 2-week intervals for a total of 4 time points, with mean tumor sizes across 8 mice at 69.8 (30.9) mm³, 190.3 (91.9) mm³, 718.9 (380.9) mm³, and 1548.0 (804.6) mm³, respectively (see online Supplemental Fig. S1). After collecting mouse sera, we processed a total of 120 sera samples (triplicates of 40 serum samples, including sera collected from 8 mice over the course of 5 time points) on NPS chip fractionation, and then tested all of the eluates on one 192-well MALDI-TOF MS plate. Each MALDI-TOF MS spectrum contained about 150 monoisotopic peaks in the range of 800 to 3500 Da (see online Supplemental Fig. S2A). The data were imported into the MarkerView software for *t*-test analysis. Compared to the normal controls collected before tumor implantation, different peaks were found in the diseased samples, after applying the primary (*P* < 0.05) and secondary (fold change >2) criteria (see online Supplemental Fig. S2B). Sequence information obtained from high-performance liquid chromatography–tandem mass spectrometry revealed 6 differential peptides, including 5 from C3f and 1 from BK₉, and their appearance correlated with the CPN expression. These peptides were thus chosen for further study (see online Supplemental Table S1). It is important to note that all 6 peptides exhibited a similar trend during the first 4 weeks after tumor induction (Fig. 4), with the quantity peaking at week 2 (the first tumor growth measurement) and then decreasing dramatically by week 4. Interestingly, the quantity of nearly all C3f peptides increased again slightly at week 6 and week 8. Exceptions included the peptide C3f_R1304-L1319, with an *m/z* of 1821.95, which increased again only at week 6 before declining for the second time at week 8, and the BK₉ peptide, which remained steady at week 6 before decreasing at week 8. The absolute intensities (indicator of concentration) of C3f_R1310-L1319 were higher than those of the other fragments.

![Fig. 4. The vertical scattering plot of the normalized peak intensities in MALDI TOF MS for the potential peptide signatures in serum samples collected from tumor-bearing animals (breast cancer mouse model).](image)

The mass-to-charge ratio and sequence identification is listed in each panel. Mouse number, *n* = 8; Student *t*-test, *P* < 0.05, **P** < 0.01, and ***P*** < 0.001.
CLINICAL VALIDATION

We expected our findings in the breast cancer mouse model to be reflected in clinical samples collected from patients with breast cancer, positioning our approach for clinical translation. To provide unequivocal evidence, we obtained a commercial tissue array slide that contains 110 human breast tissue sections, including tissue samples from 100 breast cancer cases (pathologic stages I–III) and 10 samples of adenosis/cancer-adjacent normal breast tissues as controls (see array details at http://www.biomax.us/tissue-arrays/Breast/BC081116a). Analysis by IHC using these commercial arrays revealed that indeed CPN expression was generally increased in samples taken from breast cancer patients regardless of disease stage (Fig. 5A). When we scored these 110 sections on the basis of a scoring system reported by Zhang et al. (30), 100% of the controls received a score of 1+ or less, which meant that CPN was weakly or not expressed in controls. However, across all stages of breast cancer represented on the array, 85% of the samples received scores higher than 1+ and 70% received scores higher than 2+ (Fig. 5B and online Supplemental Fig. S3). Conversely, the amount of CPN in plasma remained constant between breast cancer patients and the control cohort (Fig. 5C).

For further validation, circulating peptides of interest were profiled in 58 human plasma samples, including samples from healthy controls (n = 10) and from patients with stage I breast cancer (BC-I, n = 11), stage II breast cancer (BC-II, n = 12), stage III breast cancer (BC-III, n = 15), and stage IV breast cancer (BC-IV, n = 10). MALDI-TOF MS data showed that the BK₆ species appeared more abundant in stage I and II breast cancer (BC-I and BC-II) compared to healthy controls (BC-I vs control, P < 0.001; BC-II vs control, P < 0.05) (Fig. 6 and online Supplemental Table S2). Perhaps most striking, the peptide species C₃f_R₁₃₁₀⁻L₁₃₁₉, C₃f_R₁₃₀₉⁻L₁₃₁₉, C₃f_R₁₃₁₈⁺⁻L₁₃₁₉, C₃f_R₁₃₀₅⁻L₁₃₁₉, and C₃f_R₁₃₀₄⁻L₁₃₁₉ presented prominently in samples of BC-I and BC-II compared to samples from the healthy cohort (P < 0.001) (Fig. 6), results that are consistent with those reported by Profumo et al. (10). Overall, the patterns of these CPN-catalyzed products in the clinical samples from breast cancer patients matched strongly with our observations in the breast cancer mouse model. As shown in our results, the expression levels of the 6 CPN-specific peptides showed no significant difference between mouse serum and plasma before we processed the validation experiment on human plasma samples (see online Supplemental Fig. S4).

Discussion

There have been numerous studies to find biomarkers for early disease diagnosis, but frustratingly, we still lack definitive candidates that can be used to predict early disease onset and to do so with great precision and specificity. The often poor diagnosis and prognosis is due in part to the absence of an approach that can identify markers with such features in a manner that is non-invasive, reproducible, and conducive to rapid therapeutic processes (31). Because it is known that certain proteases/peptidases play important roles in several
cancers, a deeper understanding of certain proteolytic events, particularly in the tumor microenvironment, will facilitate the identification of tumor-derived products that are eventually secreted into circulation (8, 11, 13, 32). Profiling the serum proteome then provides a “map” that we can use to trace cancer-specific metabolic or immunological signatures indicative of early-stage tumor progression (6, 10, 12, 14).

In this study, we examined the activity of CPN, a protease known to play a role in several types of cancer, within the tumor microenvironment as well as in the blood circulation of mice (breast cancer mouse model) and patients diagnosed with breast cancer (different stages). The peptide fragments generated by tumor-resident CPN and released into the bloodstream—their quantities and identities are presented in our comprehensive analyses here—strongly correlate with enzymatic expression and reflect the organism’s pathophysiological state. Therefore, we advocate their use as distinct and precise biomarkers of early breast cancer diagnosis and risk assessment, certainly to be detected and identified before metastasis and perhaps even before the tumor presents with any observable characteristics commonly used in the clinic.

A previous in vitro study has shown that CPN can cleave the C-terminal arginine of the C3f_S1304-R1320; however, it was unclear whether CPN was the only specific peptidase able to do so in that particular assay condition (21). To make such a conclusion, or otherwise, we depleted CPN activity in protease assays that contained NIF, TIF, and CM, before adding the synthetic C3f peptide. The resulting MALDI-TOF MS spectra revealed that cleavage to C3f_R1310-L1319 was indeed terminated in both interstitial fluid and CM and confirmed the specificity of the CPN to the C-terminal arginine of C3f. We know that it was CPN but not other proteases, for example carboxypeptidase E (CPE), that was the specific enzyme for this cleavage event because the previous study had shown that MDA-MB-231 cells can secrete both CPN and CPE (33). In addition, the strong m/z intensities of C3f_R1310-L1319 fragments observed in TIF demonstrate the higher activity of CPN in the tumor microenvironment than its counterpart in the normal breast tissue. The immunoblotting study displayed 2-fold higher CPN activity in TIF than in NIF, further confirmed by IHC analysis of normal and tumor tissues. Therefore, it is very clear that CPN is highly expressed in tumor tissue and secreted into TIF

Fig. 6. Profiles of the potential peptide signatures in plasma from healthy women with breast cancer of different pathological stages.

Sample number: n = 10, healthy controls; n = 11, BC-I; n = 12, BC-II; n = 15, BC-III; n = 10, BC-IV; Student t-test, *P < 0.05, and ***P < 0.001.
in the early stage of the breast cancer. The physiological and oncolgical significance of this event needs to be further studied. Although CPN is weakly expressed in the glandular cells and highly expressed in myoepithelial cells, both cell types are considered the minority population in normal breast tissue. In adipocytes, the majority population in normal breast tissue, we did not observe any detectable expression of CPN. The cell line MDA-MB-231 used here belongs to the epithelial cell lineage, as do myoepithelial cells. Besides its high expression and secretion of CPN, MDA-MB-231 cells had a rapid growth rate and caused the poor differentiation of the tumor tissue, which probably explain the increased CPN activity observed in TIF compared to NIF.

It was somewhat surprising to find that the level of CPN expression did not differ significantly in the sera of control and diseased subjects, whether mouse or human. This observation suggests that due to dilution, any additional leakage of CPN from the tumor microenvironment may not be readily detectable above the background concentration of CPN in the serum. As described by other investigators, active CPN is secreted into circulation as a 280-kDa molecule, comprising 2 regulatory 83-kDa subunits and 2 catalytic 50-kDa subunits (16, 19, 22). We concluded that the increased concentrations of circulating CPN-catalyzed peptides presented here resulted from cleavage by CPN resident in the tumor microenvironment rather than from enzymes circulating in blood. We firmly believe it is precisely this observation that makes the idea of using CPN-catalyzed circulating peptides as disease biomarkers much more appealing and feasible.

Because the MALDI-TOF MS spectra featured the peptide signatures being expressed more prominently in the blood samples of animals and patients with breast cancer, one would expect to see some changes (increases) of these peptides in TIF compared to NIF in tumor-bearing mice. In fact, their signals or peaks were not detectable by MALDI-TOF MS. The low abundance of these peptides in interstitial fluid is likely due to their continuous release from the tumor microenvironment rather than from enzymes circulating in blood. Nevertheless, whether this explanation reflects the actual physiological process remains to be proven.

The expression levels of circulating peptides can be balanced by the activities of multiple proteases/peptidases, some of which promote proteolytic production, whereas others simultaneously inhibit the production of degraded peptides (18, 21, 34). In the breast cancer mouse model examined here, the quantities of all 6 circulating peptides had dramatically decreased at week 4, after peaking at week 2. Some studies have demonstrated that angiotensin-converting enzyme and neutral endopeptidase inhibit the cleavage of BK in human plasma (18). We speculate that a peptidase other than CPN may be more active in either tissue or blood as the tumor matures, leading to a decline in peptides cleaved by CPN. On the basis of predictions made using the program PROSPER (23), we selected cathepsin G as a peptidase that could be responsible for cleaving C3f fragments at the site of “LW.” Other studies have suggested an important role for cathepsin G in tumor progression, especially in metastasis (35, 36). As such, cathepsin G seems likely to be involved in the declining abundance of the C3f fragments in blood at midstage tumor progression. Because the peptides reported in this study are not directly released from the tumor, their abundance in blood may be regulated by other enzymes in addition to CPN, such as cathepsin G. Thus, these peptides may not correlate entirely with tumor burden.

Cumulatively, our results represent a first demonstration, to our knowledge, that clearly links the proteolytic activity of CPN, particularly at tumor sites, to the cleavage patterns of its catalytic substrates C3f and BK in the blood, by means of a rapid, reproducible, sensitive, precise, and noninvasive approach. It would certainly be naïve to think that CPN-catalyzed peptides and the enzyme itself are the only biomarkers specific for breast cancer diagnosis. We do, however, argue that they can be used as a complementary measure for very early diagnosis of tumor growth, particularly because CPN activity within the tumor at this point is already detectable by blood sampling. Such sensitivity together with all of the features mentioned above lend well to their translation to the clinic, for breast cancer and perhaps other pathologies.
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