PRECLINICAL STUDY

Clinical significance of CYLD downregulation in breast cancer

**Mitsuhiro Hayashi · Hirofumi Jono · Satoru Shinriki · Takuya Nakamura ·
Jianying Guo · Aiko Sueta · Mai Tomiguchi · Saori Fujiwara ·
Mutsuko Yamamoto-Ibusuki · Kei-ichi Murakami · Satoshi Yamashita ·
Yutaka Yamamoto · Jian-Dong Li · Hirotaka Iwase · Yukio Ando**

Abstract Cylindromatosis (CYLD) is a tumor suppressor gene that is mutated in familial cylindromatosis, a rare autosomal dominant disorder associated with numerous benign skin adnexal tumors. CYLD is now known to regulate various signaling pathways, including transforming growth factor-β signaling, Wnt/β-catenin signaling, and NF-κB signaling by deubiquitinating upstream regulatory factors. Downregulation of CYLD has been reported in several malignancies; however, the clinical significance of CYLD expression in many malignancies, including breast cancer, remains to be elucidated. This study investigated the clinical significance of CYLD in breast cancer and its roles in tumor progression. We evaluated CYLD expression in matched normal breast tissue samples and tumor breast tissue samples from 26 patients with breast cancer and in a series of breast cancer cell lines. In addition, by means of immunohistochemistry, we investigated CYLD protein expression and its clinical significance in 244 breast cancer cases. We also analyzed the effects of CYLD repression or overexpression on breast cancer cell viability, cell migration, and NF-κB activity with or without receptor activator of NF-κB ligand (RANKL) stimulation. Breast cancer tissues demonstrated significantly reduced CYLD mRNA expression compared with normal breast tissues. Downregulation of CYLD promoted cell survival and migratory activities through NF-κB activation, whereas CYLD overexpression inhibited those activities in MDA-MB-231 cells. As an important finding, CYLD overexpression also inhibited RANKL-induced NF-κB activation. Our immunohistochemical analysis revealed that reduced CYLD protein expression was significantly correlated with estrogen receptor negativity, high Ki-67 index, high nuclear grade, decreased disease-free survival, and reduced breast cancer-specific survival in primary breast cancer. Moreover, reduced CYLD expression was an independent factor for poor prognosis in breast cancer. CYLD downregulation may promote breast cancer metastasis via NF-κB activation, including RANKL signaling.

M. Hayashi · A. Sueta · M. Tomiguchi · S. Fujiwara ·
M. Yamamoto-Ibusuki · K. Murakami · Y. Yamamoto ·
H. Iwase
Department of Breast and Endocrine Surgery, Graduate School
of Medical Sciences, Kumamoto University, Kumamoto, Japan

H. Jono
Department of Pharmacy, Kumamoto University Hospital,
Kumamoto, Japan

S. Shinriki (✉)
Department of Laboratory Medicine, Graduate School
of Medical Sciences, Kumamoto University, 1-1-1 Honjo,
Chuo-ku, Kumamoto, Kumamoto 860-8556, Japan
e-mail: satorus@fc.kuh.kumamoto-u.ac.jp

T. Nakamura
Department of Oral and Maxillofacial Surgery, Graduate School
of Medical Sciences, Kumamoto University, Kumamoto, Japan

J. Guo · S. Yamashita · Y. Ando
Department of Neurology, Graduate School of Medical Sciences,
Kumamoto University, Kumamoto, Japan

J.-D. Li
Center for Inflammation, Immunity and Infection
and Department of Biology, Georgia State University,
Atlanta, GA, USA

Abbreviations

CYLD	Cylindromatosis
RANKL	NF-κB ligand
HER2	Human epidermal growth factor receptor 2
ER	Estrogen receptor
PgR	Progesterone receptor
HMEC	Human mammary epithelial cells

Introduction

The cylindromatosis gene (*CYLD*) was initially identified
as a mutated gene in familial cylindromatosis, a rare
autosomal dominant disorder associated with numerous
benign skin adnexal tumors [1]. *CYLD* encodes a protein
that possesses an ubiquitin C-terminal hydrolase domain,
which allows the protein to function as a deubiquitinating
enzyme [2]. CYLD is now known to regulate various sig-
naling pathways, including transforming growth factor-β
(TGF-β) signaling, Wnt/β-catenin signaling, and NF-κB
signaling [3–5]. In the NF-κB signaling cascade, CYLD
can negatively regulate various steps by deubiquitinating
TNF receptor-associated factors 2 and 6, NF-κB essential
modulator, receptor interacting protein 1, and TGF-β-
activated kinase 1, which allows it to have important roles
in inflammatory and immune reactions [2, 6–8]. Down-
regulation or mutation of *CYLD* has been reported in sev-
eral malignancies [1, 9, 10], and *Cyld* knockout mice were
shown to be highly susceptible to chemically induced skin
tumors [11]. However, the clinical significance of CYLD
expression in many malignancies, including breast cancer,
remains to be elucidated.

Breast cancer, one of the most common human cancers,
is a heterogeneous disease characterized by various
molecular subtypes, which have distinct molecular features
and clinical behaviors [12, 13]. Although some of these
molecular features have been identified as biomarkers and
drug targets, novel prognostic biomarkers and clarification
of molecular mechanisms underlying the development and
progression of breast cancer are still needed.

Constitutive activation of NF-κB is often observed in
breast cancer tissues across the current major breast cancer
subtypes (luminal, HER2, and triple negative) and cell lines
with the more aggressive phenotypes [14–17]. Accumulat-
ing evidence has shown that activation of NF-κB promoted
the development of breast cancer and resistance to conven-
tional therapies such as endocrine therapy, cytotoxic che-
motherapy, and radiotherapy [18–22]. Interestingly, recent
studies showed that NF-κB signaling contributed to expan-
sion of breast tumor stem cells and mammary tumorigenesis
through cell-autonomous and non-cell-autonomous mecha-
nisms [23–28]. In addition, receptor activator of NF-κB
ligand (RANKL) and its receptor RANK, which activate the
NF-κB pathway, have received much attention as key mol-
ecules in the promotion of breast cancer development and
progression. Today, a drug targeting RANKL, denosumab, is
registered to prevent skeletal-related events in advanced
breast cancer patients with bone metastases in breast cancer
treatment. However, no biomarker exists to identify tumors
that are sensitive to the drug or to detect NF-κB activation,
and the precise mechanisms underlying constitutive activa-
tion of NF-κB are not fully understood.

In this study, we investigated the clinical significance of
CYLD expression in breast cancer and the roles of CYLD
in breast cancer progression as related to NF-κB activation.

Materials and methods

Patients and tissues

Included in this study were paraffin-embedded breast
cancer specimens from 244 women with histologically
confirmed breast cancer (consecutive cases) who had
received treatment at Kumamoto University Hospital
between 2001 and 2008. Breast cancer oncologists chose
the systemic treatments used, such as hormonal therapy,
cytotoxic chemotherapy, and/or anti-HER2 therapy,
according to the clinical evidence on tumor biology and
clinical staging that was available at that time [29]. Our
mRNA dataset of clinical breast cancer cases included 26
matched cases whose tumor tissues and adjacent normal
breast tissues could both be evaluated. The ethics com-
mittee of Kumamoto University Graduate School of
Medical Sciences approved the study protocol. Informed
consent was obtained from all patients. In patients with
primary invasive breast cancer (stages I to III, $n = 230$),
89.1 % of patients received any systemic adjuvant treat-
ment (endocrine therapy 72.6 %; chemotherapy 37.4 %).
Among them, patients with luminal/HER2-negative breast
cancer ($n = 162$) received endocrine therapy (93.8 %) and/
or chemotherapy (25.9 %). Regarding triple-negative cases
($n = 32$), 62.5 % of patients received chemotherapy, and
36.1 % of 36 patients with HER2-positive breast cancer
received adjuvant trastuzumab.

Immunohistochemistry

Expression of CYLD in breast cancer samples was ana-
lyzed by using immunohistochemical methods with anti-
CYLD antibody produced in rabbits (Sigma-Aldrich, St.

Louis, MO). Expression of estrogen receptor (ER) α (SP1; Ventana Japan, Tokyo, Japan), progesterone receptor (PgR; 1E2; Ventana Japan), HER2 (Dako Japan, Tokyo, Japan), and Ki-67 (MIB-1; Dako Japan) was also examined according to the manufacturers' recommended protocols. Experienced pathologists at our institution determined the nuclear grade according to nuclear pleomorphism and frequency of cell mitosis. The CYLD status was considered positive when cytoplasmic staining was ≥10 %. ER or PgR status was considered positive when nuclear staining was ≥1 % [30]. HER2 positivity was indicated by immuno-histochemical staining score of 3+ (HercepTest, Dako) or by fluorescent in situ hybridization with a threshold ratio of more than 2.2 [31]. The Ki-67 index was scored according to the percentage of cells with nuclear staining of all cancer cells in the hot spots, at a ×400 high-power field, with at least 500 tumor cells counted.

Cell culture

Human breast adenocarcinoma cell lines (MDA-MB-231, MDA-MB-468, T47D, MCF7, ZR-75-30, MDA-MB-453, SK-BR-3, and HCC1569) and human mammary epithelial cells (HMEC) were purchased from American Type Culture Collection (Manassas, VA) and were passaged in our laboratory for less than 6 months after receipt or resuscitation. Cells were grown in Dulbecco's modified Eagle medium (Gibco, Life Technologies Corporation, Carlsbad, CA) (MDA-MB-231and MDA-MB-453) or RPMI 1640 (MDA-MB-468, T47D, MCF7, ZR-75-30 and HCC1569) or McCoy's 5a medium (SK-BR-3) supplemented with 10 % FBS (Gibco) under adherent conditions in 5 % CO_2 at 37 °C. The details of these cell lines' subtypes are as follows: basal-like subtype, MDA-MB-231 and MDA-MB-468; luminal subtype, MCF7 and T47D; luminal-HER2 subtype, ZR-75-30; and HER2 subtype, MDA-MB-453, SK-BR-3, and HCC1569.

RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells by using the TRIzol Reagent (Life Technologies Corporation) and from tissue specimens by means of the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturers' instructions. Total RNA was quantified via the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and was reverse transcribed to cDNA by using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. All qRT-PCR reactions were performed with 2 μM cDNA and each primer at 0.2 μM via the LightCycler System (Roche

Diagnostics, Tokyo, Japan) with SYBR Premix DimerE-raser (Takara Bio Inc.).

Transfection with siRNA

Cell lines were transfected with CYLD-specific siRNA by using Lipofectamine 2000 (Life Technologies Corporation) according to the manufacturer's protocol. Each analysis, such as total RNA extraction, protein extraction, and reporter assay, was performed after more than 48 h of incubation. Silencer Negative Control siRNA (Applied Biosystems, Life Technologies Corporation) was used as the control. CYLD-specific siRNA was sense 5′-GAU-UGUUACUUCUAUCAAAtt-3′ and antisense 5′-UUU-GAUAGAAGUAACAAUCtt-3′ (Applied Biosystems, Life Technologies Corporation).

Protein extraction and immunoblotting

Cells were washed once in ice-cold PBS (Gibco) and were then lysed by adding CelLytic M Cell Lysis Reagent (Sigma-Aldrich) containing a fresh Protease Inhibitor Cocktail (Sigma-Aldrich), 50 mM NAF, and 1 mM Na_3VO_4. After incubation for 15 min on a shaker on ice, cell lysate was removed from the dishes and was centrifuged at $15,000 \times g$ for 10 min to remove insoluble material. The protein concentration was determined by using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacturer's instructions. Equal amounts of protein were fractionated via SDS-PAGE and fractions were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Primary antibodies used for immunoblotting were as follows: rabbit anti-CYLD antibody (Sigma-Aldrich), mouse anti-maspin antibody (Santa Cruz Biotechnology Inc., TX), and mouse anti-β-actin antibody (Sigma-Aldrich). Blots were visualized by using ECL Prime Western Blotting Detection Reagent (GE Healthcare Japan, Tokyo, Japan) according to the manufacturer's instructions.

Reporter assay

Cell lines transfected with siRNA (negative control or CYLD) or plasmid (pcDNA or wild-type CYLD) were sequentially cotransfected with NF-κB reporter plasmid (pGL4b vector; Promega, Madison, WI) and control plasmid (phRG-TK vector; Promega). After a 24-h incubation in serum-free medium, cells were treated with recombinant human soluble RANKL (Peprotech, Rocky Hill, NJ) for 4 h or were untreated. Luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega).
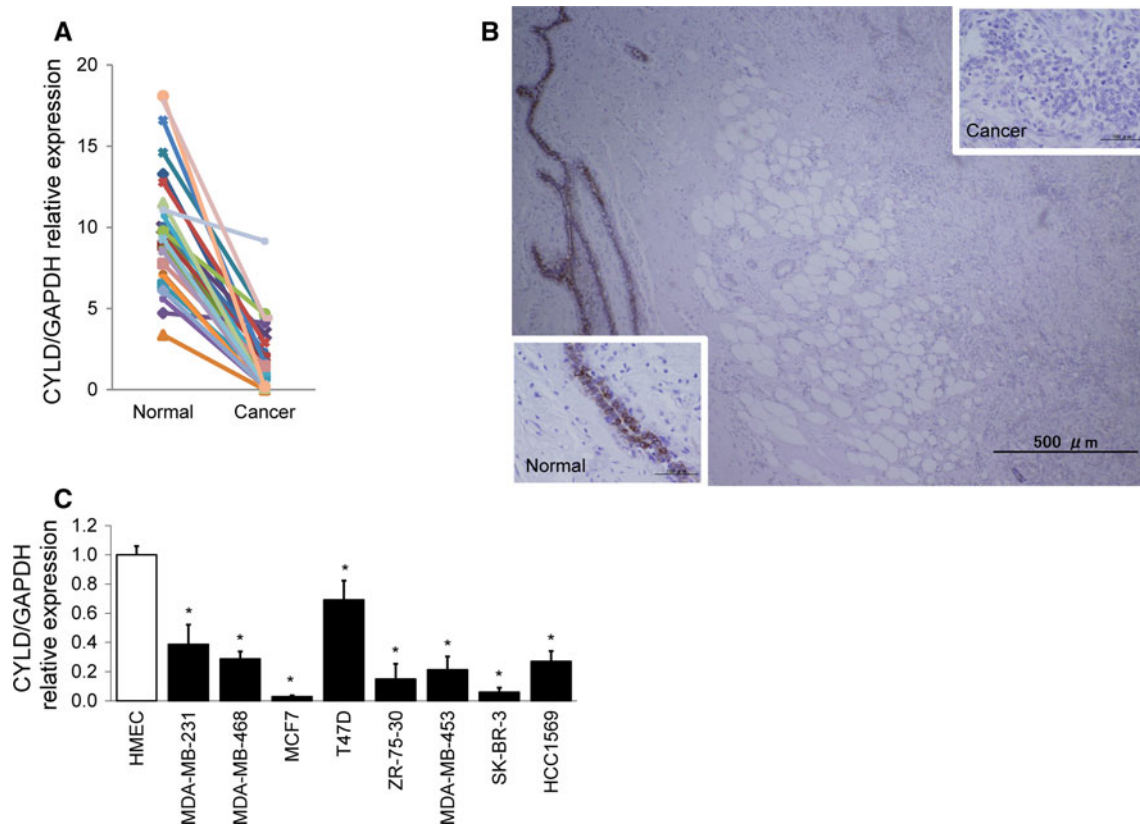
Fig. 1 CYLD expression in breast cancer. a CYLD mRNA expression in breast cancer tissue and the surrounding normal breast tissue of the same patients ($n = 26$). b Representative tissue section of breast cancer with CYLD downregulation. c CYLD mRNA expression in breast cancer cell lines and non-malignant HMEC. CYLD mRNA expression level was measured by means of qRT-PCR *$P < 0.05$ compared with HMEC in Student's t test. Values are mean ± SD of triplicate samples

Cell viability, apoptosis, and migration assays

Cell viability was evaluated by means of the MTS assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) or Luna automated cell counter (Logos Biosystems, Annandale, VA). To evaluate apoptosis, we used the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Tokyo, Japan), according to the manufacturer's protocol, and we performed the analysis with the FACSCalibur Flow Cytometer (BD Biosciences). We used the wound-healing assay to evaluate cell migration and quantified the results with ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

We analyzed the data, presented as mean ± SD, by using Student's t test or ANOVA. The means and SD were generated from triplicates in one experiment and then we confirmed the same results from two or three independent experiments. We utilized the nonparametric Wilcoxon, Fisher's exact, and χ^2 tests for statistical analysis of the associations between CYLD status and clinicopathological factors. Disease-free

survival (DFS) and breast cancer-specific survival (BCSS) curves were calculated according to the Kaplan–Meier method and were verified by means of the log-rank test. Univariate and multivariate analyses of prognostic values were performed with the Cox proportional hazards model. Statistical significance was defined as two-sided $P < 0.05$. JMP software version 8.0 for Windows (SAS Institute Japan, Tokyo, Japan) was used for all statistical analyses.

Results

CYLD expression in breast cancer

We first analyzed CYLD mRNA expression in matched tumor tissues and adjacent normal breast tissues from 26 patients with breast cancer. Breast cancer lesions had significantly lower expression of CYLD mRNA than did normal lesions [cancer: median 1.25 (25th–75th percentile 0.17–3.05); normal: median 9.60 (25th–75th percentile 7.08–11.82), paired Student's t test, $P < 0.0001$] (Fig. 1a). Our immunohistochemical analysis of breast cancer tissues showed that CYLD expression occurred predominantly in
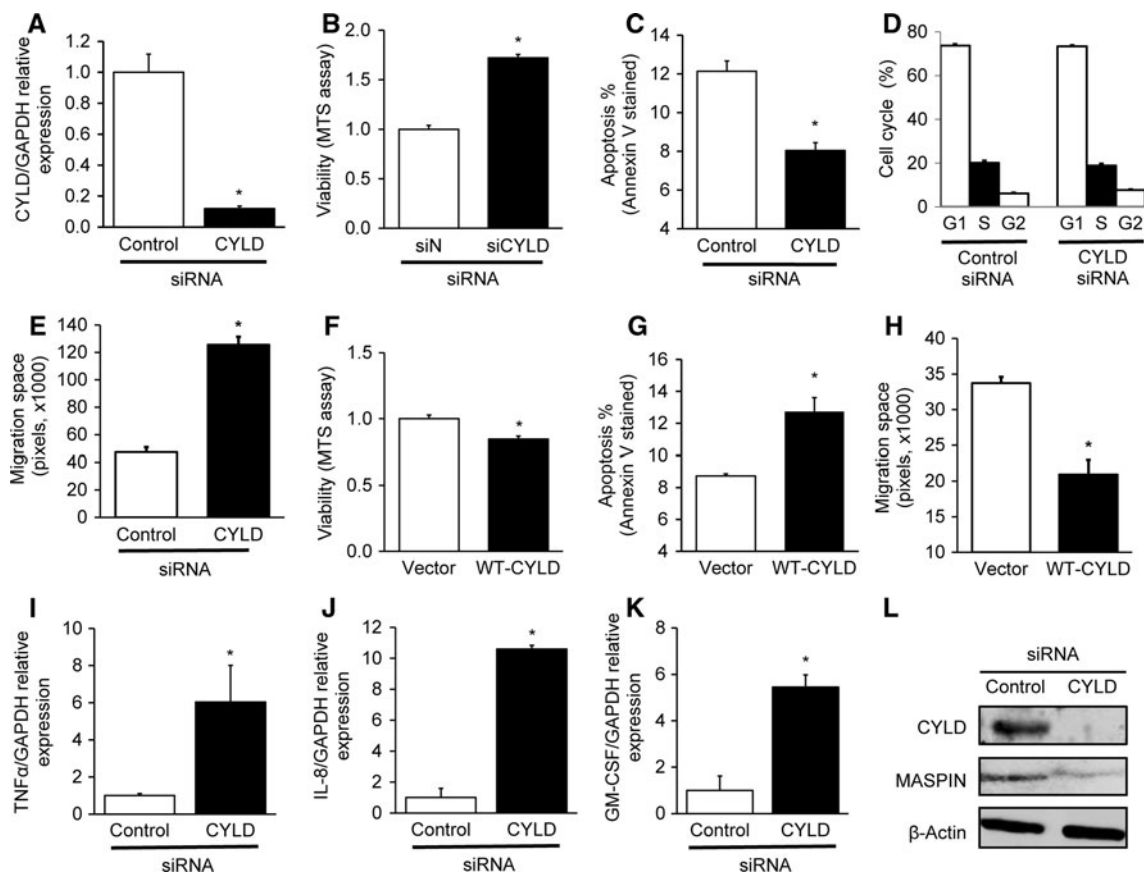
Fig. 2 Effects of CYLD expression on cell survival and migration in MDA-MB-231 cells. **a** Knockdown efficiency of CYLD mRNA by siRNA in MDA-MB-231 cells. **b** Cell viability in serum-free medium was evaluated by MTS assay 72 h after transfection with siRNA. **c** Anti-apoptotic property in serum-free condition was evaluated by Annexin V assay 96 h after transfection with siRNA. **d** Cell cycle distribution in serum-starved condition 72 h after siRNA transfection was analyzed by flow cytometry following staining with propidium iodide. **e** Cells were wounded at 72 h after siRNA transfection. After incubation for 48 h in serum-free medium, cell migration was

analyzed. Effects of CYLD overexpression on cell viability (**f**) and apoptosis (**g**) were assessed by MTS assay and Annexin V assay, respectively, 96 h after transfection with wild-type CYLD plasmid. **h** Cells were wounded at 96 h after transfection with wild-type CYLD plasmid. After incubation for 24 h in serum-free medium, cell migration was measured. **i**, **j**, and **k** Expression level of TNFa (**i**), IL-8 (**j**), or GM-CSF (**k**) mRNA was measured by means of qRT-PCR 72 h after siRNA transfection. *$P < 0.05$ in Student's t test. Values are mean ± SD of triplicate samples. **l** Maspin protein expression was evaluated via western blotting 72 h after siRNA transfection

normal appearing mammary epithelial cells, and reduced CYLD expression was found in cancer cells, as seen in a representative tissue section (Fig. 1b). All breast cancer cell lines also demonstrated significantly reduced CYLD mRNA expression, independent of molecular subtypes, compared with HMEC (Fig. 1c). These data indicate that CYLD mRNA expression was repressed in breast cancer.

CYLD repression increased viability and migration of breast cancer cells

To determine the involvement of CYLD repression in breast cancer progression, we used siRNA to study the effects of CYLD knockdown on cell viability and motility. CYLD mRNA level was reduced by approximately 90 % 48 h after transfection of CYLD-specific siRNA in MDA-MB-231

cells (Fig. 2a). With MDA-MB-231, we observed increased cell viability (Fig. 2b), with decreased apoptosis in serum-free conditions (Fig. 2c), after transfection with CYLD-specific siRNA. No significant change in the cell cycle occurred (Fig. 2d). In addition, CYLD knockdown in MDA-MB-231 cells stimulated migration (Fig. 2e). The same results were also obtained in MDA-MB-468 cells (data not shown). In contrast, overexpression of wild-type CYLD resulted in decreased cell viability, increased apoptosis in serum-free condition and reduced migration (Fig. 2f–h). Furthermore, we observed enhanced expression of various genes encoding TNFα, interleukin-8, and granulocyte–macrophage colony-stimulating factor, which are regulated by NF-κB and associated with breast cancer progression, after CYLD knockdown (Fig. 2i–K). We also found that downregulation of CYLD led to reduced expression of
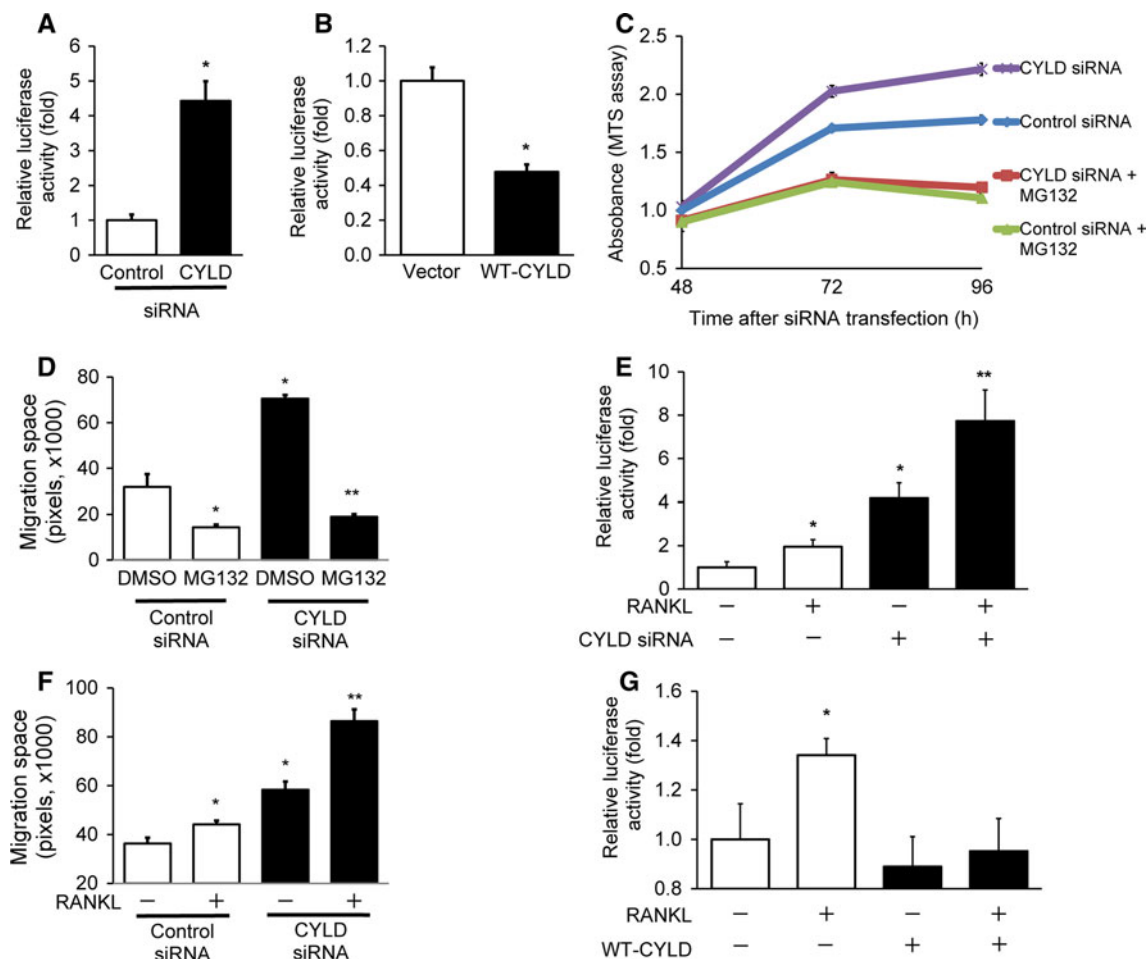
Fig. 3 CYLD as a negative regulator of NF-κB signaling including RANKL in MDA-MB-231 cells. **a** and **b** NF-κB activity was evaluated by luciferase reporter assay 96 h after transfection with siRNA or vectors in serum-free condition. *$P < 0.05$ in Student's *t* test. **c** NF-κB inhibitor, MG132 (0.2 μM), was added to the cells at 48 h after siRNA transfection, followed by incubation for up to 48 h in serum-free condition. Cell viability was evaluated by MTS assay. **d** Cells were wounded at 72 h after siRNA transfection. After incubation with 1 μM MG132 for 24 h, cell migration was analyzed. *$P < 0.05$ compared with control siRNA treated with DMSO, and **$P < 0.05$ compared with CYLD siRNA treated with DMSO in Student's *t* test. **e** Recombinant RANKL (500 ng/ml) was added to the

cells 96 h after siRNA transfection. After incubation for 4 h in serum-free condition, NF-κB activity was evaluated by luciferase reporter assay. **f** Cells were wounded at 96 h after siRNA transfection. After incubation with recombinant RANKL (500 ng/ml) for 24 h, cell migration was analyzed. *$P < 0.05$ compared with control siRNA and mock treatment, and **$P < 0.05$ compared with CYLD siRNA and mock treatment in Student's *t* test. **g** Recombinant RANKL (500 ng/ml) was added to the cells 96 h after transfection with wild-type CYLD plasmid. After incubation for 4 h in serum-free condition, NF-κB activity was evaluated by luciferase reporter assay. *$P < 0.05$ compared with all the other conditions in Student's *t* test

maspin, which was reportedly a crucial suppressor of breast cancer metastasis (Fig. 2l) [32, 33].

Negative regulation by CYLD of NF-κB activity including RANKL-RANK signaling in breast cancer

To determine whether CYLD expression was associated with NF-κB activity in breast cancer, we utilized the reporter assay. CYLD knockdown in MDA-MB-231, MDA-MB-468, and T47D cells stimulated NF-κB activity (Fig. 3a and Supplementary Fig. S1), whereas overexpression of wild-type CYLD suppressed it (Fig. 3b). In line with the above

finding, the increased cell viability and migratory activity induced by CYLD knockdown were abolished by adding MG132 as an inhibitor of NF-κB signaling (Fig. 3c, d).

Recent studies also showed that RANKL-NF-κB signaling had crucial roles in the development and progression of breast cancer other than bone events [34–38]. Thus, we next investigated whether CYLD was involved in RANKL-induced NF-κB activation in MDA-MB-231 cells. We found that RANKL enhanced not only NF-κB activity (Fig. 3e) but also migration of both control and CYLD knockdown cells (Fig. 3f). An important finding was that RANKL-induced NF-κB activation was inhibited by overexpression of wild-
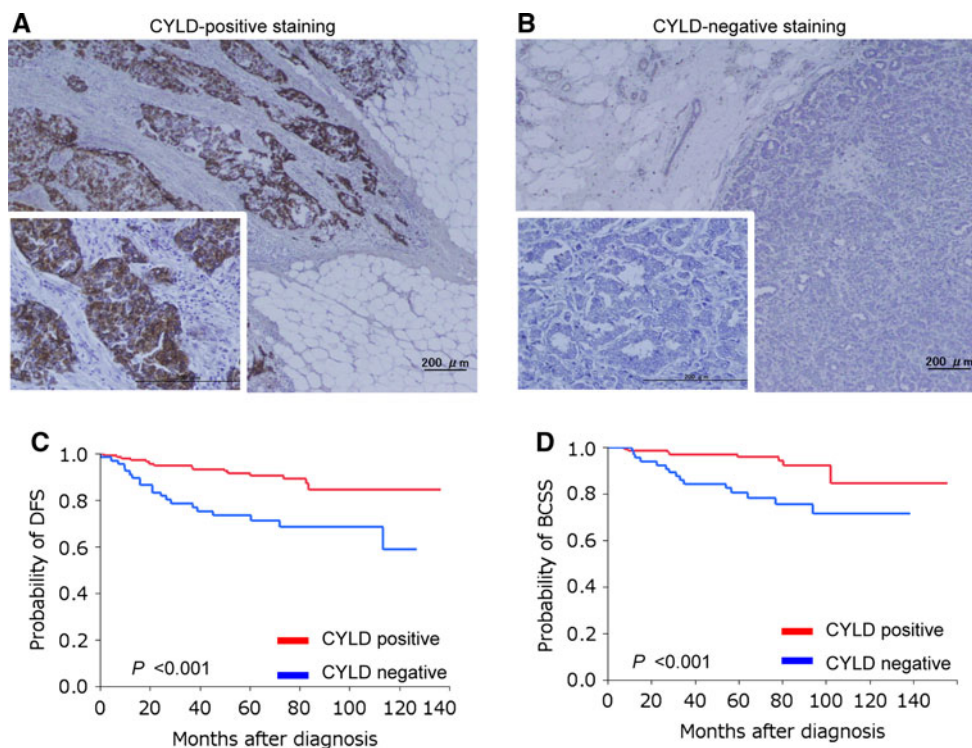
Fig. 4 Relationship between CYLD expression and prognosis in primary invasive breast cancer. CYLD protein expression in primary invasive breast cancer tissues ($n = 244$) was analyzed by immunohistochemistry. **a** and **b** Representative examples of CYLD-positive breast cancer tissue (**a**) and CYLD-negative tissue (**b**). Note that normal mammary epithelial cells or breast cancer cells mainly expressed CYLD. **c** and **d** Kaplan–Meier plots for disease-free survival (DFS) (**c**) and breast cancer-specific survival (BCSS) (**d**) according to CYLD status (negative, $N = 70$; positive, $N = 160$) were constructed in invasive primary breast cancer cases ($n = 230$) except in situ and metastatic cases. Statistical significances were evaluated using log-rank test

type CYLD (Fig. 3g). These data indicate that CYLD was a negative regulator of NF-κB activation, including RANKL signaling, in breast cancer cells.

CYLD was an independent prognostic factor in primary breast cancer

We also investigated correlations between CYLD protein expression and clinicopathological factors and prognosis in breast cancer. Our immunohistochemical analysis ($n = 244$) indicated CYLD-negative results in 73 cases (29.9 %) and CYLD-positive results in 171 cases (70.1 %) (Fig. 4a, b). CYLD-negative status occurred more frequently in ER-negative (Fisher's exact test, $P = 0.024$), PgR-negative (Fisher's exact test, $P = 0.008$), or triple-negative (χ^2 test, $P = 0.032$) breast cancer tissues [12] than did CYLD-positive status (Table 1). CYLD-negative status also correlated with a high Ki-67 index (χ^2 test, $P = 0.009$) and a high nuclear grade (χ^2 test, $P < 0.001$).

Kaplan–Meier survival analysis ($n = 230$) revealed that, with a median follow-up of 65.7 months, reduced CYLD expression correlated strongly with poor DFS (log-rank test, $P = 0.0005$, Fig. 4c) and poor BCSS (log-rank test, $P = 0.0009$, Fig. 4d) in patients with primary invasive breast cancer (stages I–III). The 5-year DFS and BCSS rates were 74.9 and 82.6 % in patients with CYLD-negative tumors compared with 91.0 and 95.3 % in patients with CYLD-positive tumors, respectively. Similar results were obtained for each breast cancer subtype (luminal A, luminal B, and triple negative) (Supplementary Fig. S2), except HER2 subtype [12]. With regard to CYLD expression and DFS, we obtained the same results when we evaluated CYLD mRNA expression (data not shown). Furthermore, our multivariate analysis using the Cox proportional hazards model revealed that CYLD expression was an independent prognostic factor for DFS (CYLD-negative status, hazard ratio HR 2.43; 95 % confidence interval CI 1.16–5.26, $P = 0.019$) (Table 2). Multivariate analysis also showed a tendency for a strong relationship between CYLD expression and BCSS (Cox proportional hazards model, $P = 0.057$, Supplementary Table S1).

Discussion

In this study, we demonstrated for the first time that reduced CYLD expression was an independent factor for poor prognosis in breast cancer. Loss of CYLD stimulated

Table 1 Correlations between CYLD expression and clinicopathological factors in breast cancer

Factor	No. of patients (%)		P
	CYLD negative (n = 73)	CYLD positive (n = 171)	
Age (y)			
Median	58	60	0.813
Range	30–86	27–93	
Menopausal status			
Pre/peri	18 (25)	43 (25)	1.000
Post	55 (75)	128 (75)	
Tumor size			
<30 mm	54 (77)	136 (81)	0.595
≥30 mm	16 (23)	32 (19)	
Lymph node metastasis			
Positive	30 (41)	52 (30)	0.139
Negative	43 (59)	119 (70)	
ER[a]			
Positive	47 (64)	135 (79)	0.024
Negative	26 (36)	36 (21)	
PgR[b]			
Positive	38 (52)	120 (70)	0.008
Negative	35 (48)	51 (30)	
HER2			
Positive	14 (19)	25 (15)	0.446
Negative	59 (81)	146 (85)	
Ki-67 index[c]			
High	45 (62)	69 (40)	0.009
Low	27 (37)	99 (58)	
Unknown	1 (1)	3 (2)	
Nuclear grade			
1	26 (36)	105 (61)	<0.001
2	16 (22)	33 (19)	
3	31 (42)	33 (19)	
Subtype[d]			
Luminal A	10 (14)	45 (26)	0.032
Luminal B	37 (51)	90 (53)	
HER2	10 (14)	18 (11)	
Triple negative	16 (22)	18 (11)	

ER estrogen receptor, *PgR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a] Positive ≥1 %

[b] Positive ≥1 %

[c] Cutoff value >31 % (median)

[d] With reference to St. Gallen International Expert Consensus 2011 [12]

breast cancer cell viability and migration by means of NF-κB activation. In addition, CYLD negatively regulated RANKL-induced NF-κB activation and migration. Our findings thus suggest that CYLD downregulation promotes

breast cancer metastasis via activation of the NF-κB pathway including RANKL signaling.

Despite recent data showing that CYLD regulated diverse signaling pathways including the NF-κB pathway, the clinical significance of CYLD expression in malignancies has remained largely unknown. This report is the first to provide analyses of CYLD expression in breast cancer, with reduced CYLD expression being associated with poor prognosis. These findings are consistent with data from a public database of gene expression arrays (Supplementary Fig. S3A) [39]. Our data and the database finding that CYLD had a more significant prognostic value in patients who had had systemic treatment of any kind compared with those who had not had such treatment (Supplementary Fig. S3B and C). Further investigation focused on association between CYLD and drug resistance for chemotherapy or endocrine therapy may provide beneficial information about breast cancer treatment.

Reduced CYLD expression was significantly associated with more aggressive clinicopathological parameters such as ER-negative status, high Ki-67 index, and high nuclear grade, which indicates the tumor-suppressive effects of CYLD. Our multivariate analysis demonstrated that reduced CYLD expression was an independent prognostic factor for DFS. These findings suggest that downregulation of CYLD commonly has tumor-promoting effects in breast cancer, in a manner similar to that of activated NF-κB [14–17]. Previous clinical studies showed that NF-κB activation in breast cancer occurred more frequently in ER-negative cases or high-risk ER-positive cases, although the mechanisms of the NF-κB activation remain unknown [15, 16]. Additional studies to evaluate NF-κB activity in breast cancer tissues in relation to CYLD expression may clarify the relevance of the NF-κB pathway in breast cancer progression. Although the mechanisms of CYLD downregulation remain to be elucidated, our clinical findings suggest that CYLD is a useful prognostic factor and a potent tumor suppressor in breast cancer.

Our clinical results are supported by in vitro data showing that downregulation of CYLD expression led to acquisition of more aggressive features of breast cancer, including increased survival and migration of breast cancer cells. Consistent with previous studies [2, 7, 8, 11, 40], our analysis indicated that CYLD negatively regulated NF-κB activity. Our data thus suggest that CYLD downregulation promotes metastasis and/or recurrence of breast cancer through induction of NF-κB activation.

Various cytokines and growth factors activate NF-κB [18]. Certain studies showed that RANKL-RANK signaling had crucial roles in development and progression of breast cancer. The RANKL-RANK system was originally shown to be essential for osteoclastogenesis and physiological bone remodeling and to promote bone metastasis in

Table 2 Cox proportional hazards analysis of DFS (stages I–III, $n = 230$)

Variable	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	P	HR (95 % CI)	P
Menopausal (post vs. pre/peri)	0.67 (0.34, 1.39)	0.268		
Tumor size (≥30 vs. <30 mm)	3.34 (1.65, 6.70)	0.001	2.53 (1.21, 5.25)	0.014
Lymph node metastasis (positive vs. negative)	2.86 (1.47, 5.76)	0.002	1.53 (0.72, 3.34)	0.268
CYLD (negative vs. positive)	3.09 (1.59, 6.16)	0.001	2.43 (1.16, 5.26)	0.019
ER (positive vs. negative)	0.19 (0.10, 0.37)	<0.001	0.35 (0.14, 0.87)	0.025
PgR (positive vs. negative)	0.30 (0.15, 0.59)	<0.001		
HER2 (positive vs. negative)	1.36 (0.55, 2.94)	0.483		
Ki-67 index (high vs. low)[a]	4.09 (1.89, 10.19)	<0.001	1.56 (0.59, 4.44)	0.379
Nuclear grade (high vs. low)[b]	3.35 (1.70, 6.55)	0.001	1.15 (0.48, 2.76)	0.753

DFS disease-free survival, *ER* estrogen receptor, *PgR* progesterone receptor, *HER2* human epidermal growth factor receptor 2

[a] Cutoff value >31 % (median)

[b] High, nuclear grade 3; low, nuclear grades 1 and 2

malignancies including breast cancer [41, 42]. Recently, in advanced breast cancer, anti-RANKL antibody, denosumab, is approved in prevention of skeletal-related events in patients with bone metastases from solid tumors. However, more recent studies revealed that RANKL signaling in breast epithelial cells themselves promoted breast carcinogenesis and metastasis to sites other than bone in vivo [35–37] and induced stemness and epithelial-mesenchymal transition [34–38], which suggests broader than expected effects of denosumab on breast cancer progression [43]. Biomarkers that predict a dependence on RANKL signaling should hold promise for treatment, but the clinical significance of RANKL-RANK expression in breast cancer is still controversial [44, 45]. As an important result in our study, CYLD inhibited RANKL-induced NF-κB activation even in breast cancer cells, a finding that is consistent with another result reported for osteoclasts [46]. In addition, downregulation of CYLD enhanced RANKL-induced migration of breast cancer cells. Additional studies to clarify the exact roles of CYLD in breast cancer, especially in RANKL-induced malignant phenotypes, may contribute to our understanding of the molecular mechanisms underlying this disease, including dysregulated NF-κB activation, and the development of improved anti-RANKL therapy.

In conclusion, reduced expression of CYLD was an independent factor for poor prognosis in breast cancer. CYLD downregulation may contribute to breast cancer metastasis through activation of NF-κB including RANKL signaling. Elucidation of CYLD functions in malignant tumors is therefore warranted.

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