Repression of cathepsin E expression increases the risk of mammary carcinogenesis and links to poor prognosis in breast cancer

Tomoyo Kawakubo1, Atsushi Yasukochi1,2, Tatsuya Toyama3, Satoru Takahashi4, Kuniaki Okamoto5, Takayuki Tsukuba4, Seiji Nakamura2, Yasuhiko Ozaki6, Koichi Nishigaki1, Hiroko Yamashita1,2 and Kenji Yamamoto1,8

1Proteolysis Research Laboratory, Graduate School of Pharmaceutical Sciences and 2Department of Oral and Maxillofacial Surgery, Graduate School of Dental Science, Kyushu University, Fukuoka 812-8582, Japan, 3Department of Oncology, Immunology and Surgery and 4Department of Experimental Pathology and Tumor Biology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan, 5Department of Dental Pharmacology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki 852-8588, Japan, 6Department of Obstetrics and Gynecology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan and 7Department of Functional Materials Sciences, Faculty of Engineering, Saitama University, Saitama 338-8587, Japan
8Present address: Department of Breast and Endocrine Surgery, Hokkaido University Hospital, Kita-ku, Sapporo 060-8648, Japan

© The Author 2013. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Introduction

Breast cancer (BC) is one of the most occurring cancers in women worldwide. Despite advances in diagnosis and treatment for BC, the prognosis and survival for most patients have not dramatically changed. Histological analyses of surgical specimens from BC patients are widely used to evaluate the tumor invasion and metastasis status, but seem not to be cost- and labor-effective. Therefore, more convenient diagnostic and prognostic methods for identifying a particular subgroup of patients with potentially poor prognosis are required to improve the overall outcome of BC. The circulating blood holds great promise as a reservoir of disease information and serves to provide a large variety of disease markers. We have thus been searching for new serum prognostic markers for BC.

Cathepsin E (CatE) is an intracellular aspartic proteinase, which is predominantly expressed in certain cell types including the immune system cells and the rapidly regenerating cells (1). The intracellular localization of CatE varies with different cell types (1). The strategic expression and localization suggest that CatE is associated with specific functions of each cell type. A role of CatE in immune responses against tumor cells has especially received special attention, because host defense against cancer cells is an important factor determining tumor evolution. It is also interesting to note that CatE displays a potent anticancer activity through manifold functions (1) and regulates the expression of proteins related to signaling, development, differentiation and proliferation in mouse mammary gland (2). Loss of CatE was also shown to induce precancerous conditions in this tissue (2). Meanwhile, recent studies suggest the clinical utility of CatE as a potential biomarker in certain human cancers, although its clinical significance is controversial (3–7). To our knowledge, endogenous CatE inhibitors have not so far been identified in mammals. On the other hand, it has previously been reported that there are two CatE genes in normal and cancer cells in a different proportion (8) or two isozymic forms of CatE derived from the same gene (9,10) and the abnormal molecular size enzyme in the pancreatic juice of the patient with ductal adenocarcinoma (11). These findings strongly suggest that some of CatE proteins produced in cancer cells are non-functional. Given that most of the previous studies were performed by immunological analyses such as immunohistochemistry, tissue microarray and enzyme-linked immunosorbent assay (ELISA) or by gene expression profiling analyses such as quantitative reverse transcription–polymerase chain reaction (RT–PCR) or in situ hybridization to assess the total CatE expression levels, it seems likely that the conflicting reports seem to arise from differences in expression levels of some non-functional CatE between cancer cell types.

In this study, we show that reduction of the serum level of CatE activity is associated with poor prognosis in BC patients. The association of CatE expression with mammary carcinogenesis was further supported by results of in vivo and in vitro studies using four different genotypes of syngeneic multiparous mice showing different levels of CatE expression. This study shows that repression of CatE expression increases the risk of mammary carcinogenesis and links to poor prognosis in BC.

Materials and methods

Patients and samples

The study included 293 consecutive Japanese women with BC, newly diagnosed at Nagoya City University Hospital between July 1999 and July 2008, and 107 control Japanese women who visited our hospital because of abnormal mammograms. The study protocol was approved by the institutional review boards and confirmed with the guidelines of the 1996 Declaration of Helsinki. Histopathological examinations were performed to confirm the diagnosis of BC. The histopathological stage was classified according to the tumor–node–metastasis (TNM) classification system. The clinical stage information was obtained from the hospital information system and records of the patients were reviewed to determine the tumor size, lymph node metastasis, and distant metastasis. The patients were classified into two groups, according to the tumor size and lymph node metastasis. Local treatment was performed using surgery or preoperative chemotherapy combined with radiotherapy. The patients were followed up as outpatients every 4 months until the end of the study period (June 2009). The median follow-up period was 130 months. The disease-free survival (DFS) and overall survival (OS) rates were calculated using the Kaplan–Meier method. The association between the serum CatE activity and clinicopathological characteristics was examined using the log-rank test and the univariate and multivariate Cox proportional hazards regression models. The categorical variables were compared using the χ2 test or Fisher’s exact test.

The study included 293 consecutive Japanese women with BC, newly diagnosed at Nagoya City University Hospital between July 1999 and July 2008, and 107 control Japanese women who visited our hospital because of abnormal mammograms. The study protocol was approved by the institutional review boards and confirmed with the guidelines of the 1996 Declaration of Helsinki. Histopathological examinations were performed to confirm the diagnosis of BC. The histopathological stage was classified according to the tumor–node–metastasis (TNM) classification system. The clinical stage information was obtained from the hospital information system and records of the patients were reviewed to determine the tumor size, lymph node metastasis, and distant metastasis. The patients were classified into two groups, according to the tumor size and lymph node metastasis. Local treatment was performed using surgery or preoperative chemotherapy combined with radiotherapy. The patients were followed up as outpatients every 4 months until the end of the study period (June 2009). The median follow-up period was 130 months. The disease-free survival (DFS) and overall survival (OS) rates were calculated using the Kaplan–Meier method. The association between the serum CatE activity and clinicopathological characteristics was examined using the log-rank test and the univariate and multivariate Cox proportional hazards regression models. The categorical variables were compared using the χ2 test or Fisher’s exact test.

Abbreviations: 3D, three-dimensional; BC, breast cancer; CatE, cathepsin E; CatEoverexpressing transgenic; CatE−/−, CatE-deficient heterozygous; CatE−/−, CatE-deficient homozygous; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IgG, immunoglobulin G; mRNA, messenger RNA; PBS, phosphate-buffered saline; PLA, proximity ligation assay; RT–PCR, reverse transcription–polymerase chain reaction; sFRP1, secreted Frizzled-related protein-1; TBS-T, Tris-buffered saline containing 0.05% Tween-20; Wnt-1, Wnt-1-induced secreted protein-1; WT, wild-type.
Helsinki. All BC patients except for those with Stage IV disease underwent surgical treatment (mastectomy or lumpectomy). Patients received adequate endocrine or chemotherapy for adjutant or metastatic diseases.

**Mice**

All animals were maintained according to the guidelines of the Japanese Pharmacological Society in a specific pathogen-free facility at the Kyushu University School for Collaborative Research. All animal experiments were approved by the Animal Research Committee of the Graduate School of Pharmaceutical Sciences, Kyushu University. Wild-type (WT), CatE-overexpressing transgenic (CatE+), CatE-deficient heterozygous (CatE+/−) and CatE-deficient homozygous (CatE−/−) mice on the C57BL/6 background were used as described previously (2,12,13).

**Assay**

The assay of CatE activity in sera was performed using KYS-1 as a substrate by a slight modification of the method of Yasuda et al. (14). Reaction mixtures contained 80 μl of 50 mM sodium acetate buffer, pH 4.0, 10 μl of 200 μM substrate solution and 10 μl of sample solution containing 1–5 μl of sera and 0.1% Triton X-100. After 10 min incubation at 40°C, the reaction was terminated by adding 2 ml of 10 mM Tris–HCl buffer, pH 8.6.

**Enzyme-linked immunosorbent assay**

Two different monoclonal antibodies against human CatE were custom made at GenoSta Co. (Tokyo, Japan). The first monoclonal antibody was added to 96-well plates and incubated at 4°C overnight. After washing with Tris-buffered saline (TBS), pH 7.4, containing 0.05% Tween-20 (TBS-T), the plates were incubated with 1% Blocking One (Nacalai Tesque, Tokyo, Japan)/ TBS-T at 37°C for 1 h. After washing, serum (15 μl) was added to the plate and incubated for 2 h at room temperature. After washing, 100 μl of the biotin-labeled secondary monoclonal antibody (2 μg/ml in 1% Blocking One/TBS-T) was added to each plate and incubated for 1 h at room temperature. After washing, 100 μl of avidin-conjugated peroxidase (2 μg/ml in 1% Blocking One/ TBS-T) was added to the plates and incubated for 30 min at room temperature. Then the plates were washed and incubated with 100 μl of 3,3’,5,5’-tetramethylbenzidine (TMB) for 20 min at room temperature in dark. The reaction was terminated by adding 100 μl of 1 N hydrochloric acid.

**Immunohistochemical analyses**

Immunohistochemical analysis was performed on mouse mammary tissue sections that had been formalin fixed and paraffin embedded, essentially according to the method described previously (15). For immunoblotting, various antibodies including purified polyclonal antibodies to human CatE (15), human CatE propeptide (16) and rat CatE (16) were used. Polyclonal anti-Wnt5a antibodies raised in rabbits against the synthetic peptide corresponding to the sequence between amino acids 190 and 240 of human Wnt5a were obtained from Abcam plc (Cambridge, UK). All other antibodies used were purchased from various commercial sources.

**Primary culture of mammary cells**

Primary mammary cells were prepared from 8- to 12-week-old virgin female mice, essentially according to the method described previously (17). Briefly, mammary glands were digested for 6h at 37°C in EpicPlt-B medium (StemCell Technologies, Tukwila, WA) containing 5% fetal bovine serum, 300 U/ml collagenase, 100 U/ml hyaluronidase, 10 ng/ml recombinant human epidermal growth factor, 10 ng/ml recombinant human basic fibroblast growth factor and 0.0004% heparin. After lysis of the red blood cells, the resultant organoid pellet was subjected to sequential dissociation by gentle pipetting in TryplE Express (GIBCO) for 2 min and in 5 mg/ml Dispase II (Godo Shusei Co., Ltd, Chiba, Japan) plus 1 mg/ml DNase for 1 min. The cell suspension was then filtered through 40 μm cell strainer and suspended in EpicPlt-B defined medium.

**Mammosphere assay**

Mammosphere formation was performed in 24-well plates coated with Matrigel (Growth Factor Reduced Matrigel, BD Biosciences), essentially according to the method described previously (18). In vitro formation assay of capillary-like structure was performed according to the method described previously (19).

**Quantitative RT–PCR analysis**

Quantitative RT–PCR was performed essentially according to the method described previously (20). Amplonc sizes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 75 bp), secreted Frizzled-related protein-1 (sFRP1; 69 bp), Wnt-1 induced secreted protein-1 (Wisp-1; 68 bp) and β-catenin (68 bp). Primers used to amplify the transcripts are as follows: GAPDH: (forward) TGT CGG TCG TGG ATG TCA C (reverse) CCT GCT TCA CCA CCT TCT TG; sFRP1: (forward) GTG GTT CAA CAT GTG CTC CCA, (reverse) TCA TAG GAG CCA ACA TGC; Wisp-1: (forward) AAA GGG CAT GTG CAT TAT CC, (reverse) AAG CAT GCT GTA AGC TAG TTC TGA and β-catenin: (forward) GCA GCA GCA GTT TGT GGA, (reverse) TGT GGA GAG CTC CAG TAC ACC. Light Cycler Universal Probe Master (Roche Diagnostics GmbH) specific for each sequence was applied to number (80 for GAPDH and sFRP1), number (65 for Wisp-1) and number (25 for β-catenin). All reactions were performed in triplicate for three dependent experiments. GAPDH was used as an endogenous control.

**Transfection**

Mouse CatE complementary DNA (cDNA) was subcloned from the pBlue-script SK plasmid containing full-length mouse CatE cDNA (21) into pCDNA3 and maxiA vector. The construct was verified by DNA sequence analysis and restriction enzyme digestion analysis. Primary cultures of CatE−/− mammary cells were transiently transfected with pcDNA4/HisMaxA-C-mCatE or pcDNA4/HisMaxA-MOCK (empty vector) using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics GmbH) following the manufacturer’s protocol. For RNA interference, primary cultures of WT mammary cells were transiently transfected with control siRNA-A (sc-37007; Santa Cruz Biotechnology, Santa Cruz, CA) or CatE siRNA (sc-41474; Santa Cruz Biotechnology), using siRNA Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer’s protocol. After 72h, the cells were washed with phosphate-buffered saline (PBS) three times and then harvested with ice-cold radioimmunoprecipitation assay buffer (Nacalai Tesque, Kyoto, Japan) containing phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO) and protease inhibitors and then lysed and centrifuged at 10,000g for 10 min. The supernatant was used as the cell extract. The cultured medium was also concentrated to a final volume of 500 μl by using Amicon Centiprep YM-3.

**Proximity ligation assay analysis**

Proximity ligation assay (PLA) was performed using the Duolink® II Kit (Olink Bioscience, Uppsala, Sweden) according to the manufacturer’s instruction. This kit is based on the use of two unique and bifunctional probes called PlaTM, each probe consisting of a secondary antibody attached to a unique synthetic oligonucleotide that acts as a reporter. After a 30 min fixation with 4% paraformaldehyde and blocking with PBS containing 5% Blocking One/TBS-T, sections of primary mammary cells were stained with one or two primary antibodies depending on the experiment (single protein detection or detection of interacting proteins). After washing, the sections were incubated with the secondary oligonucleotide-linked antibodies (PLA probes) provided in the kit. After hybridization for 1 h at 37°C, ligation for 30 min at 37°C and amplification for 100 min at 37°C, the oligonucleotides bound to the antibodies were detected using a fluorescent probe (Detection Kit 563). The products were visualized with fluorescently labeled oligonucleotides and the sections counterstained using Duolink II Mounting Medium with 4’,6-di-aminidino-2-phenylindoline. When two PLA probes were in close proximity to each other (<40nm), the products were seen as fluorescent dots.

**Statistical analysis**

Student’s t-test was used for comparison of two data sets. A P-value <0.05 was accepted to deny the null hypothesis. Estimation of disease-free and overall survival was performed using the Kaplan–Meier method, and differences between survival curves were assessed with the log-rank test. Cox’s proportional hazards model was used for univariate and multivariate analyses of prognostic values.

**Results**

Prognostic value of CatE activity in the sera of BC patients

To explore the clinical significance of CatE expression in BC, we first analyzed the serum levels of CatE activity in BC patients using MOC-Ac-Gly-Ser-Pro-Ala-Phex-Leu-Ala-Lys(Dnp)-d-Arg-NH2, termed KYS-1, as a substrate (14). Besides the insusceptibility of this substrate to other plasma proteases (1-4), almost all of the KYS-1-hydrolyzing activity in the sera of healthy women was immunoprecipitated with anti-human and anti-rat CatE immunoglobulin G (IgG), but not with normal rabbit IgG and antihuman CatE propeptide IgG (Figure 1A), indicating that the serum KYS-1-hydrolyzing activity is attributed to CatE. To further assess whether the activity levels of CatE were consistent with the protein levels, the sera of five healthy women were analyzed by ELISA using two different monoclonal anti-human CatE IgG. Although the expression levels of this protein in sera of healthy women seemed to be roughly correlated with the

---

715

Downloaded from https://academic.oup.com/carcin/article-abstract/35/3/714/2463276 by guest on 09 May 2018
activity levels (Figure 1B), those in the sera of BC patients were not necessarily consistent with those of the activity (Figure 1C). This was further substantiated by a detailed comparison of the serum CatE levels in BC patients with recurrence and distant metastasis between the activity measurement and ELISA (Supplementary Figure 1, available at Carcinogenesis Online).

Correlation between the serum CatE activity and clinicopathological parameters was analyzed in consecutively followed up BC patients. The serum CatE activity was significantly decreased in BC patients compared with control women according to the stages and progression of BC (Figure 1D). Statistically significant differences in the serum levels of CatE activity were also found between BC patients with and without recurrence or distant metastasis. A clinical meaningful cutoff point of the serum CatE activity for disease prognosis analysis was determined by Kaplan–Meier method and verified by the log-rank test and estimated to be 3.3 U/ml. Then the serum samples were thus divided into the subgroups based on this cutoff value. Kaplan–Meier analysis demonstrated that the serum levels of CatE activity were positively correlated with the disease-free and overall survival of the patients with recurrence or lymph node metastasis (Figure 1E and F). The reduced CatE activity, evident in 89% of the invasive ductal carcinoma, was significantly associated with the Stage IV disease, the lymph node metastasis and absence of estrogen receptor expression (Table I). Although univariate survival analysis showed a significant association between all the clinical parameters tested and disease-free and overall survival of BC patients, multivariate analysis revealed that only either genotype of virgin female mice but also multiparous WT and CatE<sup>−/−</sup> mice did not develop spontaneous mammary tumors at all. In contrast, multiparous CatE<sup>−/−</sup>- and CatE<sup>+/−</sup>- mice spontaneously developed mammary tumors at a high frequency in an age-dependent manner (Figure 2B). The incidence of mammary tumors in CatE<sup>−/−</sup>- mice was significantly higher than that in CatE<sup>+/−</sup>-littermates. Remarkably, more than 95% of multiparous CatE<sup>−/−</sup>-mice spontaneously developed mammary tumors at 78 weeks of age. All of the CatE<sup>−/−</sup>- mice bearing mammary tumors showed lung metastasis (Figure 2B, insert) and died within 4 weeks after the tumor was first identified. No significant relationship between the number of pregnancies and the incidence of BC was observed. Histologically, the mammary tumors formed in the CatE<sup>−/−</sup>-mice are likely to display the increased infiltration and mitotic activity of tumor cells (Figure 2C). Histological analysis also revealed that WT virgin mice at 10 weeks of age had a number of developed or developing mammary gland structures, whereas the corresponding CatE<sup>−/−</sup>- mice possessed a small number of mammary gland structures with atrophied small lumens (Figures 2D). Immunohistochemical analysis showed that CatE was mainly localized in the mammary epithelial cells of WT mice (Figure 2E, bottom). An in situ PLA using anti-CatE IgG further revealed that WT mammary cells showed punctate staining over the whole cytoplasm. This pattern appeared to be characteristic of vesicular protein expression. In addition, WT cells showed the perinuclear reticular staining characteristic of proteins retained in Golgi complex and the endoplasmic reticulum, besides the nuclear staining (Figure 2F). Western blot analysis revealed that WT cell lysates contained an intense 42 kDa and a minor 46 kDa band, whereas the culture supernatant mainly contained a 44 kDa band (Figure 2G). Unlike 42 kDa protein, the 46 and 44 kDa proteins were rapidly converted to the 42 kDa form by acid treatment at pH 4.0 and 37°C for 10 min.
**Cathepsin E in mammary gland**

**Fig. 1.** Relationship between the serum levels of CatE expression and clinical characteristics or prognosis in BC patients. Blood samples were collected from control and BC patients at the first medical examination, besides healthy women, and centrifuged, and then the separated sera were stored at −20°C before use. (A) Immunoprecipitation of KYS-1-hydrolyzing activity in sera of four healthy women with various purified antibodies. Serum samples (50 μl) were diluted four times with PBS and incubated with various purified antibodies indicated in 40 μl PBS (lane 1: normal human IgG, 44 μg; lane 2: antihuman CatE propeptide IgG, 40 μg; lane 3: antihuman CatE IgG, 40 μg; lane 4: antirat CatE IgG, 40 μg) at 37°C for 10 min, followed by at 4°C overnight. After addition of protein A-Sepharose beads and incubation at 4°C for 5 h under gentle stirring, the samples were centrifuged at 10 000 g for 10 min and then the KYS-1-hydrolyzing activity in the supernatants was determined. Data are mean values from two independent experiments for each person that were performed in triplicates for each antibody. (B) A comparison of CatE expression levels in sera of five healthy women determined by activity measurement with those by ELISA. (C) The relationship between the activity and protein levels of CatE expression in sera of BC patients. Data are mean values from three independent experiments performed in triplicates for each assay. Dotted lines show the cutoff value of 3.3 U/ml for serum CatE activity of all patients. (D) Comparison of the serum CatE activity levels with the clinical characteristics of control and BC patients. Data represent the mean ± SEM. (E) Comparison of Kaplan–Meier curves of disease-free and overall survival for BC patients with invasive carcinoma categorized according to serum levels of CatE activity: low (≤3.3 U/ml), intermediate (3.3–6.0 U/ml) and high (>6.0 U/ml). High CatE activity is significantly associated with improved survival in human BC. The P-value was determined using the log-rank test. **P < 0.0001, high versus low levels of serum CatE activity. (F) Kaplan–Meier curves of disease-free and overall survival for BC patients without lymph node metastasis. *P < 0.01, BC patients with high CatE activity (>6.0 U/ml) versus to those with low CatE activity (≤3.3 U/ml).

As it is known that pro- and intermediate forms of CatE are rapidly converted to mature CatE by a brief acid treatment, whereas the mature enzyme does not undergo any alteration (22), the 46 and 44 kDa proteins are considered to be the pro- and intermediate forms of CatE, respectively, and the 42 kDa is the mature enzyme. The mouse mammary gland extract and serum also showed major 42 kDa and minor 46 kDa forms of CatE (Supplementary Figure 2B, available at Carcinogenesis Online).
Human or rodent mammary cell cultures have been shown to promote morphogenetic differentiation on this culture. To address whether primary mammary cells isolated from 10-week-old WT and CatE−/− virgin mice contain the stem cells that can regenerate new mammary tissue-like structures and possess self-renewal activity, a mammosphere formation assay was performed using an in vitro 3D Matrigel culture was composed of two layers: an underlying layer of primary mammary cells suspended in Matrigel and an overlying layer of the culture medium. The number of mammospheres formed by a combination between CatE−/− mammary cells and the corresponding conditioned medium was significantly less than that by a combination between WT cells and their conditioned medium (Figure 3A). The reduced mammosphere formation ability of CatE−/− cells with their conditioned medium was markedly restored with replacement of the cells by WT cells and significantly with replacement of the conditioned medium by that of WT cells. Further, an in vitro capillary-like structure formation assay showed that WT cells had a low proliferation activity and a high capillary-like structure formation activity, whereby the size and number of the cells were stably maintained. In contrast, CatE−/− cells exhibited the enhanced cell proliferation whereby large spheroids with a number of aberrant capillary-like structures and branching processes were formed (Figure 3B). These results indicate that WT mammary cells hold the stem cell activity, whereas CatE−/− cells display the impaired mammary growth and differentiation.

Epithelial–mesenchymal transition (EMT) of tumor cells is considered a crucial factor in cancer metastasis. Aberrant formation of mammospheres and capillary-like structures induced by CatE deficiency, therefore, is likely associated with misregulation of EMT. Indeed, the mRNA levels of N-cadherin, sFRP1, and Wisp-1 in whole CatE−/− mammary glands were ~1.7-, ~1.9- and ~1.76-fold, respectively, those in WT counterparts (Figure 3C). The mRNA levels of E-cadherin and vimentin in these tissues tended to be significantly, but not significantly, decreased by CatE deficiency (data not shown). In contrast, β-catenin mRNA level in the former was ~0.4-fold the level in the latter (Figure 3C). Consistent with this, western blot analysis revealed that N-cadherin protein was significantly increased in CatE−/− mammary glands compared with the WT cells, whereas β-catenin was apparently decreased by CatE deficiency (Figure 3D). Despite the increase of sFRP1 mRNA in...
CatE−/− mammary glands, its protein level in the supernatant was significantly lower than that of WT tissues. Immunohistochemical analysis revealed that there were no significant differences in the expression and localization of β-catenin in various breast skin regions other than the subcutaneous tissue, such as epidermis, hair follicle and muscle layer, between WT and CatE−/− mice (Figure 3E, top, Supplementary Table 1, available at Carcinogenesis Online). However, the overall expression of β-catenin in subcutaneous tissues of CatE−/− breast skin was significantly decreased compared with that of WT cells, where the ratio of the cytoplasmic/nuclear staining pattern appeared to be decreased (Figure 3E, middle and bottom). Of note, although the expression of β-catenin in the whole mammary glands was significantly decreased by CatE deficiency, the expression in single mammary cells was not significantly different between WT and CatE−/− mice, except for the translocation and accumulation of β-catenin in the nuclei of CatE−/− mammary cells. These data suggest that EMT-related alterations in the CatE−/− breast skin were specific for mammary epithelial cells. Given that Wnt/β-catenin signaling links with induction of EMT during development and tumor progression (25–27), the translocation and accumulation of β-catenin in the nuclei of CatE−/− mammary cells should induce the upregulation of Wnt/β-catenin pathway, thereby increasing the risk of mammary carcinogenesis in multiparous CatE−/− mice through aberrant EMT activation.

Interaction of CatE and Wnt5a in mammary epithelial cells

Differing from canonical Wnts, non-canonical Wnt5a is known to be strictly regulated during the period from the pubescent development to the pregnant and lactation in the postnatal mouse mammary gland (28) and to inhibit the β-catenin pathway (29). Further, loss of Wnt5a was shown to be associated with early relapse of invasive BC, increased metastasis and poor survival in humans and mice (28,30,31) and acceleration of mouse mammary gland development (32). These findings suggest that this protein acts as a tumor suppressor in mammary carcinogenesis. To explore a functional link between CatE and Wnt5a in mammary gland development, the impact of CatE deficiency on the expression and localization of Wnt5a in mammary tissues was first analyzed by immunohistochemistry. Although Wnt5a was widely detected in a number of mammary cells in the subcutaneous tissue of WT breast skin regions, the number of Wnt5a-positive cells was markedly decreased in the subcutaneous tissue of CatE−/− breast skin regions with decrease in the number of mammary gland structures (Figure 4A). Importantly, however, the expression of Wnt5a protein in single mammary epithelial cells was not significantly

![Graphs and images]

(A) Relative KLN-192 activity

(B) Frequency of spontaneous mammary tumors

(C) Histological analysis of CatE−/− mammary glands

(D) CatE+/+ vs. CatE−/−

(E) CatE+/+ vs. CatE−/−
The first finding of this study is that the serum CatE activity is a novel independent prognostic marker for BC. The fundamental question changed by CatE deficiency (Figure 4A), although the protein was mainly localized in the nuclei of CatE−/− cells (Figure 4B). PLA further revealed that Wnt5a in WT mammary cells displayed punctuate granular and reticular staining patterns and was colocalized mostly with CatE (Figure 4B) and partly with organelle marker proteins for the endoplasmic reticulum (KEDL), the Golgi complex (GM130), the late endosome or lysosome (M6PR) and early endosomes (EEA1) (Figure 4C).

Like other Wnt family proteins, Wnt5a is known to be dynamically modified in its intracellular transport processes (33,34). Western blot analysis revealed that there were no significant differences in the quantity and electrophoretic profiles of Wnt5a in cell lysates between WT and CatE−/− mammary cells (Figure 4D). Strikingly, the amount of Wnt5a proteins secreted in the culture supernatant of WT cells was markedly decreased compared with CatE−/− cells. In addition, a few minor bands having Mr ~30 000 and ~20 000 found in the culture supernatant of WT cells were barely detectable compared with CatE−/− cells. When treated with purified CatE at various pH values and 37°C for 3 h, recombinant mouse Wnt5a was specifically degraded at pH 5.0–5.5 to generate four major protein bands with Mr ~43 000, ~30 000, ~20 000 and ~18 000, whose N-terminal amino acid sequences were found to start with Asp1, Thr12, Asn14 and Met19 of the sequence of the recombinant protein, respectively (Supplementary Figure 2, available at Carcinogenesis Online). Therefore, Wnt5a proteins with apparent Mr ~45 000, ~40 000, ~30 000 and ~20 000 in the culture supernatant of WT cells appear to correspond to its processed or degraded forms.

To further assess whether the impaired processing and secretion of Wnt5a, besides its translocation to the nucleus, affects Wnt5a-dependent signaling, the impact of CatE deficiency on Wnt5a-triggered Ror2 signaling was analyzed. Ror2, a member of the Ror-family receptor tyrosine kinases, is known to act as a receptor or coreceptor for Wnt5a to mediate various Wnt5a-induced cellular functions (33,35). Wnt5a is also known to directly enhance Ror2 tyrosine kinase activity, whereby Wnt5β/catenin signaling is inhibited (36). To the contrary, loss of Ror2 is shown to enhance Wnt5β/catenin signaling (36). Although a basal expression level of Ror2 was not significantly different between WT and CatE−/− cells, Ror2 immunoprecipitated by phosphotyrosine antibodies was significantly lower in CatE−/− cells than in WT cells (Figure 4E), indicating that loss of CatE impairs Wnt5a/Ror2 signaling. To provide additional evidence for association between CatE action and the trafficking, maturation and secretion of Wnt5a in mouse mammary epithelial cells, CatE gene was transiently expressed in CatE−/− cells using pcDNA4/HisMax6A-mCatE vector (Figure 4F). The expression of CatE protein in CatE−/− mammary transfectants significantly enhanced the secretion and production of mature and/or processed Wnt5a in the culture medium comparable to that in WT cells. To the contrary, repression of the endogenous expression of CatE protein by transfection with CatE siRNA in WT cells, which retained only 5–10% of the endogenous CatE, resulted in a significant decrease in the secretion and production of mature and/or processed form of Wnt5a in the culture medium (Figure 4G). This study thus provide the first evidence for a functional linkage between CatE and Wnt5a in mammary cells and the first to show that CatE is required for Wnt5a to regulate normal growth and differentiation of mammary glands.

Discussion

The first finding of this study is that the serum CatE activity is a novel independent prognostic marker for BC. The fundamental question...
Cathepsin E in mammary gland

(A) Mammospheres/Field

(B) CatE<sup>+/+</sup> vs. CatE<sup>-/-</sup>

(C) Relative ratio of mRNA levels

(D) Western blot analysis of N-cadherin, β-catenin, and Anti-sFRP1
Fig. 3. Effect of CatE deficiency on differentiation and proliferation of mouse mammary gland. (A) In vitro mammosphere formation assay using Matrigel to detect mammosphere-initiating cells (mammary progenitor cells). Primary mammary cells (1.0 × 10^5) from each genotype were mixed in Matrigel and added to 24-well plates with an overlay of 50 μl solidified Matrigel. Then EpiCult-B medium (1 ml) was replenished to each well and cultured for 2 days at 37°C in 5% CO₂. After the following 3 days, the medium was changed into the indicated conditioned media that were cultured for 3 days separately and concentrated to 25% with Amicon Centriprep YM-3 (Millipore, Billerica, MA). The number of spheres (>50 μm diameter) was measured in four replicate wells per condition and counted in four fields per well. Data represent the mean ± SEM. *P < 0.001 versus the values from a combination between WT cells and the corresponding conditioned medium. (B) In vitro 3D culture on Matrigel to assess the differentiation potential of primary mammary cells. Primary mammary cells (1.5 × 10^5) from each genotype of virgin mice were suspended in 1 ml EpiCult-B medium containing 5 ng epidermal growth factor (StemCell Technologies) and 2% Matrigel and seeded as a single-cell suspension onto the 24-well plates coated with 100% Matrigel. The plates were cultured for 3 days at 37°C in 5% CO₂. The data are representative of the results with six mice for each genotype. Scale bars: 100 μm. (C) The mRNA levels of EMT-related molecules in WT and CatE⁻/⁻ breast tissues were analyzed by quantitative RT–PCR. The relative expression levels of the genes normalized to the amount of the transcripts in WT samples. Data represent the mean ± SEM. *P < 0.001 versus the value of WT samples. (D) Western blot analysis for EMT-related molecules in the cell lysate and sFRP1 in the culture media of mammary cells from WT and CatE⁻/⁻ mice. (E) Immunohistochemical localization for β-catenin in breast skin regions of WT and CatE⁻/⁻ virgin mice at 10 weeks of age. Top: The breast skin of CatE⁻/⁻ mice was characterized by significantly reduced subcutaneous tissue and hair follicles. β-Catenin was detected in most of the mammary glands of WT mice, whereas this protein was found in a small number of mammary glands of CatE⁻/⁻ mice. Of note, there are no significant differences in β-catenin staining patterns in breast skin regions other than subcutaneous tissues, including epidermis (black arrows) and muscle layers (yellow arrows) between WT and CatE⁻/⁻ mice. Middle: β-Catenin in the subcutaneous tissues was detected in most of the developing or developed mammary cells of WT mice, whereas this protein was found in a small number of mammary cells of CatE⁻/⁻ mice. Bottom: A higher magnified view of the images in the middle panels. β-Catenin was diffusely detected in the mammary cells of WT mice, whereas this protein was granularly stained in a small number of mammary cells of CatE⁻/⁻ mice. The data are representative of the results with six mice for each genotype.
whether the serum levels of CatE activity determined with KYS-1 would be attributed to this protein was substantiated by immunoprecipitation experiments with anti-CatE IgG. The serum levels of CatE activity were negatively associated with the stage, recurrence and lymph node metastasis of BC and positively correlated with the overall survival of the patients. Our data also showed that the activity levels of CatE in the sera of BC patients with recurrence and lymph node metastasis were not consistent with the protein levels determined by ELISA, indicating that CatE expression at protein levels in sera of BC patients is unable to serve as a plasma marker for prognosis and metastasis. Although it still remains unclear why the diagnostic and prognostic significance of CatE expression in human cancers is controversial in the literature, the present findings strongly suggest that some of CatE proteins produced in some types of cancer cells
Fig. 4. Expression and cellular localization of Wnt5a in WT and CatE−/− breast tissues. (A) Immunohistochemical staining for Wnt5a in primary mammary cells from WT and CatE−/− virgin mice at 10 weeks of age. Simple Stain Mouse MAX-PO (Rabbit) (Nichirei Biosciences, Tokyo, Japan) was used for immunohistochemistry with anti-Wnt5a antibody (1:100 dilution). The photographs in the bottom panels represent higher magnification views of the images (square) in the upper panels. Wnt5a is widely distributed in both WT and CatE−/− epithelial cells, although the number of mammary gland structures is markedly different between the two genotypes. White and black arrows indicate CatE-positive epidermis and -immunocompetent cells, respectively. The data are representative of the results with six mice for each genotype. (B) PLA for detecting Wnt5a as well as CatE in mammary epithelial cells of WT or CatE−/− mice. For visualization of Wnt5a, primary mammary cells of each genotype were treated with rabbit anti-Wnt5a polyclonal antibodies and then with a pair of anti-rabbit PLUS and MINUS probes. Wnt5a exhibited punctuate and perinuclear staining in WT cells, whereas this protein was exclusively confined to the nuclei of CatE−/− mice. For analyzing the colocalization between CatE and Wnt5a, the WT cells were treated with mouse anti-CatE monoclonal antibody and rabbit anti-Wnt5a polyclonal antibody, followed by the addition of anti-mouse PLA MINUS and anti-rabbit PLA PLUS, respectively. PLA images clearly indicate the colocalization of CatE and Wnt5a in the cells. (C) PLA for analyzing colocalization of Wnt5a with organelle marker proteins for the endoplasmic reticulum (KEDL), the Golgi complex (GM130), the late endosome or lysosome (M6PR) and early endosomes (EEA1) in WT cells, using rabbit polyclonal anti-Wnt5a antibodies and mouse monoclonal antibody for each marker protein. Nuclei were displayed by 4′,6-diamidino-2-phenylindole (blue). Wnt5a was found to colocalized with all the markers. (D) Western blot analysis of Wnt5a in the cell lysate and culture supernatant fractions of primary mammary epithelial cells from WT or CatE−/− virgin mice. Equal amounts of proteins for the corresponding fractions of each genotype were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and immunoblot analyses. (E) Western blot analyses of total and phosphorylated Ror2 in primary mammary cells from each genotype of virgin mice. The cell lysates were incubated with phosphotyrosine antibody bound to protein G-Sepharose beads in the presence of phosphatase inhibitor cocktail. The beads were washed three times with the radioimmunoprecipitation assay buffer and boiled in sodium dodecyl sulfate sample buffer. The solubilized samples were subjected to immunoblot analysis using rabbit polyclonal antibody against phosphotyrosine (P-Tyr-100). Lysates were proved with anti-GAPDH to ensure equal loading of samples. (F) The expression and secretion of Wnt5a in CatE−/− mammary epithelial cells transiently transfected with an expression vector carrying the cDNA of CatE or with an empty vector. The cell lysates and concentrated culture supernatants of each cell culture were separated and the equal amounts of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected using anti-Wnt5a. Lysates were proved with anti-CatE and anti-GAPDH to ensure the expression of CatE and equal loading of samples, respectively. (G) Downregulation of CatE expression in WT mammary epithelial cells transfected with control siRNA-A (sc-37007) or CatE siRNA (sc-41474) using siRNA Transfection Reagent. Lysates were proved with anti-CatE and anti-GAPDH to ensure downregulation of CatE expression and equal loading of samples, respectively. Other details were the same as described above.
Cathepsin E in mammary gland

Interaction between CatE and Wnt5a in mammary cells was validated by knockdown and restoration of CatE expression, besides PLA data indicating their colocalization. Our data thus indicate that CatE is a novel protein essential for the molecular events underpinning the processing, trafficking and secretion of Wnt5a in the mammary epithelial cells.

Supplementary material
Supplementary Figures 1–3 and Table 1 can be found at http://carcin.oxfordjournals.org/

Funding

Conflict of Interest Statement: None declared.

References
The opposing roles of Wnt-5a in cancer.

T.Kawakubo et al.


Received July, 19, 2013; revised October 28, 2013; accepted November 6, 2013