Abstract

Patients who undergo surgical extirpation of a primary liver carcinoma followed by radiotherapy and chemotherapy leading to complete remission are nevertheless known to develop cancerous metastases 3–10 years later. We retrospectively examined the blood sera collected over 8 years from 30 patients who developed bone metastases after the complete remission of liver cancer to identify serum proteins showing differential expression compared to patients without remission. We detected a novel RGD (Arg-Gly-Asp)-containing peptide derived from the C-terminal portion of fibrinogen in the sera of metastatic patients that appeared to control the EMT (epithelial-mesenchymal transition) of cancer cells, in a process associated with miR-199a-3p. The RGD peptide enhanced new blood vessel growth and increased vascular endothelial growth factor levels when introduced into fertilized chicken eggs. The purpose of this study was to enable early detection of metastatic cancer cells using the novel RGD peptide as a biomarker, and thereby develop new drugs for the treatment of metastatic cancer.

Background

Liver cancer is the second leading cause of cancer-related deaths, and the fifth most common form of cancer worldwide [1]. It is most common in Asia, and hence is of particular concern in Japan. Liver cancer typically takes upward of 30 years to develop following infection with hepatitis C virus or other pathogens. In most Japanese cases, the incidence of disease can be linked to the country’s hepatitis C outbreak in the years immediately following the Second World War [2–4]. This has led to the current epidemic of liver cancer among...
Methods

**Ethical Guidelines**

The study protocol was approved by Kurume University Hospital (Approval Number 2547). An anonymous retrospective study was conducted, following Freezerworks validation procedures, as per US Food and Drug Administration guidelines, on 47 Japanese cancer patients during a follow-up period from 1994 to 2002 (see Supplemental Study Protocol). Before conducting an analysis of any patient serum samples, we obtained informed consent on the serum survey, including the use of all preserved blood for measurement in this study, and we also received written consent from other cancer patients and healthy volunteers. Patients started treatment after the diagnosis of metastasis was confirmed by the quantification of CFMP in serum and imaging diagnosis. Standard treatments were administered, which were not affected by the decision of the patient’s participation in the study. Samples were collected using Venoject II 7-mL tubes equipped with a clot activator and gel separator (VP-P073K; Terumo, Tokyo, Japan), and the serum was then separated by centrifugation followed by preservation at −80 °C.

**Animal Welfare and Management**

Strain SHO SCID mice (Crlj:SHO-Pkdscid HrHr, 4 weeks old) were purchased from Charles River, Yokohama, Japan. Severity assessment in laboratory animals is an important issue with respect to implementing the 3Rs concept in biomedical research, and is a pivotal feature of current Japanese regulations [8]. All experiments were approved and permitted by Kurume University. Chicken eggs and the SCID mice were monitored daily for health and weight. All procedures, including the euthanasia of morbidly ill mice and chicken embryos, were performed under isoflurane (minimum alveolar concentration at approximately 50% atm; MAC50, 1.41%) and ketamine anesthesia (80 mg/kg) according to standard protocols and the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) [9]. On the basis of the pain classification of the Scientists Center for Animal Welfare (SCAW), tumor diameters >20 mm and high levels of cancer markers were defined as humane endpoints and noninvasive imaging analysis was performed (narcotics handler license: 230,263/2011–2012, 250,263/2013–2014, 270,263/2015–2016, 170,263/2017) [10]. The procedures were conducted in accordance with the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms; the protocol was approved under Type 2 Regulations Concerning the Use of Living Modified Organisms of Kurume University (approval no. 22–7) and was carried out following International Council for Laboratory Animal Science (ICLAS) approval (approval nos. 18–2/2010, 43/2011, 190/2012, 165/2013, 54–1/2014, 20/2015 45/2016 and 136/2017) [11,12]. Routine microbiologic monitoring according to recommendations of the Federation of ICLAS did not reveal any evidence of infection with common urine pathogens [13]. The SCID mice were maintained in specific-pathogen-free (SPF) Level P1A rooms with a controlled environment: 21 ± 2 °C; relative humidity 55 ± 5%; 12:12 h light:dark cycle (lights on at 7:00 a.m.). There were no significant adverse events noted in the SCID mice experiments.

**Cell Lines**

The human non-small cell lung cancer cell line H460, the breast cancer cell line BT474, the bone metastatic prostate cell line PC-3, and the osteosarcoma MNNG/HOS cell line were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). H460-GFP, osteosarcoma HNNG/HOS-GFP, BT474-GFP, and PC3-GFP cell lines were purchased from AntiCancer Inc. (San Diego, CA, USA). HMVEC-L; human lung microvascular endothelial cells) and mesenchymal stem cells purchased from Lonza Inc. (Basel, Switzerland).

**Design**

We carried out a proteomics analysis to identify serum proteins that are differentially expressed between liver cancer patients who had complete remission and those who did not. Our sample set was composed of 47 Japanese patients (25 men, 22 women; mean age: 55 years; age range: 52–68 years), of which 30 (17 men, 13 women; mean age: 61 years; age range: 21–60 years) developed bone metastasis in 2002, following complete remission in 1994. Bone metastases for each patient were confirmed using positron emission tomography computed tomography (PET-CT) as part of the patients’ standard of care. In order to investigate the effect on racial differences, we also obtained serum from Analytical Biological Services Inc. (Wilmington, DE, USA). The sera were obtained from five Caucasian females (39–66 years old) who had breast cancer with bone metastasis, with infiltrating ductal carcinoma or infiltrating lobular carcinoma, based on their histological diagnosis (Supplemental Carcinoma Data). As controls, we also examined the serum proteins from two groups of adult volunteers (1) 21 healthy controls (9 men, 12 women; mean age: 51 years; age range: 21–60 years) with no malignant tumors identified during health check-ups or by PET-CT, and (2) 17 patients with rheumatoid arthritis (2 men, 15 women; mean age: 54 years; age range: 43–63 years, American College of Rheumatology; ACR, class I; 4, class II; 4, class III; 4, and class IV; 5 [https://www.rheumatology.org/Practice-Quality/Clinical-Support/Clinical-Practice-Guidelines] Supplemental Study Protocol).

For patients who developed bone metastasis later on, this occurred after complete remission of their liver cancer via surgical extirpation of a primary carcinoma followed by radiotherapy and chemotherapy.
We had previously performed a proteomic analysis of their sera obtained as early as 1994, a full 8 years prior to the occurrence of bone metastasis. For these patients, complete remission was defined according to tumor guidelines [14], using low values of tumor markers and diagnostic imaging as indicators. We also performed a similar investigation for seven patients with breast cancer who expressed the novel RGD-containing peptide, described later, after complete remission following radiation treatment and chemotherapy.

Among the 47 patients with complete remission of liver cancer, 30 patients who developed bone metastases and 17 patients, who did not develop bone metastases, were retrospectively investigated. Healthy individuals and patients with rheumatoid arthritis were examined as controls and after the amino acid sequence of the peptide was identified, metastases other than bone metastasis, were investigated.

**Targeting Proteomics**

Traditional discovery proteomics [15] using modern Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [16,17] (as previously described) usually progresses directly to the clinical validation stage where large cohorts of patients are evaluated using well-established, validated methodologies (such as enzyme-linked immunosorbent assays (ELISAs) prior to clinical implementation [18]. For SELDI, a weak cation exchange chip (CM10), a strong cation exchange chip (Q10), a copper modified chip (IMAC30), and a negative-phase chip (H50) were used. For catenation and wash buffers, 100 mM sodium acetate (pH 4) and 50 mM HEPES (pH 7) were used for CM10, 50 mM Tris–HCl (pH 8) and 100 mM sodium acetate (pH 5) were used for Q10, 100 mM sodium phosphate +0.5 M NaCl (pH 7) was used for IMAC30, and 50 mM HEPES (pH 7) was used for H50. First, after adding 100 mM CuSO₄ to the IMAC30 chip and shaking for 10 minutes, the chip was washed with ultra-pure water (twice, 2 minutes/wash) and copper was fixed onto the chip’s surface. After adding 0.1 M sodium acetate (pH 4) and shaking for 5 minutes, the chip was washed for 2 minutes with ultra-pure water. The four types of chips were then catenated and washed with a wash buffer (twice, five minutes/wash), and the chip surfaces were equalized. Following this, 100 μL of a five-fold dilution of the sample and wash buffer was added and the chip was shaken for 30 minutes. After adding the catenation and wash buffer, shaking, and washing the chip surface (three times, 5 minutes/wash), desalination was performed using ultra-pure water. After air-drying the chip, 2 μL of a 50% saturated solution of sinapinic acid was added and air-dried (twice). In addition, porcine dynorphin (2147.5 m/z), human ACTH (β-chain) (3495.9 m/z), human insulin (5807.7 m/z), recombinant hirudin (6963.5 m/z), hirudin BHVK (7033.6 m/z), bovine carbonic anhydrase (29,023 m/z), equine cytochrome C (12,230.9 m/z), recombinant hirdin (6963.5 m/z), hirdin BHVK – molecular field data and 10,000 PCS4000 (Bio-Rad) and data was obtained through CiphergenExpress™ Data Manager version 3.0 (Bio-Rad). The measurement ranges were 0–100,000 m/z (Focus mass/SPA-Low; 6500) as the low molecular field data and 10,000–200,000 m/z (Focus mass/SPA-High; 20,000) as the high molecular field data. All measurements were performed in duplicate. Data analysis was performed using CiphergenExpress™ Data Manager version 3.0. After baseline correction of the measured spectra, normalization was conducted between the spectra by means of molecular weight calibration and normalization. The peak of the analysis target was a signal/noise (s/n) ratio >2.5. In regards to the relative expression intensity for the peaks in cancerous and non-cancerous areas, a significance test was performed using a Mann–Whitney U test, and a P value below .05 was determined to be a significant difference. In addition, a Receiver Operating Characteristic (ROC) plot was done, the area under curve was calculated, and a single marker analysis was assessed.

**Results**

**Protein Identification and Characterization**

Of the 47 patients with primary liver cancer who achieved remission, we conducted a retrospective proteomic analysis of the sera from 30 patients who developed bone metastasis over 8 years. Purified fractions from HPLC were analyzed by TOF-MS, which revealed a target peak of interest in the range of m/z 2000–100,000. LC–MS/MS analysis narrowed the location of the peak of interest down to m/z 5813. We named the 5813 Da peptide circulating fibrinogen metastatic peptide (referred to hereinafter as CFMP, a 41-aa peptide). The peak at MW 5813 was detected before the onset of bone metastasis in all patients, and its levels increased over time. Expression of CFMP peaked at the time of the definitive diagnosis of bone metastases by bone scintigraphy, and decreased after the start of subsequent chemotherapy and radiation treatment (Figure 1, A and B). No expression was detected in the serum of healthy controls or patients with rheumatoid arthritis (Figure S1A). The peptide was an RGD-containing peptide comprising 41 amino acids (CQFTSSTSYNRDSTTFESKYKMADEAGSEADHEGTHSTK) (which corresponds to FGA amino acids 582–621). Immunohistochemical staining demonstrated that both cancer cells and CFMP were detected in the blood vessels of human chondroblastic osteosarcoma tissue (Figure 1C) derived from a 13-year-old boy. In situ hybridization for CFMP revealed that it was expressed in numerous cancer tissues in patients, as well as in the liver of healthy individuals (Figure 1D).

**Functional Characteristics of CFMP**

In order to investigate whether CFMP has a role in the formation of new blood vessels, as one of the characteristics in cancer metastasis, we injected either water or a synthetic CFMP peptide into fertilized chicken eggs, and allowed the eggs to hatch in an incubator for 20 days, with egg rotation every 8 hours. We then obtained the vascular tissue surrounding the embryos before hatching (Figure 1E) in order to examine the presence of neo-vessels and measure the VEGF levels (Figure 1F). Significantly more new blood vessels were observed surrounding the twenty embryos injected with CFMP compared with those surrounding the five control embryos (P < .001).

H460 cells were cultured with CFMP along with normal human lung microvascular endothelial cells, or mesenchymal stem cells, on membranes containing 8.0-μm pores (Figure 2A). After 24 h of incubation, scanning electron microscopy showed that the cancer cells in the lower layer had migrated upward through the 8.0-μm pores but only when the upper layer contained mesenchymal stem cells, but not when it contained normal human lung microvascular epithelial cells (Figure 2B). RNA was extracted from the gel shown in Figure 2A and real time PCR was performed to examine the expression of GATA-2, with the data showing a high level of GATA-2 expression of CFMP peaked at the time of the definitive diagnosis of bone metastases by bone scintigraphy, and decreased after the start of subsequent chemotherapy and radiation treatment (Figure 1, A and B).
bone resorption, and its expression level is known to be regulated by the parathyroid hormone (PTH), retinoic acid and insulin-like growth factors. RNA was also isolated from the blood of healthy individuals as well as from breast cancer patients, and samples with a relative integrity number (RIN) of 8.3 were subjected to qRT-PCR analysis to measure GATA2 and MMP-13 levels, each of which is highly expressed in cancer metastasis. As a result, breast cancer patients were found to exhibit lower GATA2 levels compared with control individuals (Figure 2).

As mentioned previously, we synthesized a peptide encompassing the 576–628 aa region of FGA. The CFMP was removed and the amino acid sequence without the coiled-coil secondary structure was used as a negative control peptide to which we raised antibodies (15-aa antibody). Breast cancer BT474-GFP cells (AntiCancer Inc., San Diego, CA) stably expressing GFP and the labeled 15-aa negative peptide, comprising a sequence distinct from that of CFMP (HyLyte Fluor 555 C2 maleimide-CYGTGSETESPRNPSS) (FGA amino acid sequence 277–291 aa), were injected into nine female SCID SHO mice via the tail vein. We then examined the localization of the CFMP peptide in mouse tissues by fluorescent imaging, and Optix MX2 images were obtained at both 24 hours and 4 days after injection (Figures 3A and S3F). After 4 days, fluorescence appeared in the cranial region. After 10 days, the entire body was fluorescent, and enhanced cancer marker visibility using an Optix MX2 Imager (ART Advanced Research Technologies, Inc., Montreal, Canada) was observed. Fourteen days after the CFMP peptide was introduced into the mouse, we confirmed that the tumors occurred in the head and neck area, and we observed the formation of new-vascular vessels and bone tissues in the tumors using a 3D-CT analysis (Figure 3B).
tumors were confirmed to be adenocarcinoma by hematoxylin and eosin staining. Similar data were seen in all nine of the mice examined (Figure 3C).

We created thin-slices of tissues taken from the entire mouse 10 days after CFMP injection (5 μg/mL; 20 μL/g) (Figure 3A and Supplemental Methods), and examined these tissues for GFP-tagged cancer cells and the CFMP peptide using laser microscopy (Figure S3F). As shown in Figure 3D, whole body preparations of mice injected with BT474-GFP breast cancer cells and the CFMP peptide in Figure 3A were prepared and examined for the expression of GFP and Fluor 594-tagged CFMP using confocal laser microscopy. In breast cancer patients, metastasis typically occurs a long period of time (5 to 10 years) after chemotherapy or radiotherapy, and the reason for this delay is thought to be due to the fact that cancer cells can lie dormant in tissues. The aim of this analysis was to identify the tissues in which cancer cells and the CFMP peptide co-exist. As a result of this experiment, we discovered that the BT474-GFP breast cancer cells and the CFMP peptide were present in the same vascular tissue (Figure 3D).

Because miR-21 has previously been shown to be associated with cancer cell development [23–25], we performed in situ hybridization of miR-21 and observed its expression in vascular tissue (Figure 3E). Since there was no probe capable of quantitating the C-terminal region of FGA, we designed multiple primers capable of quantitating the CFMP nucleotide sequence. Two sets of primers were designed representing sequences inside and outside the sequence encoding CFMP. These primers are referred to FGA inner and FGA outer.

FGA inner FW 5′-GTTCTCATCACCCTGGGATAGC-3′, FGA inner RV 5′-AGCTCTTGCTTTCAAATGTGGAG-3′, FGA outer FW 5′-TGGCATCTTCACAAATACAAAGG-3′, FGA outer RV 5′-ACTTAGTCTAGGGGGACAGGGAAG-3′ (Figure 3F) to detect FGA in the vascular tissues in the cancer metastasis model, as well as vascular tissues in the same model.

The two primer PCR assay indicated that there was a lower CFMP peptide level in an anti-CFMP antibody-administered mouse. mRNA was extracted from the blood of control mice or from the cancer metastasis model mice that had been administered antibodies against CFMP (Figure 3G), and subjected to qRT-PCR to measure the expression of the C-terminal FGA CFMP nucleotide sequence using the primers shown in Figure 3F. When anti-CFMP antibodies (7 mg/kg) were administered, the expression of CFMP, as well as the tumor incidence was suppressed (Figure 3J). Each tissue excised from the mouse cancer metastasis model was subjected to western blotting using antibodies to CFMP, integrin β3, p21, VEGF, and β-actin (Figure 3H).

Next, BT474 cells, with or without CFMP, were transplanted into mice. Metastasized head and neck tissues were excised and used to determine the relative expression levels of FGA, miR-21, and
ICAM-1 by qRT-PCR. Transplantation of cancer cells along with CFMP induced higher levels of expression of miR-21 and VEGF, than did cancer cells without the peptide (Figure 3). We also performed a survival analysis to investigate the effect of the anti-CFMP peptide antibody (Supplemental Methods). CFMP by itself had no influence on in vivo survival, but in the presence of

Figure 3. In vivo model of cancer metastasis. (A) A representative image taken four days after incubating the CFMP peptide with BT474 breast cancer cells and transplanting them into a mouse is shown. BT474 breast cancer cells labeled with GFP and Fluor 594-tagged CFMP (HiLyte Fluor 594 C2 maleimide-CQFTSSTSYNRGDSTFESKSYSKMFADEAGSEADHEGHTHSTKR) were implanted via the tail vein in twenty-one female SCID Hairless Outbred (SHO) mice, and fluorescence was observed over time using an Optix MX2 Imager (ART Advanced Research Technologies, Inc., Montreal, Canada). After 4 d, fluorescence appeared in the cranial region. After 10 d, the entire body was fluorescent, and enhanced CFMP visibility was observed. (B) 3D-CT imaging confirmed the formation of new blood vessels and bony tissue in a mouse with cancer metastasis. (C) Hematoxylin and eosin staining of potentially metastasized tissue revealed the presence of carcinoma. Magnification, 20 ×. (D) Co-localization of cancer cells and CFMP. Confocal laser scanning microscopy confirmed the results seen in mice 10 days after injection of the CFMP peptide (see panel A) (Olympus FluoView FV 1000-D; Tokyo, Japan). The peptide was observed in the vascular tissue. Magnification, 10 ×. (E) In situ hybridization of miR-21. Nuclear Fast Red was used as a comparative stain. Bar: 20 μm. (F) PCR primers were designed to amplify regions of the mRNA encoding the CFMP molecule (41 aa), an external sequence (FGAc-out; nucleotides 1683–1972) and the internal sequence (FGAc-inner; nucleotides 1712–1831), and were confirmed to be useful for PCR amplification. (G) After injection of an anti-CFMP antibody (7 mg/kg) into mice implanted with cancer cells and CFMP peptides (5 μg/mL; 20 μL/g), the suppression of FGAc expression was examined by real-time PCR. (H) Western blotting analysis of SCID mice tissues following injection of CFMP peptide (5 μg/mL; 20 μL/g) and/or H460 cells into the tail vein after 2 weeks. The expression of CFMP, p21, VEGF, and integrin β3 were examined using Western blotting with an anti-CFMP antibody, an anti-p21 antibody (ab16767), an anti-VEGF antibody (TA500289) and an anti-integrin β3 antibody (ab7166), respectively. An anti-beta actin antibody (ab8227) was used as the loading control. Far left lane: Molecular weight markers. Lane 1: Lung tissue after transplantation with human lung cancer (H460) cells. Lane 2: Right lobe of liver tissue after transplantation with H460 cells. Lane 3: Left maxillary tumor tissue after transplantation with H460 cells. Lane 4: Intestinal tissue after transplantation with H460 cells and CFMP peptide. Lane 5: Left lobe of liver tissue after transplantation with H460 cells. Lane 6: Left lung tumor tissue after transplantation with H460 cells. Lanes 7: Right lung tumor tissue after transplantation with H460 cells. Lanes 8: Left lung tumor tissue after transplantation with MNNG/HOS human osteosarcoma cells. Lanes 9: Culture supernatant derived from H460 cells. Lane 10: Culture supernatant derived from MNNG/HOS human osteosarcoma cells. Lanes 11–14 are vascular tissues from mice implanted with H460 cells and CFMP peptides. (I) Quantitative analysis of VEGF values over a time course in SCID mice with metastatic cancer, and corresponding quantitative analysis by real-time PCR of the target. (J) All mice received an equivalent volume of cancer cells through their caudal (tail) vein. Thereafter, mice were injected with CFMP peptide (5 μg/mL; 20 μL/g) or an anti-CFMP antibody (7 mg/kg) for survival analyses. P-value: 0.0017, hazard ratio (HR) 3.37, 95% confidence interval (CI) 5.66–12.18 (H460 cf. H460 + anti-CFMP antibody), P-value: 0.073, HR: 1.31, 95% CI 0.64–1.75 (H460 cf. H460 + CFMP peptide).
cancer cells the mean mouse survival time was 1 week, compared with 13 weeks for mice injected with the anti-CFMP antibody (Figure 3). In addition, we also used an anti-CD51/CD61 (platelet glycoprotein GPIIb/IIIa) antibody from a previous study[26,27] to examine its expression using immunofluorescence. Figure 4 shows the layout of the primary metastatic cancer tissues (i) metastatic squamous epithelial cancer tissue, male Caucasian, aged 60 years. (ii) Metastatic lung cancer tissue, male Caucasian, aged 67 years. (iii) Metastatic breast cancer tissue, female Caucasian, aged 58 years. (iv) Metastatic ovarian mucinous cystadenoma carcinoma tissue, female Caucasian, aged 33 years. All images are at a magnification of 40 ×. Fluorescent staining of metastatic cancer tissue arrays (Z7020073; https://www.biochain.com/search/?fwp_search_bar=Z7020073, Z7020074: https://www.biochain.com/search/?fwp_search_bar=Z7020074, Bio Chain, Institute Inc., Newark, CA, USA) was observed with an Olympus fluorescence microscope BX 50 microscope and analyzed with the Image Pro-plus 4.5 software. (B) In situ hybridization for CFMP was performed in the metastatic lung cancer tissue of a male Caucasian, aged 60 years. Bar: 50 μm. (C) Cancer markers were analyzed before and after tumor extraction from prostate cancer (PSA) and breast cancer (ErbB2) patients. (D) A follow-up of breast cancer patients with bone metastasis showed that although CFMP peptide serum levels decreased postoperatively as shown from an ELISA analysis, high CFMP peptide levels were observed 1 year postoperatively. Bone metastasis was confirmed approximately 7 years postoperatively by PET-CT and 8 years postoperatively by bone scintigraphy.

A relative quantitative analysis of FGA, miR-21, and ICAM-1 in the blood revealed higher levels of these genes among patients with breast cancer and prostate cancer, both before and after operation. Moreover, CFMP levels, measured by mass spectrometry, as well as measurements of the prostate cancer marker PSA and the breast cancer marker ErbB2, revealed that levels of these markers decreased following tumor excision (Figure 4C).

Even if a high level of CFMP peptide is confirmed, a follow-up survey is important for the definitive diagnosis of a cancer metastasis site using image diagnosis. The serum from a 51-year-old female patient who underwent pectoral muscle preservation mastectomy in 1997, due to breast cancer, was examined to detect the appearance of CFMP. Following a request from the PET center of our university hospital, we initiated a prospective study around CFMP in May.
2005. At this time, cancer metastasis was not confirmed by PET-CT, but ELISA measurements did confirm a high level of CFMP, and that cancer metastasis was suspected. Therefore, we conducted a retrospective study going back to before the 1997 surgery, which confirmed bone metastasis by bone scintigraphy in October 2005. Although cancer markers (such as CEA, CA15–3, NCC-ST-439, and ALP) had been measured since 1997, they do not enable an early diagnosis like the one that CFMP has made possible (Figure 4D).

Discussion

In the present study, we retrospectively investigated the sera obtained from patients who developed bone metastases after liver cancer treatment. We discovered a novel 41-aa peptide, which we refer to as CFMP, in the sera, which was also expressed in cancer metastases, but was absent from osteoclasia in control rheumatoid arthritis samples. Rheumatoid arthritis is a systemic inflammatory disease caused by an immune abnormality, and it primarily targets joints. It is characterized by proliferation in the synovial tissue, which leads to inflammatory synovial granulation and destruction of the Pannus tissue [28]. The mechanism of its onset is suggested to be different from the bone destruction caused by bone metastasis due to cancer. We therefore also examined the expression of CFMP in patients classified according to the U.S. rheumatoid arthritis (ACR) diagnostic criteria, and we found that a class 2 patient and class 4 (severe) patient did not express CFMP.

Therefore, we suggest that this CFMP peptide is specifically related to the mechanism of bone destruction in cancer metastasis and not to the bone destruction in rheumatoid arthritis (Figure S1A and Supplemental Mass Data).

We also examined follow-up data in breast cancer patients. We also observed fluctuations in the levels of CEA, CA15–3, NCC-ST-439, ALP, and CFMP by ELISA in the serum (Figure 4D) following the removal of breast cancer tissue; bone metastasis was not confirmed in the 3D–CT imaging performed in July 2005. As a result, we measured the serum CFMP levels in these patients. However, since the levels were already 0.32 ± 0.04 μM (P < .05), we conducted a prospective study. Bone metastasis was confirmed using diagnostic imaging through bone scintigraphy in July 2006 and we resumed treatment with radiation therapy and chemotherapy. As a result, we examined our previously stored serum, and found that in 1998, the CEA, CA15–3, NCC-ST-439, and ALP markers were within normal levels after surgical removal of breast cancer tissue, but their CFMP peptide levels were 0.09 ± 0.04 μM (P < .05), demonstrating an increase. This level of CFMP was not observed in healthy subjects, or patients who did not develop metastasis. A high level of CFMP was observed at the time of bone metastases confirmation by bone scintigraphy; in addition, we observed a decline in the levels of this peptide after treatment initiation.

We also investigated the potential functions of the CFMP peptide. Early detection of breast cancer is relatively easy and the treatment methods are well established, but the question remains as to why after a long period of time (5 to 10 years) do patients then develop bone metastasis? This is puzzling since chemotherapy and radiotherapy are usually clinically effective; one possible explanation is that cancer cells continue to remain in some areas of the body. Therefore, we wanted to understand where these cancer cell depots are located. Because CFMP appears to function by causing the EMT, and therefore is important in the metastases process, we reasoned that the CFMP may be co-localized with cancer cells. As a result, we discovered that CFMP and breast cancer cells were both localized to vascular tissues. Since the CFMP peptide is not expressed in healthy subjects, or in patients with rheumatoid arthritis.

Importantly, the CFMP peptide was present in patients with bone and tumor metastasis, but absent from healthy individuals (Supplemental Mass Data). When it was administered in vivo, it caused EMT-related infiltration, angiogenesis, and neovascularization. During EMT, epidermal cells lose their epithelial characteristics and acquire those of mesenchymal cells. Cell-to-cell adhesion is also disrupted, while cell movement and invasive capacity are enhanced. Cells that differentiate into mesenchymal cells promote tissue fibrosis and contribute to the transformation into cancer cells [29]. An miRNA array analysis of migrating cancer cells in vitro revealed a high level of expression of miR-199a-3p in mesenchymal stem cells (Table S1), whereas no change in miR-199a-3p was observed in the endothelium of blood vessels found on the membrane (Figure 2A).

We concluded that the RGD motif in CFMP was responsible for promoting EMT and cancer invasion. Arginine–glycine–aspartic acid (RGD) is a cell adhesion motif that can aid in the adhesion of cancer cells. It has been identified in fibronectin, osteopontin, and laminin [30,31], and is capable of binding integrin αvβ3. This integrin is produced at high levels in the endothelial cells of new blood vessels in cancer tissues; indeed, the radiotracer 18F-galacto-RGD is frequently used as a radioactive drug target for PET imaging of tumors to verify angiogenesis in cancer [32].

CFMP was also shown in the present study to induce the expression of VEGF and ICAM-1 in the vascular tissues of a mouse cancer metastasis model. To confirm the presence of mesenchymal stem cells, western blotting with antibodies to CD90 [33] and p21 was conducted, revealing the expression of p21 in the vascular tissues of these mice. This suggests that CFMP has detected in blood vessels, which is consistent with the finding that human cancer metastasis occurs 5–10 years after the onset of cancer [34]. Based on advances in oncology, primary cancer has become almost controllable. Together with our current findings, this indicates treatment should aim to prevent metastasis to distant organs.

Numerous studies have demonstrated an association between cancers and fibrinogen, a precursor to a blood clotting factor [35–40]. Trouseau’s syndrome is a paraneoplastic disorder that was described in 1865, by Armand Trouseau as migratory superficial thrombophlebitis [41]. Previous research reported that a fibrinogen-deficient mouse showed significantly reduced cancer metastases, suggesting that fibrinogen plays an important role in the process of cancer metastasis [41–45]. Moreover, the blood of cancer patients is often in a hypercoagulable state that causes thrombosis [46,47]. In the present study, we observed that CFMP originated from C-terminal region of FGA (576–628 aa) and that it is involved in several important processes, including platelet aggregation to form tumor thrombi around cancer cells, and cancer cell adhesion to the vascular endothelial beds of metastasis target organs, thereby facilitating extravascular invasion through fibrinogen receptors and metastasis formation; to our knowledge, these mechanisms have not previously been elucidated. Fibrinogen is known to promote the adhesion of leukocytes to vascular endothelial cells via an interaction with ICAM-1 and to further mediate migration across the vascular endothelium [48]. However, fibrinogen itself has no physiological activity and is degraded in vivo into various fragments by proteases such as thrombin. Consequently, it is unclear why this novel peptide is cleaved at this specific site.
Ogah et al. [49], previously reported that highly metastatic tumor-derived vascular endothelial cells have stem cell-like properties, which might explain our finding that CFMP exists in the blood vessels of patients with metastatic cancer, causes EMT, and moves towards the mesenchymal stem cells. In our in vitro model of metastatic invasion, miR-199-3a expression was detected in the membrane between the cancer cells and the upper cell layer, whereas miR-21 expression was observed in the 3D gel. This experiment also revealed that plasmin activity was decreased, whereas p21 was up-regulated, when H460 cells and vascular endothelial cells were co-cultured in the presence of CFMP, with no changes being observed in the absence of the peptide. This was presumably because in the period following excision of the primary tumor, during which a small number of residual cancer cells generate new blood vessels and were hidden in thrombi. We also found that tumor cells were confined to a net-like structure within the metastatic tissue and were obscured inside the blood vessels or at the vessel wall.

Two variants of FGA result from alternative splicing. The CFMP 41-aa sequence identified in this study is present in both isoforms, but obscured inside the blood vessels or at the vessel wall.

Confined to a net-like structure within the metastatic tissue and were observed in the period following excision of the primary tumor, during which a small number of residual cancer cells generate new blood vessels and were hidden in thrombi. We also found that tumor cells were confined to a net-like structure within the metastatic tissue and were obscured inside the blood vessels or at the vessel wall.

This would enable earlier [51,52], more specific, and sensitive approach to therapy for chronic liver disease in an HCV hyperendemic area of Japan. Hepatol Res 28, 30–35.

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Appendix A. Supplementary data

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