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*Original Article*

# Low expression of cytosolic NOTCH1 predicts poor prognosis of breast cancer patients

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**Abstract:** The incidence of breast cancer is increasing, and is one of the leading causes of cancer death worldwide. Dysregulation of NOTCH1 signaling is reported in breast cancer. In present study, bioinformatics was utilized to study the expression of *NOTCH1* gene in breast cancer from public databases, including the Kaplan-Meier Plotter, PrognoScan, Human Protein Atlas, and cBioPortal. The relationship between *NOTCH1* mRNA expression and survival of patients was inconsistent in public databases. In addition, we performed immunohistochemistry (IHC) staining of 135 specimens from our hospital. Lower cytoplasmic staining of NOTCH1 protein was correlated with cancer recurrence, bone metastasis, and a worse disease-free survival of patients, especially those with estrogen receptor-positive and human epidermal growth factor receptor 2-positive ($HER2^+$) cancers. In TCGA breast cancer dataset, lower expression of *NOTCH1* in breast cancer specimens was correlated with higher level of *CCND1* (protein: cyclin D1). Decreased expression of NOTCH1 was correlated with lower level of *CCNA1* (protein: cyclin A1), *CCND2* (protein: cyclin D2), *CCNE1* (protein: cyclin E1), *CDK6* (protein: CDK6), and *CDKN2C* (protein: p18). In conclusion, *NOTCH1* mRNA expression is not consistently correlated with clinical outcomes of breast cancer patients. Low cytoplasmic expression of NOTCH1 in IHC study is correlated with poor prognosis of breast cancer patients. Cytoplasmic localization of NOTCH1 protein failed to initial oncogenic signaling in present study. Expression of *NOTCH1* mRNA was discordant with cell cycle-related genes. Regulation of NOTCH1 in breast cancer involves gene expression, protein localization and downstream signaling.

**Keywords:** Breast cancer, NOTCH1, protein localization, recurrence-free survival, bioinformatics, cell cycle

## Introduction

Breast cancer is the second most common incident cancer in women, and 1 in 18 women will develop breast cancer over their lifetime. The incidence of breast cancer has increased by 35% from 1990 to 2017. Even with the improvement of cancer therapy, breast cancer remains one of the leading causes of cancer death worldwide [1]. The age-standardized incidence

rate of breast cancer was 70.7 per 100,000 persons in 2015 in Taiwan, with an incremental annual change of 3.5 per 100,000 [2]. Breast cancer in Taiwan is characterized by early tumor onset, with a peak age of diagnosis at 45-55 years [3]. A higher prevalence of luminal A subtype and a lower prevalence of histological Grade III tumor or basal-like subtype have been reported [4]. The age-specific incidence of breast cancer in Taiwan is entirely different from that reported in the United States [5]. Ethnic differences in the populations in the United States have been analyzed by the Surveillance, Epidemiology, and End Results (SEER) databases (n = 452,215). Asian women are more likely to be diagnosed with early stage cancer, and also have a lower risk of death, than western patients [6]. From these epidemiological studies, treatment for breast cancer patients is an urgent and important issue worldwide.

The NOTCH signaling pathway is evolutionarily conserved and has functions in embryonic development. There are four isoforms of NOTCH receptors (NOTCH-1, 2, 3, and 4) and five NOTCH ligands [7]. The NOTCH receptor is a type I single-pass transmembrane protein with extracellular epidermal growth factor-like tandem repeats and three Lin-12/NOTCH repeats; a juxtamembrane domain, RBP-jk-associated molecule (RAM); intracellular ankyrin-like repeat (ANK); and a proline-glutamate-serine-threonine (PEST) sequence [8]. Binding of ligands induces proteolysis of the NOTCH receptor by γ-secretase and release of the NOTCH intracellular domain (NICD). Nuclear localization of NICD serves to initiate transcription of downstream signaling, including cyclin D1 and other cell cycle-related genes [9]. Increased expression of nuclear NICD is detected in early phases of breast cancer development [10]. Nuclear NICD, detected by immunohistochemistry (IHC) staining, is increased in invasive ductal carcinoma compared to in adjacent non-neoplastic breast tissue. High expression of nuclear NICD is correlated with upregulated N-cadherin and downregulated E-cadherin. These results suggest a correlation between nuclear NICD and the epithelial-mesenchymal transition of breast cancer cells [11]. Furthermore, elevated expression of nuclear NICD is associated with lymph node metastasis, advanced stage, and higher histological grade [12].

However, the role of NOTCH1 in solid tumors mainly depends on interactions with other oncogenic signaling pathways. Inhibition of γ-secretase suppresses the release of NICD and activates epithelial growth factor receptor signaling to promote cell proliferation [13]. A positive feedback loop between NOTCH1 and p53 has also been detected, and suppression of NOTCH1 and p53 signaling in cervical cancer and Ewing's sarcoma is necessary to maintain carcinogenesis [14]. NOTCH4 activity is increased in breast cancer stem cells, whereas NOTCH1 activity is decreased 4-fold [15]. NOTCH1 activation induces differentiation and cell cycle arrest of skin cells, while loss of NOTCH1 results in chemically induced skin carcinogenesis due to concomitant suppression of the WNT-Hedgehog pathway [16]. The intricate interactions between NOTCH1 and other oncogenic pathways contribute to the poor response of NOTCH1 targeting or γ-secretase inhibitors in clinical trials. Indeed, the clinical benefit of an anti-NOTCH1 monoclonal neutralizing antibody was only 17% in a Phase I study [17]. In the present study, we performed IHC staining of intracellular NOTCH1 protein. Our results were correlated with the demographics and outcome of breast cancer patients. Furthermore, we collected high-throughput publicly available datasets and validated the results using bioinformatics to demonstrate that regulation of NOTCH1 signaling in breast cancer.

## Materials and methods

### *Bioinformatics analysis of NOTCH1*

The Kaplan-Meier Plotter platform was used to correlate the results of mRNA microarray and recurrence-free survival (RFS) of 3951 breast cancer patients [18]. mRNA expression was examined with Affymetrix (Santa Clara, California, United States) microarray chip using two probes for *NOTCH1*, 218902_at and 223508_at. The targeted region of two probes is located in different part of *NOTCH1* gene. The former probe is the JetSet best probe set and targeted the 3'-untranslated region (3'UTR) of *NOTCH1* gene. The latter one is not the JetSet best probe set and interacted with the sequencing coding polypeptide PEST domain at the C-terminus in Exon 34 [19]. The cut-off point of low or high expression of *NOTCH1* was based on the median mRNA level. Redundant sam-

ples and biased arrays were excluded. Survival curves were estimated using the Kaplan-Meier method, and compared using the log-rank test. Hazard ratios (HRs) with 95% confidence intervals (CIs) were used to measure the association between the expression of *NOTCH1* and survival.

PrognoScan was built using multiple published datasets from cancer microarrays, and also contained clinical annotation and information of the patients' survival [20]. Information on the array type, probe *id*, and primary study end point were provided. The cut-off point of low or high expression of *NOTCH1* was based on the default setting of the software. The optimal cut-off point for the continuous variable of gene expression was chosen based on the minimum *P* value during survival analysis, according to default setting of the software. Kaplan-Meier curves with log-rank test, HR, and 95% CI of HR were provided from the PrognoScan database.

The Human Protein Atlas contains multiple histological sections from tissue microarrays of breast cancer [21, 22]. IHC staining was performed in 1075 breast cancer samples with two primary antibody clones and labeled with DAB (3,3'-diaminobenzidine). The CAB008112 antibody was a rabbit polyclonal antibody, and CAB022466 was a mouse monoclonal antibody; the epitopes of both antibodies were directed to the intercellular domain of the NOTCH1 protein. The annotation parameters included immunoreactivity (high, medium, or low), staining intensity (strong, moderate, or weak), fraction of stained cells (> 75%, 75%-25%, or < 25%), and subcellular localization (nuclear, or cytoplasmic and nuclear). A Kaplan-Meier overall survival curve is presented, and was used to compared low or high expression of NOTCH1 in IHC using log-rank test.

The cBioPortal platform collects multidimensional cancer genomics and datasets [23, 24]. A total of 13 breast cancer datasets were selected from cBioPortal, including those of primary and metastatic cancer. Breast fibroepithelial neoplasms, xenografts of breast cancer, or adenoid cystic breast cancer were excluded due to different tumor pathophysiology and tumor growth conditions. Mutation, deletion, insertion, truncating, fusion, and copy number alteration (CNA) of *NOTCH1* genes were explored. The altered group was defined as those with mutation, fusion, amplification, deep deletion, and multiple alteration of *NOTCH1* gene. The unalted group is set for those without mutation, fusion, amplification, deep deletion, or multiple alteration. A Kaplan-Meier disease-free survival (DFS) curve is shown, and was used to compare patients with altered and unaltered *NOTCH1* by log-rank test.

RNA sequencing of 1217 breast cancer samples was performed by Illumina platform and raw data was downloaded from The Cancer Genome Atlas Breast Invasive Carcinoma in Genomic Data Commons Data Portal (GDC TCGA-BRCA) [25]. The results were re-analyzed using the latest Human Genome Assembly hg38 and re-organized by University of California Santa Cruz Xena team [26]. The upper quartile of the fragments per kilobase of transcript per million mapped reads (HTSeq-FPKM-UQ) was selected. Expression of *NOTCH1* and cell cycle-associated genes was extracted for further analysis. Survival status and times were collected for evaluating overall survival.

*Patients and materials*

A total of 135 patients were enrolled from July 2007 to December 2013 for IHC study. All patients received curative surgery for breast cancer, and the surgical specimens were preserved in the Department of Pathology and the Human Biobank in Research Center of Clinical Medicine, National Cheng Kung University Hospital (NCKUH). Written informed consent was obtained, and the protocol was approved by the Institutional Review Board of NCKUH (IRB number: A-ER-105-233). Only patients who agreed to participate and donate part of their specimens were enrolled. After surgery, all patients received standard adjuvant therapy according to suggestion from the attending physicians. The patients received regular follow-up with annual breast sonography, mammography, and radiography of the thorax. Computerized tomography or bone scintigraphy were optional in cases with clinical suspicion of metastasis. Clinical information and long-term survival were collected by a retrospective chart review. Cancer staging conformed to the American Joint Committee on Cancer tumor, node, and metastasis (AJCC TNM) classification of 2010, 7th edition [27].

*Immunohistochemistry stain of NOTCH1 in surgical specimens*

Formalin-fixed, paraffin-embed (FFPE) tissue specimens were obtained from surgical specimens and collected by the Human biobank in NCKUH. The slides were deparaffinized, rehydrated, and blocked endogenous peroxidase. Antigen retrieval was performed by heat in retrieval buffer (DAKO, Carpinteria, California) using a pressure boiler. The primary polyclonal anti-NOTCH1 antibody was synthesized corresponding to amino acid 1755-67 of intracellular NOTCH1 (Abcam, Cambridge, United Kingdom). The epitope was not accessible in the uncleaved form, and was only exposed following cleavage by γ-secretase. The slides were incubated in primary antibody under 4°C for 16 hours, then the color was developed in aminoethyl carbazole (Zymed, San Francisco, California, United States) for 5 min. The slides were counterstained in hematoxylin and mounted in coverslips. In the negative controls, the primary antibody was omitted from the above process.

All slides were evaluated by a single researcher (H.P.H.). Only one sample expressed simultaneous nuclear and cytoplasmic NOTCH1 staining, and all other samples with positive immunoreactivity only showed cytoplasmic staining. Therefore, we only evaluated the expression level of cytoplasmic NOTCH1 (cNOTCH1). The immunoreactivity of cNOTCH1 was determined using the modified immunoreactive score (mIRS). The percentage of positive cells (every 10% as a stratum from 0% to 90%), and intensity of staining (from no color to intense reaction as 0, 1, 2, to 3) were multiplied to determine the immunoreactive score (IRS) score [28]. The score ranged from 0 to 270, with a median of 20, and mean ± standard deviation of 33 ± 45. The cut-off point of cNOTCH1 staining was set as 20 (median). Negative (mIRS score, 0-10) and weak immunoreactivity (mIRS score, 11-20) were grouped as low expression of cNOTCH1; and moderate (mIRS score, 21-160) and strong immunoreactivity (mIRS score, 161-270) were defined as high expression of cNOTCH1. The IHC results correlated with the clinical information and outcome of patients.

*Statistical analysis*

All statistical analyses were performed using SPSS v17 (SPSS, Armonk, New York, United States) or software R version 4.1.2. Univariate analysis was performed using chi-square test or Fisher's exact test for categorical variables, and nonparametric Wilcoxon rank-sum or Kruskal-Wallis test for two or more groups of continuous variables. Survival curves were estimated using the Kaplan-Meier method, and compared between groups using the log-rank test. Median split was used for turning a continuous variable into binary variable to visualize the difference between groups in Kaplan-Meier method. The Cox proportional regression model was used to assess the association between cNOTCH1 expression and survival, where the hazard ratio (HR) with 95% confidence interval (CI) is the measure of effect. Spearman's rank correlation coefficient (ρ) was used to assess monotonic relationships between two variables (either continuous or discrete ordinal variables). For multiple testing, *P* value was adjusted by the Benjamini-Hochberg procedure [29]. Statistical significance was defined as a $P$ value $< 0.05$.

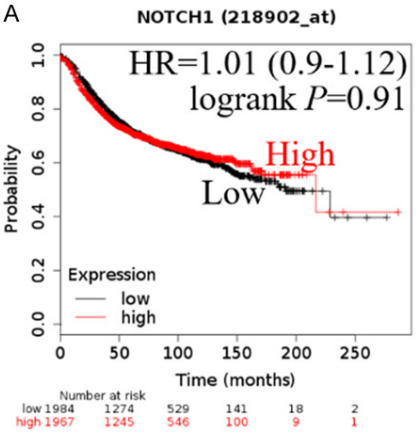
## Results

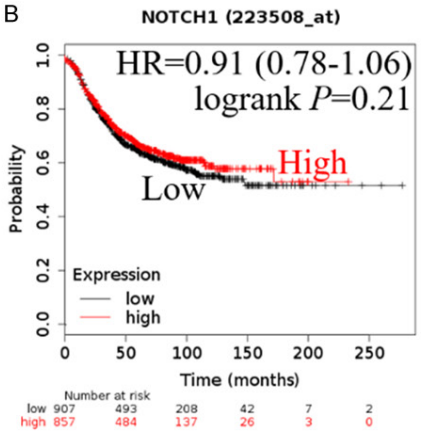
*Bioinformatics study of NOTCH1 mRNA in breast cancer*

Expression of *NOTCH1* mRNA was compared to the RFS of patients using the Kaplan-Meier Plotter. The RFS showed no correlation with either low or high expression of *NOTCH1* mRNA in all breast cancer patients (**Figure 1A**, **1B**) and $ER^+$ breast cancer patients (**Figure 1C**, **1D**). There was no significant difference of RFS between the two probes. For $HER2^+$ breast cancer patients, low expression of probe 223508_at for *NOTCH1* mRNA was correlated with a better RFS (**Figure 1F**), and low expression of probe 218902_at was correlated with a trend of better survival (**Figure 1E**). The RFS of patients with triple negative breast cancer (TNBC) demonstrated better survival with low expression of probe 218902_at (**Figure 1G**); however, this phenomenon was not observed with a different probe.

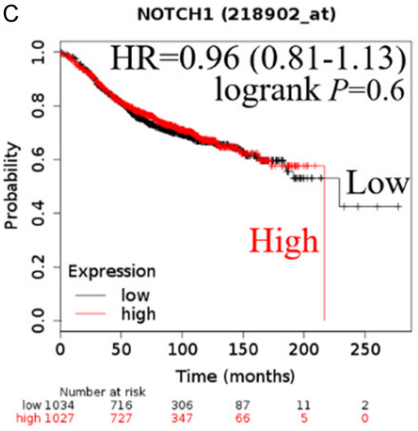
PrognoScan collected 20 mRNA microarrays of breast cancer and analyzed the correlation between expression of *NOTCH1* and survival of patients. No correlation could be established in 17 datasets, and three analyses claimed that the patients with high expression of *NOTCH1* had a higher hazard ratio (Figure S1A). The cut-

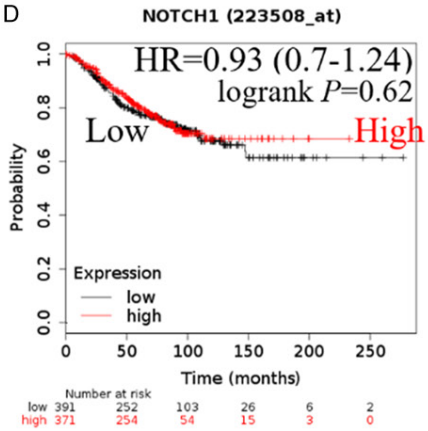
All kinds of breast cancer



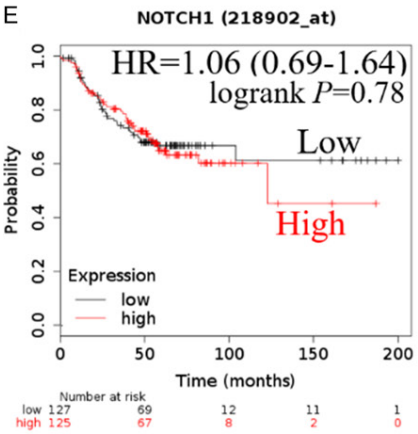



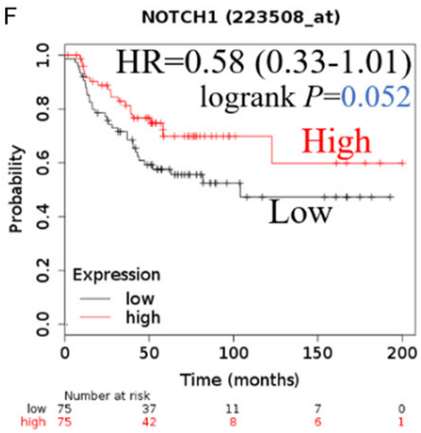

$ER^+$ breast cancer



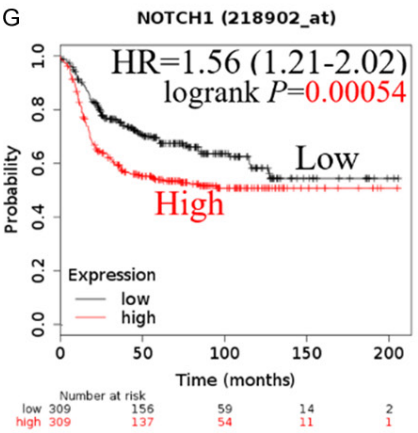




$HER2^+$ breast cancer




TNBC



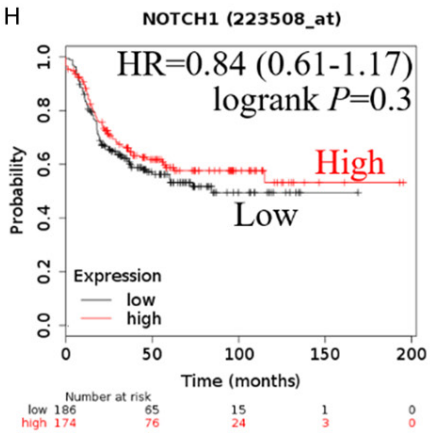



**Figure 1.** Comparison of the expression of *NOTCH1* mRNA and disease-free survival of breast cancer patients in the Kaplan-Meier Plotter. There are two probes of the *NOTCH1* gene: the 218902_at probe is targeted on the 3'-UTR, and the 223508_at probe is targeted on Exon 34 of *NOTCH1*. A, B. All breast cancer patients. C, D. ER$^+$ breast cancer patients. E, F. HER2$^+$ breast cancer patients. G, H. TNBC patients. 3'-UTR: 3'-untranslated region; ER: estrogen receptor; HER2: human epidermal growth factor receptor Type II; HR: hazard ratio; mRNA: messenger ribonucleic acid; TNBC: triple negative breast cancer.

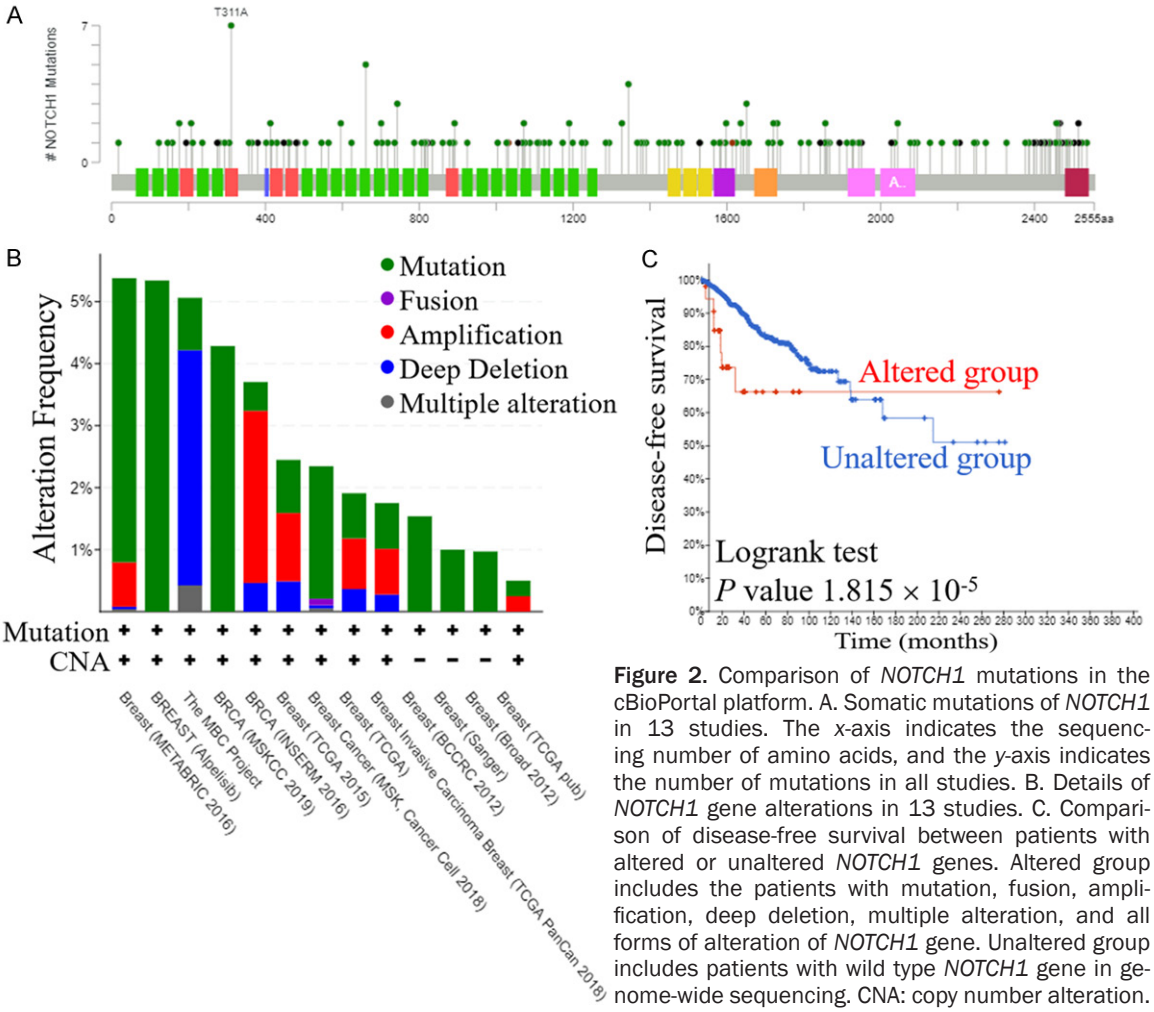



**Figure 2.** Comparison of *NOTCH1* mutations in the cBioPortal platform. A. Somatic mutations of *NOTCH1* in 13 studies. The *x*-axis indicates the sequencing number of amino acids, and the *y*-axis indicates the number of mutations in all studies. B. Details of *NOTCH1* gene alterations in 13 studies. C. Comparison of disease-free survival between patients with altered or unaltered *NOTCH1* genes. Altered group includes the patients with mutation, fusion, amplification, deep deletion, multiple alteration, and all forms of alteration of *NOTCH1* gene. Unaltered group includes patients with wild type *NOTCH1* gene in genome-wide sequencing. CNA: copy number alteration.

off point for low or high expression of *NOTCH1* was different in these datasets, which resulted in an imbalance of the number of patients in the two groups (Figure S1B-D). The detection platform and analytic parameters of survival were different in the three datasets (relapse free survival in GSE12276 [31], overall survival in GSE9893 [32], and disease specific survival in GSE3494) [33-35]. Thus, the relationship between *NOTCH1* mRNA expression and survival of patients was inconsistent, and further evaluation with other methods is necessary to determine *NOTCH1* expression in breast cancer.

*Mutation of NOTCH1 in breast cancer*

We selected combined datasets from 8,875 patients and 9,193 samples in 13 studies of the cBioPortal platform. The frequency of somatic mutations in *NOTCH1* was 2.2% in all samples, including 177 missense, 33 truncating, 3 deletion, 1 insertion, and 2 fusion with other gene (*NOTCH1-SEC16A* and *NOTCH1-CUL5*) (**Figure 2A**). Mutation and deletion were common forms of alterations, and amplification of NOTCH1 was also detected in six datasets (**Figure 2B**). The survival curve of patients with alteration of NOTCH1 (altered group,

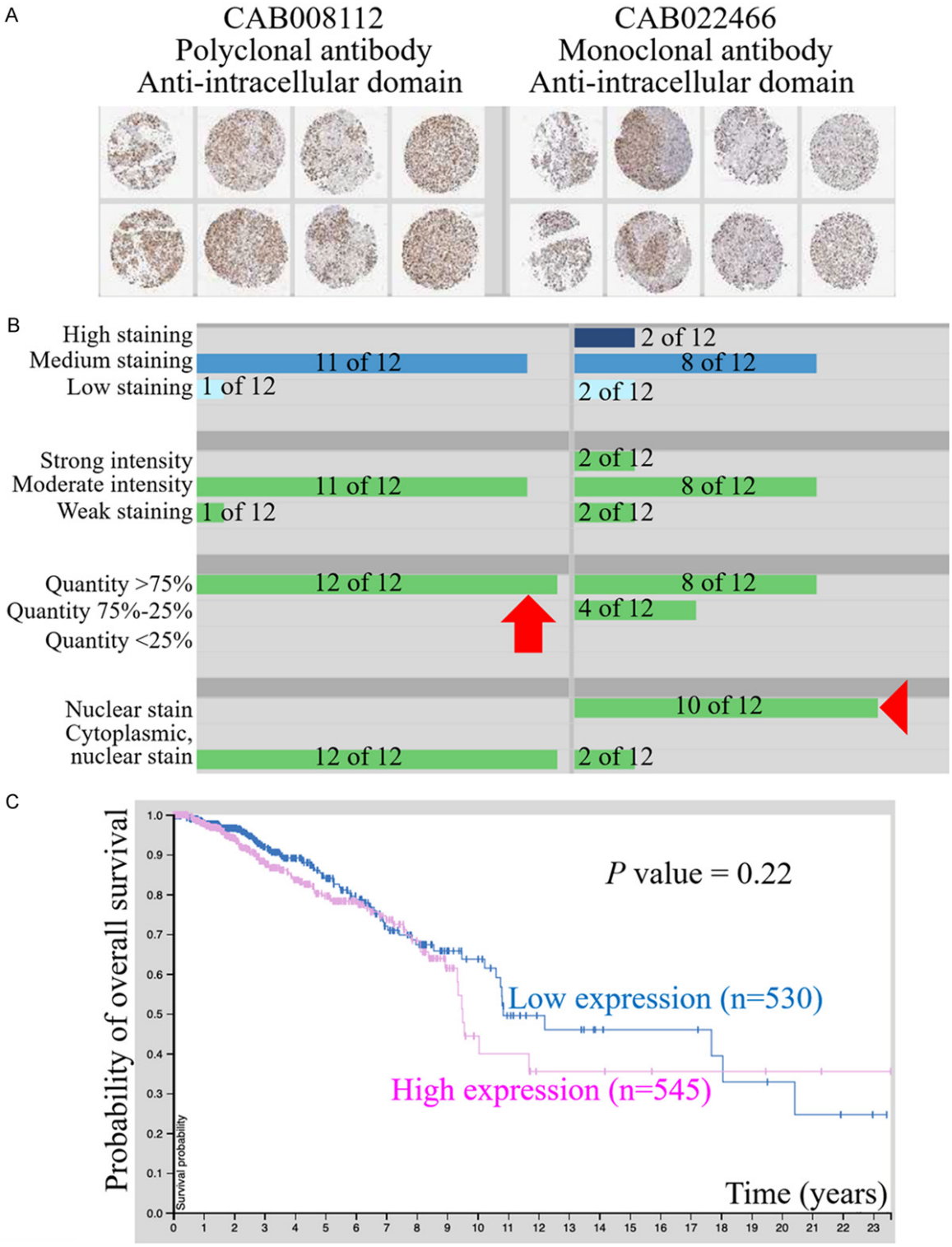



**Figure 3.** Immunohistochemistry (IHC) of NOTCH1 in Human Protein Atlas. A. IHC staining of NOTCH1 in tissue microarrays. Left block: stain with CAB008112 antibody. Right block: stain with CAB022466 antibody. B. Results of IHC of NOTCH1. Arrow: more than 75% of cells had positive NOTCH1 staining. Arrowhead: Nuclear staining of NOTCH1. C. Comparison of overall survival between patients with low or high expression of NOTCH1.

including mutation, fusion, amplification, deep deletion, multiple alteration, and all forms of alteration) was crossed with the survival curve of the unaltered NOTCH1 (**Figure 2C**), and log-

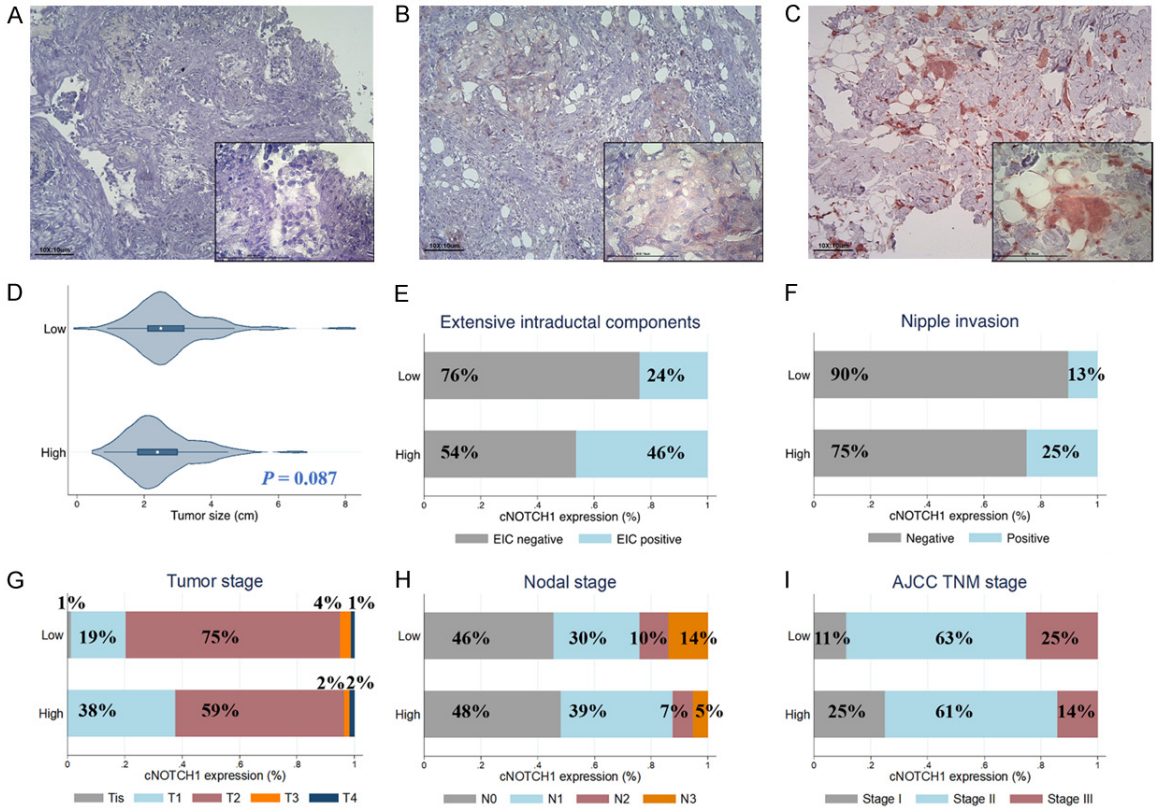



**Figure 4.** Immunohistochemistry (IHC) staining of cNOTCH1 in breast cancer. A. Negative immunoreactivity. B. Weak immunoreactivity. C. Strong immunoreactivity. The main photos are 100× magnification with a 10-μm-scale bar in the left lower corner. The inserted photos in the right lower corner are 400× magnification with a 10-μm-scale bar. The tumors with negative and weak immunoreactivity are grouped as low cNOTCH1 expression, while those with strong immunoreactivity are grouped as high cNOTCH1 expression. D-I. Comparison of different pathological factors and expression of cNOTCH1 by IHC staining. D. Tumor size ($P$ = 0.087). E. Extensive intraductal components ($P$ = 0.009). F. Nipple invasion by cancer ($P$ = 0.043). G. Tumor stage ($P$ = 0.139). H. Nodal stage ($P$ = 0.304). I. AJCC TNM stage ($P$ = 0.063). AJCC TNM stage: American Joint Committee on Cancer tumor, node, metastasis staging system; cNOTCH1: cytosolic NOTCH1 protein.

rank testing using the default setting of the website showed statistical significance ($P$ = $1.815 \times 10^{-5}$). The patients with altered NOTCH1 had a better DFS than those without alteration after postoperative 220 months.

*Immunohistochemistry staining of NOTCH1 in breast cancer*

The Human Protein Atlas includes IHC staining of NOTCH1 from multiple tissue microarrays (**Figure 3A**). The results of two anti-NOTCH1 antibodies were slightly different; the staining density was higher in the samples stained with CAB008112 antibody, while a higher proportion of nuclear staining was noted in those stained with CAB0224466 (**Figure 3B**, arrow and arrowhead, respectively). Twelve photos of IHC staining from each primary antibody were provided in the website, and we selected 8 samples of each antibody for example. However, no information about other 1,051 samples was shown in the Human Protein Atlas and the survival of patients cannot be compared according to intracellular location of NOTCH1. The results of IHC staining of two anti-NOTCH1 antibodies were merged together by default setting of the website to analyze overall survival of breast cancer patients. The expression of intracellular NOTCH1 had no clear impact on the effect of overall survival of patients (**Figure 3B**). The patients with high expression of NOTCH1 had a trend of poor survival after 10 years ($P$ = 0.22, **Figure 3C**).

Each tissue microarray sample size is around 1 mm, and a selection bias is possible. In the present study, larger FFPE sections from surgi-

**Table 1.** Characteristics of patients with breast cancer (n = 135), comparison between low and high cNOTCH1 expression was done

| Characteristic | cNOTCH1 expression | | *P* value |
|---|---|---|---|
| | Low (n = 79) | High (n = 56) | |
| Age at surgery (years)[a] | 52 (31-83) | 53 (24-82) | 0.711 |
| Female gender | 79 (100%) | 56 (100%) | > 0.99 |
| Diagnosis | | | 0.570 |
| Invasive ductal carcinoma | 78 (99%) | 54 (96%) | |
| Invasive ductal carcinoma with predominant ductal carcinoma in situ | 1 (1%) | 2 (4%) | |
| Operation methods | | | 0.973 |
| Partial mastectomy and sentinel lymph node biopsy | 5 (6%) | 4 (7%) | |
| Partial mastectomy and axillary lymph node dissection | 6 (8%) | 4 (7%) | |
| Total mastectomy and sentinel lymph node biopsy | 12 (15%) | 10 (18%) | |
| Modified radical mastectomy | 56 (71%) | 38 (68%) | |
| Tumor size (cm)[a] | 2.5 (0.2-8.0) | 2.4 (0.8-6.5) | 0.087 |
| Tumor stage | | | 0.139 |
| Tis | 1 (100%) | 0 | |
| T1 | 15 (42%) | 21 (58%) | |
| T2 | 59 (64%) | 33 (36%) | |
| T3 | 3 (75%) | 1 (25%) | |
| T4 | 1 (50%) | 1 (50%) | |
| Nodal stage | | | 0.301 |
| N0 | 36 (57%) | 27 (43%) | |
| N1 | 24 (52%) | 22 (48%) | |
| N2 | 8 (67%) | 4 (33%) | |
| N3 | 11 (79%) | 3 (21%) | |
| AJCC TNM stage | | | 0.063 |
| Stage I | 9 (39%) | 14 (61%) | |
| Stage II | 50 (59%) | 34 (41%) | |
| Stage III | 20 (71%) | 8 (29%) | |
| Adjuvant chemotherapy | 65 (82%) | 49 (88%) | 0.476 |
| Subtypes | | | 0.693 |
| ER/PR-positive, HER2-negative | 45 (57%) | 36 (64%) | |
| HER2-positive | 22 (28%) | 13 (23%) | |
| Triple negative breast cancer | 12 (15%) | 7 (13%) | |

Abbreviations: AJCC TNM stage, American Joint Committee on Cancer tumor-node-metastases (TNM) staging system; ER, Estrogen receptor; HER, Human epidermal growth receptor type II; cNOTCH1, Cytosolic NOTCH1 protein; PR, Progesterone receptor.
[a]Values are expressed as median (range).

cal specimens were used for IHC. Expression of NOTCH1 was detected in IHC staining, and was scored by mIRS (**Figure 4**; **Table 1**). Negative or weak immunoreactivity were grouped as low expression (**Figure 4A**, **4B**), while moderate or strong immunoreactivity were grouped as high expression (**Figure 4C**). One specimen with strong immunoreactivity had dual positive cytoplasmic and nuclear staining. In all other samples, only cytoplasmic staining of NOTCH1 was recognized, and the nuclear location was not detectable. Expression of cNOTCH1 was not associated with a larger tumor size (**Figure 4D**, $P = 0.087$). Tumors with low cNOTCH1 had a lower proportion of positive extensive intraductal components (**Figure 4E**, $P = 0.009$) and nipple invasion (**Figure 4F**, $P = 0.043$). There was no significant association between cNOTCH1 expression and tumor stage (**Figure 4G**, $P = 0.139$), nodal stage (**Figure 4H**, $P = 0.304$), and AJCC TNM cancer Stage II or III (**Figure 4I**, $P = 0.063$). Patients with low expres-

**Table 2.** Disease-free survival events and number of deaths, comparison between low and high cNOTCH1 expression

| Characteristic | cNOTCH1 expression | | *P* value |
|---|---|---|---|
| | Low (n = 79) | High (n = 56) | |
| Disease-free survival events[a] | 58 | 47 | 0.207 |
| Breast cancer events | 19 (24%) | 5 (9%) | 0.038 |
| Lung metastasis | 15 (75%) | 5 (25%) | 0.141 |
| Liver metastasis | 7 (9%) | 3 (5%) | 0.522 |
| Bone metastasis | 12 (15%) | 2 (4%) | 0.042 |
| Brain metastasis | 5 (6%) | 1 (2%) | 0.400 |
| Regional lymph node recurrence | 7 (9%) | 1 (2%) | 0.139 |
| Distant lymph node recurrence | 12 (15%) | 4 (7%) | 0.185 |
| Local recurrence | 3 (4%) | 2 (4%) | > 0.999 |
| Skin recurrence | 0 | 2 (4%) | 0.170 |
| Adrenal gland metastasis | 2 (3%) | 0 | 0.511 |
| Non-breast cancer deaths | 2 | 4 | 0.232 |
| Cerebrovascular accident | 0 | 2 | 0.170 |
| Heart failure | 1 | 0 | > 0.999 |
| Rectal cancer | 1 | 0 | > 0.999 |
| Endometrial cancer | 0 | 1 | 0.415 |
| Hepatocellular carcinoma | 0 | 1 | 0.415 |
| Breast cancer-related deaths | 8 | 5 | > 0.999 |

Abbreviations: cNOTCH1, Cytosolic NOTCH1 protein. [a]Excluding breast cancer-related and non-breast cancer-related events.

sion of cNOTCH1 tended to have advanced cancer, while cNOTCH1 expression had no correlation with demographics, other pathological factors, or subtypes of breast cancer (Figure S2; **Table 1**).

A total of 19 patients in the low cNOTCH1 group and 5 in the high cNOTCH1 developed cancer recurrence during follow-up (**Table 2**, $P$ = 0.038). Lung metastasis was the most common pattern of recurrence, followed by bone, distant lymph node, or liver metastasis (**Table 2**). Patients with low cNOTCH1 expression had a higher ratio of bone metastasis ($P$ = 0.042). There were two nonbreast cancer deaths in the low cNOTCH1 group, and four in the high cNOTCH1 group. The incidence of breast-cancer-related deaths was not significantly different between the two groups. Breast cancer patients with low expression of cNOTCH1 had a worse DFS (**Figure 5A**). Low expression of cNOTCH1 had a tendency to correlate with a worse DFS in patients with $ER^+$ (**Figure 5B**) or $HER2^+$ cancer (**Figure 5C**). However, expression of cNOTCH1 was not correlated with survival in TNBC patients (**Figure 5D**). Several patients refused standard chemotherapy after surgery, which may have interfered with the survival analysis. We analyzed patients who received standard chemotherapy, and low expression of cNOTCH1 was correlated with a worse DFS (**Figure 5E**). Breast cancer patients were stratified, and low expression of cNOTCH1 was correlated with a worse DFS of patients with T2 tumor stage (Figure S4B) and AJCC TNM Stage II (Figure S6B). In other subgroups, the expression of cNOTCH1 was not correlated with survival (Figure S3, by nuclear grade; Figure S4, by tumor stage; Figure S5, by lymph node metastasis; or Figure S6, by TNM stage).

*Gene expression of NOTCH1 and cell cycle proteins in breast cancer*

RNA sequencing data from 1,217 breast cancer patients were obtained from The Cancer Genome Atlas Breast Invasive Carcinoma in Genomic Data Commons Data Portal (GDC TCGA-BRCA) via the Xena platform [26]. The scatter plot for the relationship between *NOTCH1* expression and other gene expression was presented with a Spearman's rank correlation coefficient ($\rho$) and an adjusted $P$ value (**Figure 6**). Decreased expression of *NOTCH1*

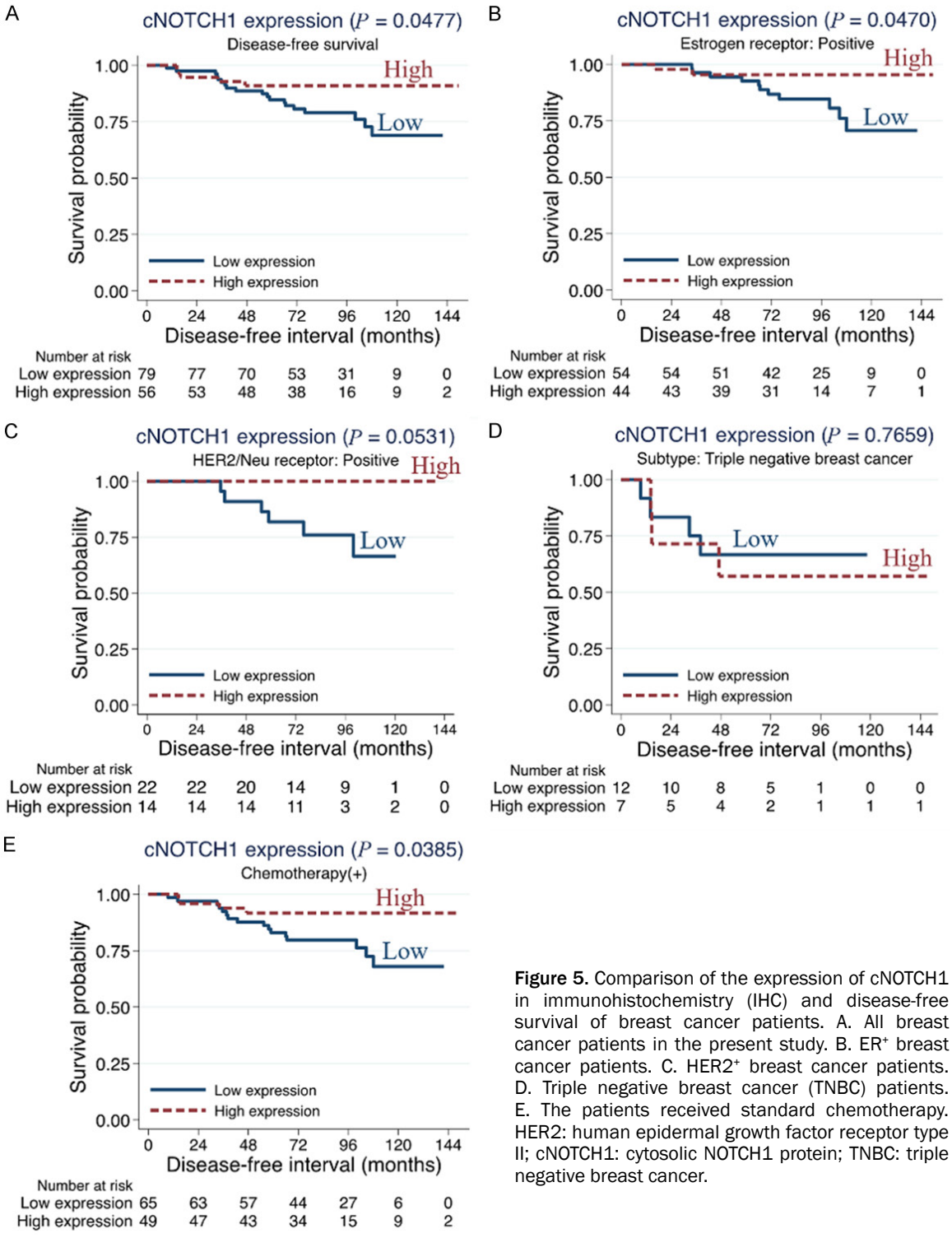



**Figure 5.** Comparison of the expression of cNOTCH1 in immunohistochemistry (IHC) and disease-free survival of breast cancer patients. A. All breast cancer patients in the present study. B. $ER^+$ breast cancer patients. C. $HER2^+$ breast cancer patients. D. Triple negative breast cancer (TNBC) patients. E. The patients received standard chemotherapy. HER2: human epidermal growth factor receptor type II; cNOTCH1: cytosolic NOTCH1 protein; TNBC: triple negative breast cancer.

was correlated with lower level of *CCNA1* (protein: cyclin A1, **Figure 6A**), *CCND2* (protein: cyclin D2, **Figure 6E**), *CCNE1* (protein, cyclin E1, **Figure 6F**), *CDK6* (protein: CDK6, **Figure 6I**), and *CDKN2C* (protein: p18, **Figure 6L**). However, decreased expression of *NOTCH1* was associated with higher level of *CCND1* (protein: cyclin D1, **Figure 6D**). Cyclin D and CDK4/6 complex triggers DNA replication and cell cycle progression. Expression of Cyclin D and CDK4/6 mRNA in *NOTCH1*$^{low}$ breast cancer was inconsistent. Moreover, expression of cell cycle

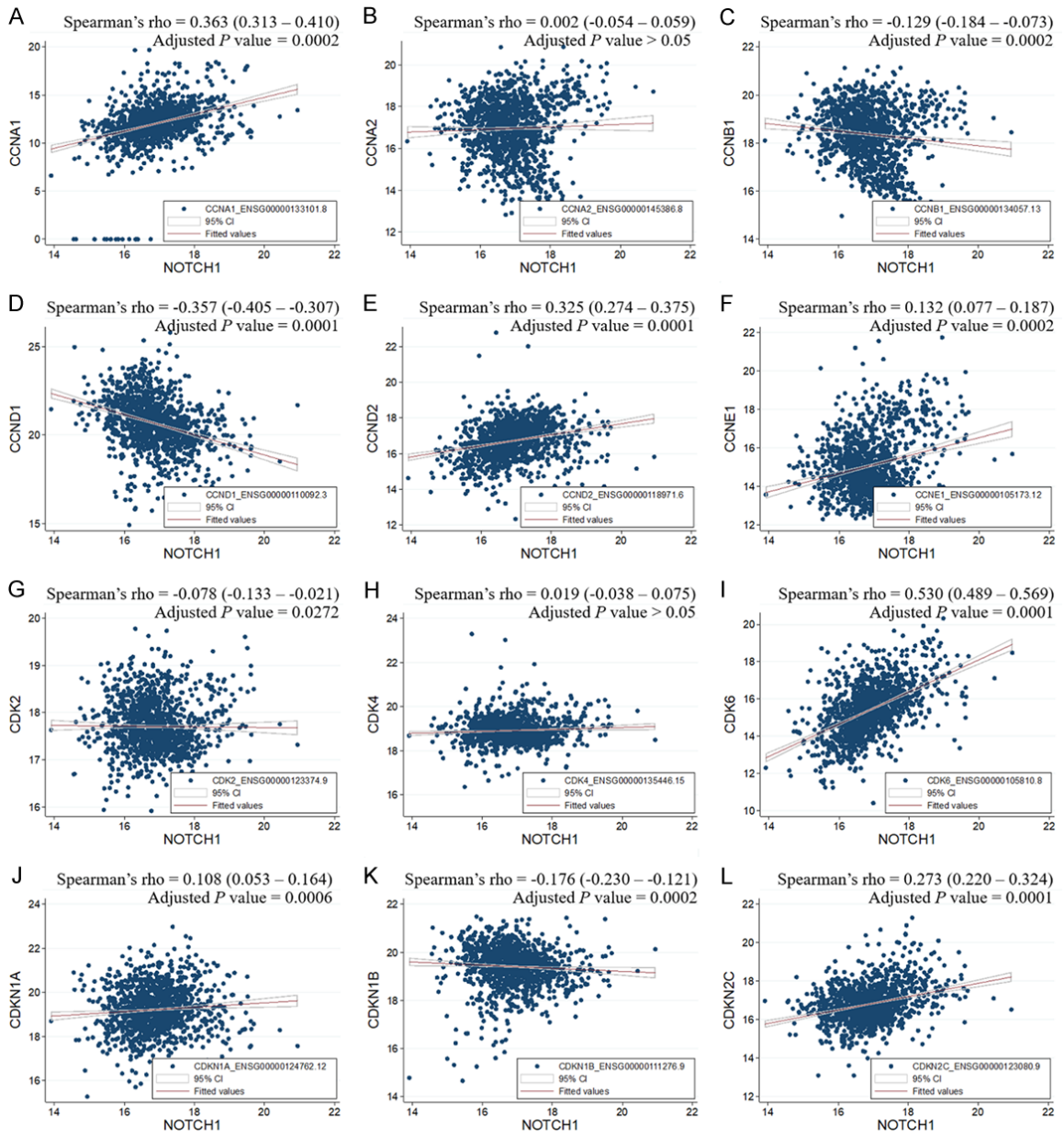



**Figure 6.** RNA sequencing data from The Cancer Genome Atlas Breast Invasive Carcinoma in Genomic Data Commons Data Portal (GDC TCGA-BRCA) via the Xena platform. Scatter plots of NOTCH1 versus (A) CCNA1 (protein: cyclin A1), (B) CCNA2 (protein: cyclin A2), (C) CCNB1 (protein: cyclin B1), (D) CCND1 (protein: cyclin D1), (E) CCND2 (protein: cyclin D2), (F) CCNE1 (protein: cyclin E1), (G) CDK2 (protein: CDK2), (H) CDK4 (protein: CDK4), (I) CDK6 (protein: CDK6), (J) CDKN1A (protein: p21), (K) CDKN1B (protein: p27), AND (L) CDKN2C (protein: p18). Adjusted *P* value was calculated by Benjamini-Hochberg procedure.

regulating *CDKN1A* (protein: p21, **Figure 6J**) and *CDKN1B* (protein: p27, **Figure 6K**) was similar in *NOTCH1*$^{high}$ and *NOTCH1*$^{low}$ breast cancer. All these results implicated the complicated regulation of cell cycle in breast cancer. The survival status was obtained from 1194 breast cancer patients and higher expression of *NOTCH1* mRNA was correlated with poorer overall survival (Figure S7, $P = 0.028$).

## Discussion

Breast cancer is the most common female malignancy in Taiwan, with increased incidence rates and early onset at middle age. In the present study, we first verified the publicly available datasets in Kaplan-Meier Plotter and PrognoScan. The correlation between high expression of *NOTCH1* mRNA and prognosis of

patients was inconsistent. The frequency of somatic mutations in *NOTCH1* was 2.2% in the cBioPortal platform. Genetic alterations along the whole *NOTCH1* gene were reported in breast cancer patients. Furthermore, IHC staining of intracellular NOTCH1 in tissue microarray in the Human Protein Atlas failed to detect the correlation between NOTCH1 expression and overall survival of patients. We employed large samples of breast cancer obtained from surgical resection to detect NOTCH1 in heterogeneous cancer. High expression of cNOTCH1 was associated with a higher proportion of positive extensive intraductal components and nipple invasion. However, breast cancer patients with low expression of cNOTCH1 had a higher incidence of bone metastasis, and a trend of worse DFS. In subtype analysis, low expression of cNOTCH1 predicted poor prognosis in breast cancer patients with $ER^+$ or $HER2^+$, but not in TNBC patients. In TCGA breast cancer dataset, higher expression of NOTCH1 in breast cancer specimens was correlated with lower level of cylin D1 and a worse prognosis of breast cancer patients. Our study provides evidence that cytoplasmic accumulation of NOTCH1 fails to correlate with advanced cancer. Dysregulation of NOTCH1 decreases transcription of cyclin D1. Therefore, comprehensive assessment of NOTCH1 signaling should be performed before giving anti-NOTCH1 agents for breast cancer patients.

Two previous reports have performed IHC staining of *NOTCH1*. Cao et al. utilized TMA samples and a rabbit polyclonal antibody against cytoplasmic components of NOTCH1 to show that increased expression of NOTCH1 in breast cancer is correlated with lymph node metastasis [11]. Moreover, Wan et al. utilized specimens from surgical resection and a rabbit polyclonal antibody against the same epitopes as our study, and showed that activated NOTCH1 is associated with lymph node metastasis, advanced cancer stage, higher nuclear grade, and worse survival of patients [12]. The Human Protein Atlas utilized two types of antibodies, rabbit polyclonal and mouse monoclonal, against the NICD, and demonstrated that the expression of NICH protein is not correlated with survival of patients [21, 22]. In the current study, we utilized specimens from surgical resection and a rabbit polyclonal antibody against the juxtamembranous, intracellular NOTCH1 protein. Our results showed that breast cancer patients with low cNOTCH1 expression had a higher ratio of advanced cancer and worse survival. Furthermore, only one of the included samples demonstrated nuclear staining, and only cytoplasmic staining of NOTCH1 was detected in the other samples with NOTCH1 immunoreactivity. Failure of NICD to localize to the nucleus is one possible explanation for our results. The accumulation of cytoplasmic NOTCH1 proteins prevents the initiation of transcription of downstream genes. Therefore, malposition of NICD proteins could not be evaluated by mRNA microarray or copy number analysis.

Somatic mutations of breast cancer are common, and somatic mutations of *TP53*, *PIK3CA*, and *GATA3* genes occur in all subtypes of breast cancer [35]. Aberrant RNA splicing and mutation of *NOTCH1* gene in cancer results in different variants or isoforms of RNA [36]. The mutation sites in *NOTCH1* spread over the whole genome, and the patient number of a specific mutation was not sufficient for analysis. Understanding the protein structure of NOTCH1 may help to speculate the phenotypes. The intracellular domains of NOTCH1 contain RAM, furin-like convertase, ANK repeats, nuclear localization signals, and the PEST domain [7]. Mutations in intracellular domains might alter the protein structure and prohibit entry into the nucleus. In the present study, function of NOTCH1 in breast cancer was investigated in mRNA and protein levels. In mRNA studies, the survival analysis of two NOTCH1 probes in Kaplan-Meier Plotter showed dissimilar results. This could result from these two probes recognized different region of *NOTCH1* gene. The survival analyses in Kaplan-Meier Plotter were different from results in the TCGA database. This could be attributed to the different platforms for analyzing the levels of *NOTCH1* mRNA. The former was performed in microarray and the latter was analyzed using RNA sequencing. In protein studies, expression of NOTCH1 protein was not correlated with survival in Human Protein Atlas while merging IHC results from two kinds of primary antibody. We used one kind of polyclonal anti-NOTCH1 antibody and found that the NOTCH1 protein was pronuced in cytoplasm but not in ther nucleus. The function of NOTCH1 in breast cancer is complicated. Gene mutations, the aberrant

splicing of mRNA, and the intracellular localization of NOTCH1 protein could dysregulate the function and downstream effectors of NOTCH1 signaling.

NOTCH1 signaling is important in mammary gland development and breast cancer carcinogenesis [37]. Activation of NOTCH signaling causes transcription of target genes, including cyclin D1, p21, and other cell cycle-associated genes [9]. Small molecule Notch pathway inhibitors targeting γ-secretase should be very powerful for NOTCH-rich cancer; however, most clinical trials are suspended or terminated currently. NOTCH signaling is essential for normal development, and γ-secretase is important for maintaining cellular function. Drugs targeting NOTCH signaling or γ-secretase induce intolerable toxicity in clinical trials. NOTCH also functions as a tumor suppressor in certain types of cancer [38, 39]. Furthermore, downstream of NOTCH1 signaling is very complicated. In then present study, higher expression of NOTCH1 in breast cancer specimens was correlated with lower level of cylin D1. The breast cancer patients with low cNOTCH1 expression had a higher ratio of advanced cancer and worse survival. Dysregulation of NOTCH1 signaling fails to initiate transcription of downstream genes in breast cancer cells, and it might cause poor efficacy of NOTCH inhibitors in clinical trials.

The limitation of the present study was the inadequate number of patients. We collected 135 specimens for IHC staining. The inadequate sample size restricted the statistical power. In survival analysis, the number of events also affects statistical power to detect the difference. We only detected a trend of better survival from high cNOTCH1 expression in IHC staining. We employed bioinformatical tools and publicly available databases to verify our findings. The information was fragmented, and the experimental methods were dissimilar in different studies. Bias from confounders was possible because of unknown patients' information. Over the past decade, several clinical trials have been conducted with γ-secretase inhibitors, although, no reliable result for breast cancer patients has yet been reported [40]. One Phase I clinical trial using anti-NOTCH1 monoclonal neutralizing antibody demonstrated that the clinical response rate is only 17% [17]. Mutation of the extracellular domains of NOTCH1 may be one reason for the poor therapeutic response. Further study is indicated to better understand the mechanism of NOTCH1 signaling in breast cancer.

## Conclusion

In conclusion, somatic mutations of *NOTCH1* spread across the whole genome. Regulation of NOTCH1 signaling depends on gene regulation and protein expression. In the present study, we demonstrated that lower cytoplasmic staining of NOTCH1 was correlated with larger tumor size, advanced stages, cancer recurrence with bone metastasis, and a worse DFS of $ER^+$ and $HER^+$ breast cancer patients. NOTCH1 signaling in breast cancer is complex, and targeted therapy against NOTCH1 should be approached cautiously.

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## Disclosure of conflict of interest

None.

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## References

[1] Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Abate D, Abbasi N, Abbastabar H, Abd-Allah F, Abdel-Rahman O, Abdelalim A, Abdoli A, Abdollahpour I, Abdulle

ASM, Abebe ND, Abraha HN, Abu-Raddad LJ, Abualhasan A, Adedeji IA, Advani SM, Afarideh M, Afshari M, Aghaali M, Agius D, Agrawal S, Ahmadi A, Ahmadian E, Ahmadpour E, Ahmed MB, Akbari ME, Akinyemiju T, Al-Aly Z, AlAbdulKader AM, Alahdab F, Alam T, Alamene GM, Alemnew BTT, Alene KA, Alinia C, Alipour V, Aljunid SM, Bakeshei FA, Almadi MAH, Almasi-Hashiani A, Alsharif U, Alsowaidi S, Alvis-Guzman N, Amini E, Amini S, Amoako YA, Anbari Z, Anber NH, Andrei CL, Anjomshoa M, Ansari F, Ansariadi A, Appiah SCY, Arab-Zozani M, Arabloo J, Arefi Z, Aremu O, Areri HA, Artaman A, Asayesh H, Asfaw ET, Ashagre AF, Assadi R, Ataeinia B, Atalay HT, Ataro Z, Atique S, Ausloos M, Avila-Burgos L, Avokpaho EFGA, Awasthi A, Awoke N, Ayala Quintanilla BP, Ayanore MA, Ayele HT, Babaee E, Bacha U, Badawi A, Bagherzadeh M, Bagli E, Balakrishnan S, Balouchi A, Bärnighausen TW, Battista RJ, Behzadifar M, Behzadifar M, Bekele BB, Belay YB, Belayneh YM, Berfield KKS, Berhane A, Bernabe E, Beuran M, Bhakta N, Bhattacharyya K, Biadgo B, Bijani A, Bin Sayeed MS, Birungi C, Bisignano C, Bitew H, Bjørge T, Bleyer A, Bogale KA, Bojia HA, Borzì AM, Bosetti C, Bou-Orm IR, Brenner H, Brewer JD, Briko AN, Briko NI, Bustamante-Teixeira MT, Butt ZA, Carreras G, Carrero JJ, Carvalho F, Castro C, Castro F, Catalá-López F, Cerin E, Chaiah Y, Chanie WF, Chattu VK, Chaturvedi P, Chauhan NS, Chehrazi M, Chiang PP, Chichiabellu TY, Chido-Amajuoyi OG, Chimed-Ochir O, Choi JJ, Christopher DJ, Chu DT, Constantin MM, Costa VM, Crocetti E, Crowe CS, Curado MP, Dahlawi SMA, Damiani G, Darwish AH, Daryani A, das Neves J, Demeke FM, Demis AB, Demissie BW, Demoz GT, Denova-Gutiérrez E, Derakhshani A, Deribe KS, Desai R, Desalegn BB, Desta M, Dey S, Dharmaratne SD, Dhimal M, Diaz D, Dinberu MTT, Djalalinia S, Doku DT, Drake TM, Dubey M, Dubljanin E, Duken EE, Ebrahimi H, Effiong A, Eftekhari A, El Sayed I, Zaki MES, El-Jaafary SI, El-Khatib Z, Elemineh DA, Elkout H, Ellenbogen RG, Elsharkawy A, Emamian MH, Endalew DA, Endries AY, Eshrati B, Fadhil I, Fallah Omrani V, Faramarzi M, Farhangi MA, Farioli A, Farzadfar F, Fentahun N, Fernandes E, Feyissa GT, Filip I, Fischer F, Fisher JL, Force LM, Foroutan M, Freitas M, Fukumoto T, Futran ND, Gallus S, Gankpe FG, Gayesa RT, Gebrehiwot TT, Gebremeskel GG, Gedefaw GA, Gelaw BK, Geta B, Getachew S, Gezae KE, Ghafourifard M, Ghajar A, Ghashghaee A, Gholamian A, Gill PS, Ginindza TTG, Girmay A, Gizaw M, Gomez RS, Gopalani SV, Gorini G, Goulart BNG, Grada A, Ribeiro Guerra M, Guimaraes ALS, Gupta PC, Gupta R, Hadkhale K, Haj-Mirzaian A, Haj-Mirzaian A, Hamadeh RR, Hamidi S, Hanfore LK, Haro JM, Hasankhani M, Hasanzadeh A, Hassen HY, Hay RJ, Hay SI, Henok A, Henry NJ, Herteliu C, Hidru HD, Hoang CL, Hole MK, Hoogar P, Horita N, Hosgood HD, Hosseini M, Hosseinzadeh M, Hostiuc M, Hostiuc S, Househ M, Hussen MM, Ileanu B, Ilic MD, Innos K, Irvani SSN, Iseh KR, Islam SMS, Islami F, Jafari Balalami N, Jafarinia M, Jahangiry L, Jahani MA, Jahanmehr N, Jakovljevic M, James SL, Javanbakht M, Jayaraman S, Jee SH, Jenabi E, Jha RP, Jonas JB, Jonnagaddala J, Joo T, Jungari SB, Jürisson M, Kabir A, Kamangar F, Karch A, Karimi N, Karimian A, Kasaeian A, Kasahun GG, Kassa B, Kassa TD, Kassaw MW, Kaul A, Keiyoro PN, Kelbore AG, Kerbo AA, Khader YS, Khalilarjmandi M, Khan EA, Khan G, Khang YH, Khatab K, Khater A, Khayamzadeh M, Khazaee-Pool M, Khazaei S, Khoja AT, Khosravi MH, Khubchandani J, Kianipour N, Kim D, Kim YJ, Kisa A, Kisa S, Kissimova-Skarbek K, Komaki H, Koyanagi A, Krohn KJ, Bicer BK, Kugbey N, Kumar V, Kuupiel D, La Vecchia C, Lad DP, Lake EA, Lakew AM, Lal DK, Lami FH, Lan Q, Lasrado S, Lauriola P, Lazarus JV, Leigh J, Leshargie CT, Liao Y, Limenih MA, Listl S, Lopez AD, Lopukhov PD, Lunevicius R, Madadin M, Magdeldin S, El Razek HMA, Majeed A, Maleki A, Malekzadeh R, Manafi A, Manafi N, Manamo WA, Mansourian M, Mansournia MA, Mantovani LG, Maroufizadeh S, Martini SMS, Mashamba-Thompson TP, Massenburg BB, Maswabi MT, Mathur MR, McAlinden C, McKee M, Meheretu HAA, Mehrotra R, Mehta V, Meier T, Melaku YA, Meles GG, Meles HG, Melese A, Melku M, Memiah PTN, Mendoza W, Menezes RG, Merat S, Meretoja TJ, Mestrovic T, Miazgowski B, Miazgowski T, Mihretie KMM, Miller TR, Mills EJ, Mir SM, Mirzaei H, Mirzaei HR, Mishra R, Moazen B, Mohammad DK, Mohammad KA, Mohammad Y, Darwesh AM, Mohammadbeigi A, Mohammadi H, Mohammadi M, Mohammadian M, Mohammadian-Hafshejani A, Mohammadoo-Khorasani M, Mohammadpourhodki R, Mohammed AS, Mohammed JA, Mohammed S, Mohebi F, Mokdad AH, Monasta L, Moodley Y, Moosazadeh M, Moossavi M, Moradi G, Moradi-Joo M, Moradi-Lakeh M, Moradpour F, Morawska L, Morgado-da-Costa J, Morisaki N, Morrison SD, Mosapour A, Mousavi SM, Muche AA, Muhammed OSS, Musa J, Nabhan AF, Naderi M, Nagarajan AJ, Nagel G, Nahvijou A, Naik G, Najafi F, Naldi L, Nam HS, Nasiri N, Nazari J, Negoi I, Neupane S, Newcomb PA, Nggada HA, Ngunjiri JW, Nguyen CT, Nikniaz L, Ningrum DNA, Nirayo YL, Nixon MR, Nnaji CA, Nojomi M, Nosratnejad S, Shiadeh MN, Obsa MS, Ofori-Asenso R, Ogbo FA, Oh IH, Olagunju AT, Olagunju TO, Oluwasanu MM, Omonisi AE, Onwujekwe OE, Oommen

AM, Oren E, Ortega-Altamirano DDV, Ota E, Otstavnov SS, Owolabi MO, P A M, Padubidri JR, Pakhale S, Pakpour AH, Pana A, Park EK, Parsian H, Pashaei T, Patel S, Patil ST, Pennini A, Pereira DM, Piccinelli C, Pillay JD, Pirestani M, Pishgar F, Postma MJ, Pourjafar H, Pourmalek F, Pourshams A, Prakash S, Prasad N, Qorbani M, Rabiee M, Rabiee N, Radfar A, Rafiei A, Rahim F, Rahimi M, Rahman MA, Rajati F, Rana SM, Raoofi S, Rath GK, Rawaf DL, Rawaf S, Reiner RC, Renzaho AMN, Rezaei N, Rezapour A, Ribeiro AI, Ribeiro D, Ronfani L, Roro EM, Roshandel G, Rostami A, Saad RS, Sabbagh P, Sabour S, Saddik B, Safiri S, Sahebkar A, Salahshoor MR, Salehi F, Salem H, Salem MR, Salimzadeh H, Salomon JA, Samy AM, Sanabria J, Santric Milicevic MM, Sartorius B, Sarveazad A, Sathian B, Satpathy M, Savic M, Sawhney M, Sayyah M, Schneider IJC, Schöttker B, Sekerija M, Sepanlou SG, Sepehrimanesh M, Seyedmousavi S, Shaahmadi F, Shabaninejad H, Shahbaz M, Shaikh MA, Shamshirian A, Shamsizadeh M, Sharafi H, Sharafi Z, Sharif M, Sharifi A, Sharifi H, Sharma R, Sheikh A, Shirkoohi R, Shukla SR, Si S, Siabani S, Silva DAS, Silveira DGA, Singh A, Singh JA, Sisay S, Sitas F, Sobngwi E, Soofi M, Soriano JB, Stathopoulou V, Sufiyan MB, Tabarés-Seisdedos R, Tabuchi T, Takahashi K, Tamtaji OR, Tarawneh MR, Tassew SG, Taymoori P, Tehrani-Banihashemi A, Temsah MH, Temsah O, Tesfay BE, Tesfay FH, Teshale MY, Tessema GA, Thapa S, Tlaye KG, Topor-Madry R, Tovani-Palone MR, Traini E, Tran BX, Tran KB, Tsadik AG, Ullah I, Uthman OA, Vacante M, Vaezi M, Varona Pérez P, Veisani Y, Vidale S, Violante FS, Vlassov V, Vollset SE, Vos T, Vosoughi K, Vu GT, Vujcic IS, Wabinga H, Wachamo TM, Wagnew FS, Waheed Y, Weldegebreal F, Weldesamuel GT, Wijeratne T, Wondafrash DZ, Wonde TE, Wondmieneh AB, Workie HM, Yadav R, Yadegar A, Yadollahpour A, Yaseri M, Yazdi-Feyzabadi V, Yeshaneh A, Yimam MA, Yimer EM, Yisma E, Yonemoto N, Younis MZ, Yousefi B, Yousefifard M, Yu C, Zabeh E, Zadnik V, Moghadam TZ, Zaidi Z, Zamani M, Zandian H, Zangeneh A, Zaki L, Zendehdel K, Zenebe ZM, Zewale TA, Ziapour A, Zodpey S and Murray CJL. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 29 cancer groups, 1990 to 2017: a systematic analysis for the Global Burden Of Disease Study. JAMA Oncol 2019; 5: 1749-1768.

[2] Kuo CN, Liao YM, Kuo LN, Tsai HJ, Chang WC and Yen Y. Cancers in Taiwan: practical insight from epidemiology, treatments, biomarkers, and cost. J Formos Med Assoc 2020; 119: 1731-1741.

[3] Huang CS, Lin CH, Lu YS and Shen CY. Unique features of breast cancer in Asian women - breast cancer in Taiwan as an example. J Steroid Biochem Mol Biol 2010; 118: 300-303.

[4] Lin CH, Liau JY, Lu YS, Huang CS, Lee WC, Kuo KT, Shen YC, Kuo SH, Lan C, Liu JM, Kuo WH, Chang KJ and Cheng AL. Molecular subtypes of breast cancer emerging in young women in Taiwan: evidence for more than just westernization as a reason for the disease in Asia. Cancer Epidemiol Biomarkers Prev 2009; 18: 1807-1814.

[5] Lin CH, Chen YC, Chiang CJ, Lu YS, Kuo KT, Huang CS, Cheng WF, Lai MS, You SL and Cheng AL. The emerging epidemic of estrogen-related cancers in young women in a developing Asian country. Int J Cancer 2012; 130: 2629-2637.

[6] Iqbal J, Ginsburg O, Rochon PA, Sun P and Narod SA. Differences in breast cancer stage at diagnosis and cancer-specific survival by race and ethnicity in the United States. JAMA 2015; 313: 165-173.

[7] Li L, Tang P, Li S, Qin X, Yang H, Wu C and Liu Y. Notch signaling pathway networks in cancer metastasis: a new target for cancer therapy. Med Oncol 2017; 34: 180.

[8] Kopan R and Ilagan MX. The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 2009; 137: 216-233.

[9] Edwards A and Brennan K. Notch signalling in breast development and cancer. Front Cell Dev Biol 2021; 9: 692173.

[10] Zardawi SJ, Zardawi I, McNeil CM, Millar EK, McLeod D, Morey AL, Crea P, Murphy NC, Pinese M, Lopez-Knowles E, Oakes SR, Ormandy CJ, Qiu MR, Hamilton A, Spillane A, Soon Lee C, Sutherland RL, Musgrove EA and O'Toole SA. High Notch1 protein expression is an early event in breast cancer development and is associated with the HER2 molecular subtype. Histopathology 2010; 56: 286-296.

[11] Cao YW, Wan GX, Sun JP, Cui XB, Hu JM, Liang WH, Zheng YQ, Li WQ and Li F. Implications of the Notch1-Snail/Slug-epithelial to mesenchymal transition axis for lymph node metastasis in infiltrating ductal carcinoma. Kaohsiung J Med Sci 2015; 31: 70-76.

[12] Wan G, Tian L, Yu Y, Li F, Wang X, Li C, Deng S, Yu X, Cai X, Zuo Z and Cao F. Overexpression of Pofut1 and activated Notch1 may be associated with poor prognosis in breast cancer. Biochem Biophys Res Commun 2017; 491: 104-111.

[13] Li T, Wen H, Brayton C, Das P, Smithson LA, Fauq A, Fan X, Crain BJ, Price DL, Golde TE, Eberhart CG and Wong PC. Epidermal growth factor receptor and notch pathways participate in the tumor suppressor function of gamma-

secretase. J Biol Chem 2007; 282: 32264-32273.
[14] Dotto GP. Crosstalk of Notch with p53 and p63 in cancer growth control. Nat Rev Cancer 2009; 9: 587-595.
[15] Harrison H, Farnie G, Howell SJ, Rock RE, Stylianou S, Brennan KR, Bundred NJ and Clarke RB. Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. Cancer Res 2010; 70: 709-718.
[16] Lobry C, Oh P and Aifantis I. Oncogenic and tumor suppressor functions of Notch in cancer: it's NOTCH what you think. J Exp Med 2011; 208: 1931-1935.
[17] Ferrarotto R, Eckhardt G, Patnaik A, LoRusso P, Faoro L, Heymach JV, Kapoun AM, Xu L and Munster P. A phase I dose-escalation and dose-expansion study of brontictuzumab in subjects with selected solid tumors. Ann Oncol 2018; 29: 1561-1568.
[18] Győrffy B. Survival analysis across the entire transcriptome identifies biomarkers with the highest prognostic power in breast cancer. Comput Struct Biotechnol J 2021; 19: 4101-4109.
[19] Li Q, Birkbak NJ, Gyorffy B, Szallasi Z and Eklund AC. Jetset: selecting the optimal microarray probe set to represent a gene. BMC Bioinformatics 2011; 12: 474.
[20] Mizuno H, Kitada K, Nakai K and Sarai A. PrognoScan: a new database for meta-analysis of the prognostic value of genes. BMC Med Genomics 2009; 2: 18.
[21] Human protein atlas. http://www.proteinatlas.org.
[22] Uhlen M, Zhang C, Lee S, Sjöstedt E, Fagerberg L, Bidkhori G, Benfeitas R, Arif M, Liu Z, Edfors F, Sanli K, von Feilitzen K, Oksvold P, Lundberg E, Hober S, Nilsson P, Mattsson J, Schwenk JM, Brunnström H, Glimelius B, Sjöblom T, Edqvist PH, Djureinovic D, Micke P, Lindskog C, Mardinoglu A and Ponten F. A pathology atlas of the human cancer transcriptome. Science 2017; 357: eaan2507.
[23] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-404.
[24] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: pl1.
[25] National Institute of Health, United States National Library of Medicine. https://clinicaltrials.gov/ct2/home.
[26] Goldman MJ, Craft B, Hastie M, Repečka K, McDade F, Kamath A, Banerjee A, Luo Y, Rogers D, Brooks AN, Zhu J and Haussler D. Visualizing and interpreting cancer genomics data via the Xena platform. Nat Biotechnol 2020; 38: 675-678.
[27] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A. AJCC cancer staging manual. 7th ed. New York: Springer; 2010.
[28] Kaemmerer D, Peter L, Lupp A, Schulz S, Sänger J, Baum RP, Prasad V and Hommann M. Comparing of IRS and Her2 as immunohistochemical scoring schemes in gastroenteropancreatic neuroendocrine tumors. Int J Clin Exp Pathol 2012; 5: 187-194.
[29] McDonald JH. Handbook of biological statistics (3rd ed.). Baltimore, Maryland: Sparky House Publishing; 2014.
[30] Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van de Vijver MJ, Gerald WL, Foekens JA and Massagué J. Genes that mediate breast cancer metastasis to the brain. Nature 2009; 459: 1005-1009.
[31] Chanrion M, Negre V, Fontaine H, Salvetat N, Bibeau F, Mac Grogan G, Mauriac L, Katsaros D, Molina F, Theillet C and Darbon JM. A gene expression signature that can predict the recurrence of tamoxifen-treated primary breast cancer. Clin Cancer Res 2008; 14: 1744-1752.
[32] Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET and Bergh J. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A 2005; 102: 13550-13555.
[33] Palazon A, Tyrakis PA, Macias D, Veliça P, Rundqvist H, Fitzpatrick S, Vojnovic N, Phan AT, Loman N, Hedenfalk I, Hatschek T, Lövrot J, Foukakis T, Goldrath AW, Bergh J and Johnson RS. An HIF-1α/VEGF-A axis in cytotoxic T cells regulates tumor progression. Cancer Cell 2017; 32: 669-683.
[34] Lundberg A, Lindström LS, Harrell JC, Falato C, Carlson JW, Wright PK, Foukakis T, Perou CM, Czene K, Bergh J and Tobin NP. Gene expression signatures and immunohistochemical subtypes add prognostic value to each other in breast cancer cohorts. Clin Cancer Res 2017; 23: 7512-7520.
[35] Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012; 490: 61-70.
[36] Sveen A, Kilpinen S, Ruusulehto A, Lothe RA and Skotheim RI. Aberrant RNA splicing in cancer; expression changes and driver mutations of splicing factor genes. Oncogene 2016; 35: 2413-2427.
[37] Chen W, Wei W, Yu L, Ye Z, Huang F, Zhang L, Hu S and Cai C. Mammary development and

breast cancer: a Notch perspective. J Mammary Gland Biol Neoplasia 2021; 26: 309-320.

[38] Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP and Radtke F. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet 2003; 33: 416-421.

[39] Wang NJ, Sanborn Z, Arnett KL, Bayston LJ, Liao W, Proby CM, Leigh IM, Collisson EA, Gordon PB, Jakkula L, Pennypacker S, Zou Y, Sharma M, North JP, Vemula SS, Mauro TM, Neuhaus IM, Leboit PE, Hur JS, Park K, Huh N, Kwok PY, Arron ST, Massion PP, Bale AE, Haussler D, Cleaver JE, Gray JW, Spellman PT, South AP, Aster JC, Blacklow SC and Cho RJ. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. Proc Natl Acad Sci U S A 2011; 108: 17761-17766.

[40] Ranganathan P, Weaver KL and Capobianco AJ. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 2011; 11: 338-51.

A

| Dataset | Endpoint | Array type | Probe id | N | Cox *P*-value | HR [95% C.I.] | Reference |
|---|---|---|---|---|---|---|---|
| GSE12276 | Relapse Free Survival | HG-U133_Plus_2 | 218902_at | 204 | 0.000335 | 1.71 [1.28 - 2.30] | [30] |
| GSE9893 | Overall Survival | MLRG Human 21K V12.0 | 3223 | 155 | 0.016167 | 2.40 [1.18 - 4.89] | [31] |
| GSE3494-GPL97 | Disease Specific Survival | HG-U133B | 223508_at | 236 | 0.001881 | 2.11 [1.32 - 3.38] | [32-34] |

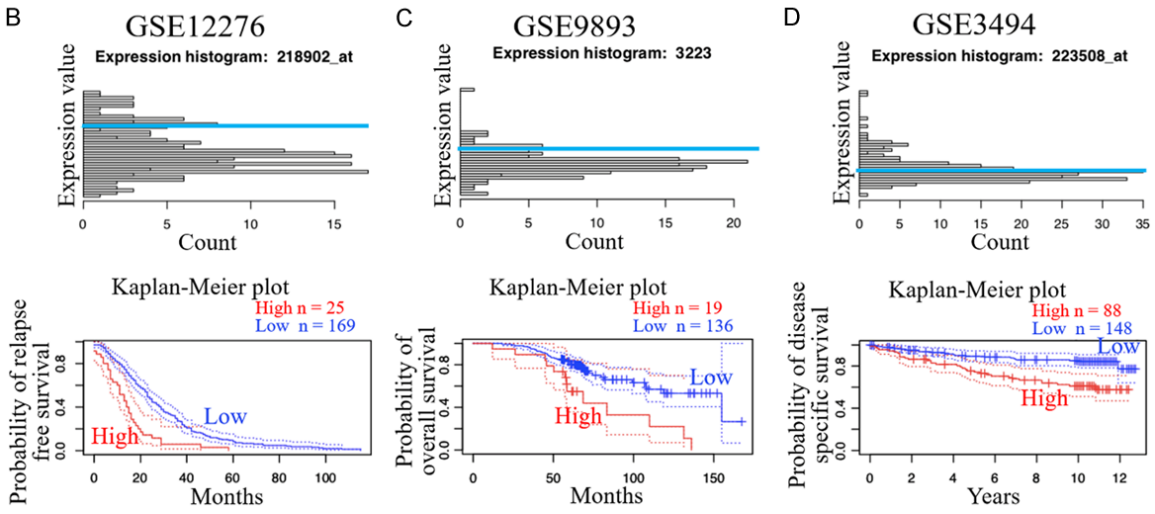


**Figure S1**. Comparison of the expression of *NOTCH1* mRNA and survival of breast cancer patients in PrognoScan. Significant correlations were detected in three datasets. A. Information on these three datasets. B. Results of GSE12276. C. Results of GSE9893. D. Results of GSE3494. Upper part: histogram of expression. The cut-off point is shown as a blue horizontal line to categorized low or high expression. Lower part: Kaplan-Meier plots of survival probability.

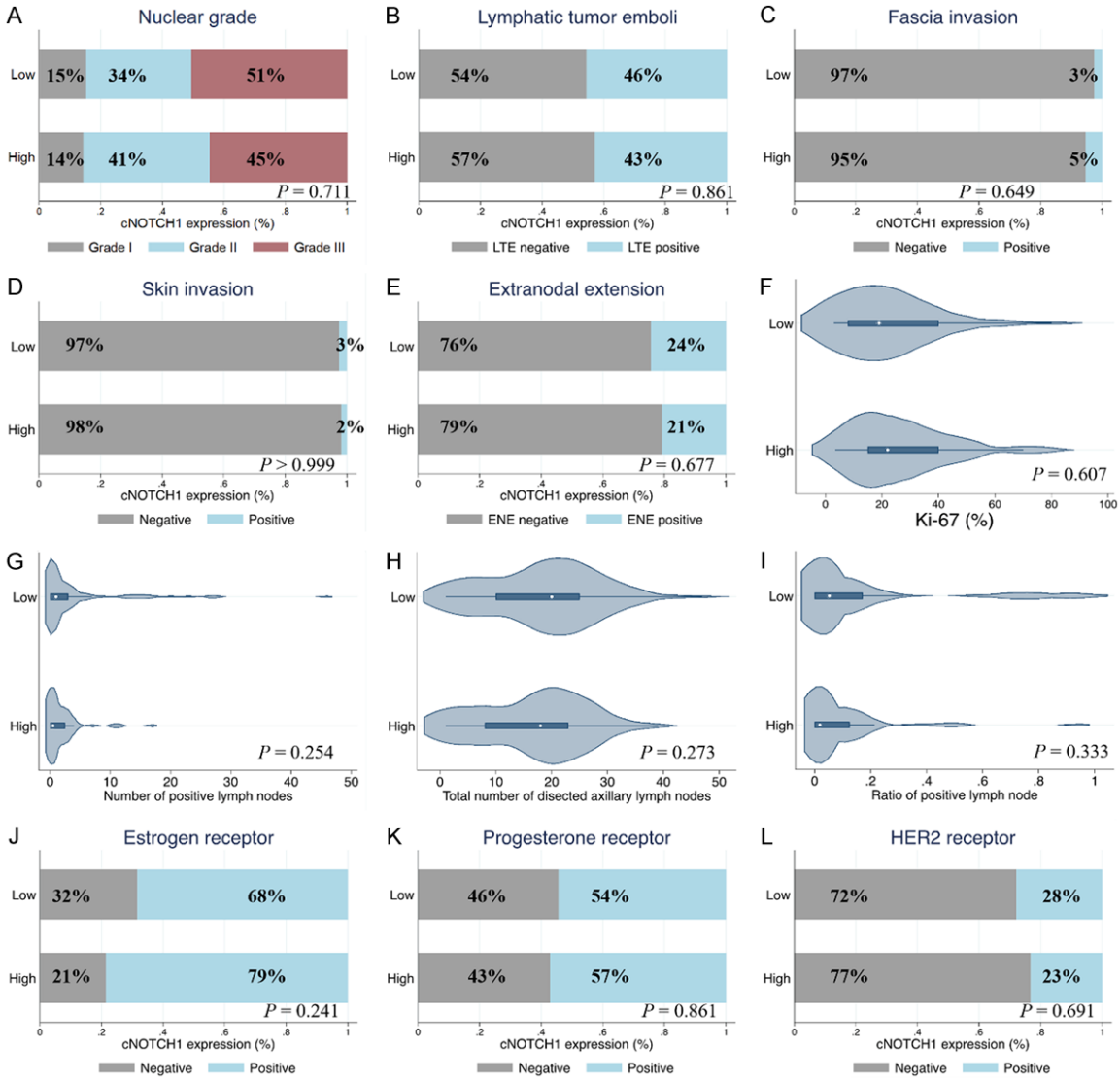


**Figure S2.** Correlation of pathological factors with immunohistochemistry (IHC) of cNOTCH1 in breast cancer. The results were compared between low and high expression of cNOTCH1. A. Nuclear grade of histological differentiation. B. Lymphatic tumor emboli. C. Fascia invasion. D. Skin invasion. E. Extranodal extension. F. Expression of nuclear protein Ki-67 in cancer cells (%). G. Number of axillary lymph nodes with metastasis. H. Number of dissected axillary lymph nodes. I. Ratio of positive lymph nodes (number of positive lymph nodes divided by number of dissected axillary lymph nodes). J. Positive expression of estrogen receptor (≥ 1% nuclear staining in IHC stain). K. Positive expression of progesterone receptor (≥ 1% nuclear staining in IHC stain). L. Positive expression of HER2 receptor (3+ in IHC stain or amplification in FISH study).

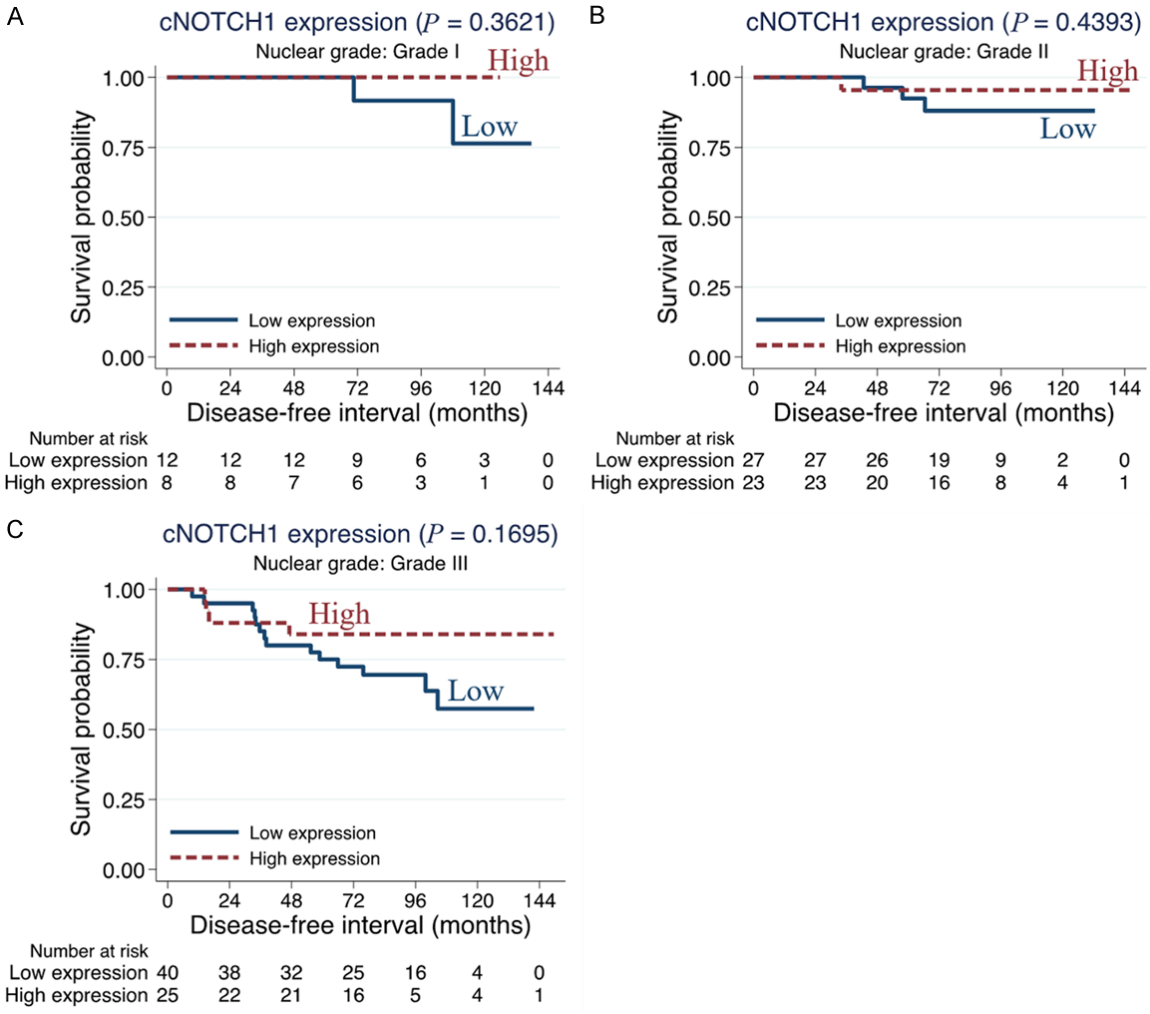


**Figure S3.** Comparison of the expression of cNOTCH1 in immunohistochemistry (IHC) and disease-free survival of breast cancer patients. A. Breast cancer patients with nuclear grade I cancer. B. Breast cancer patients with nuclear Grade II cancer. C. Breast cancer patients with nuclear Grade III cancer.

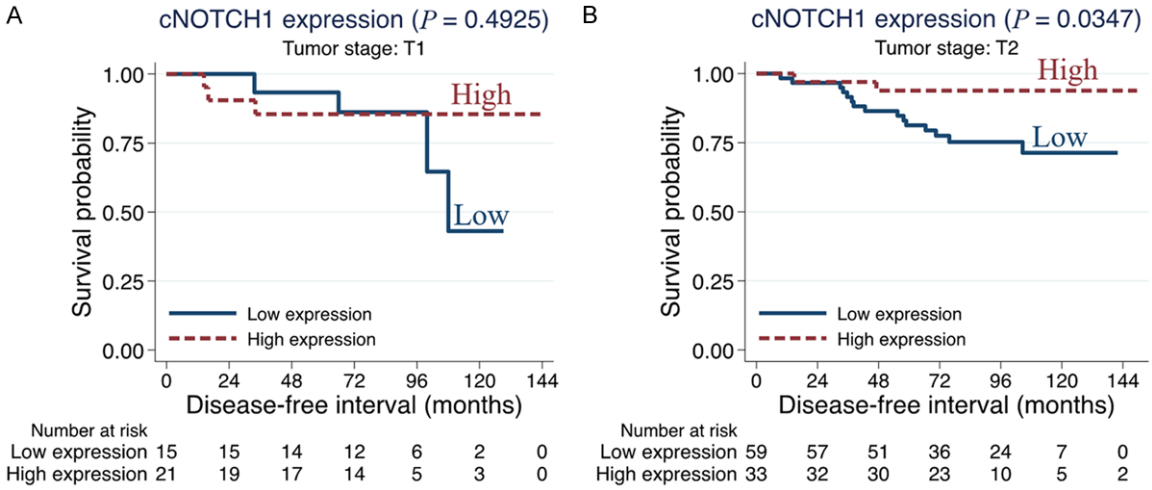


**Figure S4.** Comparison of the expression of cNOTCH1 in immunohistochemistry (IHC) and disease-free survival of breast cancer patients. A. Breast cancer patients with T1 cancer. B. Breast cancer patients with T2 cancer.

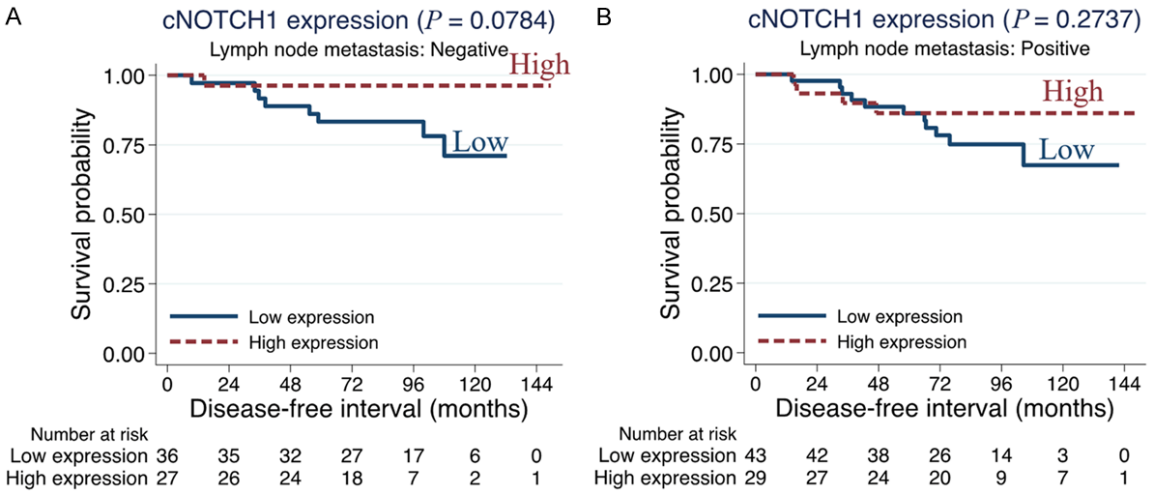


**Figure S5.** Comparison of the expression of cNOTCH1 in immunohistochemistry (IHC) and disease-free survival of breast cancer patients. A. Breast cancer patients without lymph node metastasis. B. Breast cancer patients with lymph node metastasis.

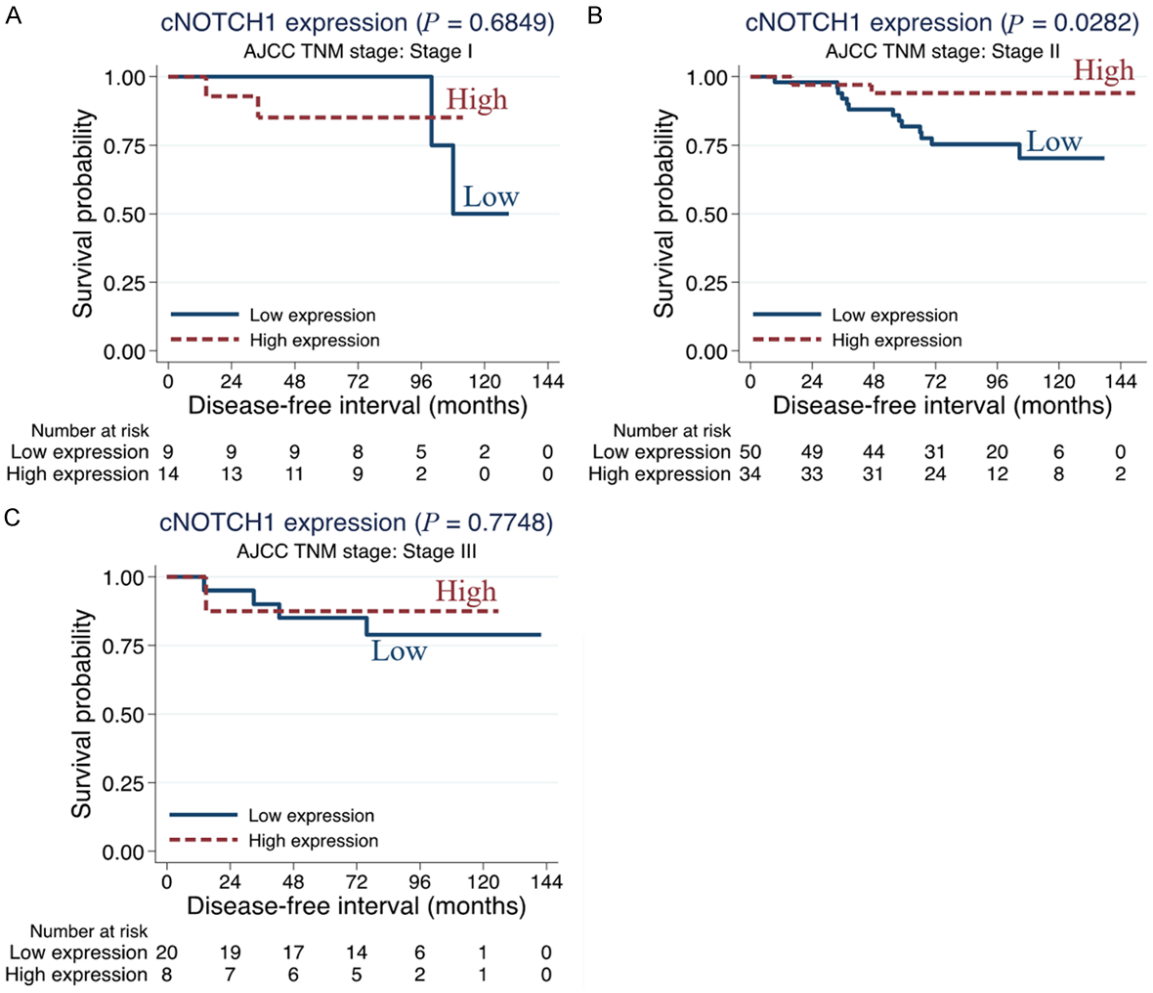


**Figure S6.** Comparison of the expression of cNOTCH1 by immunohistochemistry (IHC) and disease-free survival of breast cancer patients. A. Breast cancer patients with stage I cancer. B. Breast cancer patients with Stage II cancer. C. Breast cancer patients with Stage III cancer.

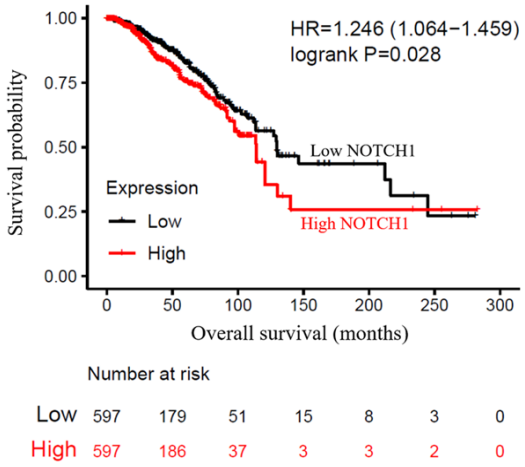


**Figure S7.** Comparison of the expression of NOTCH1 mRNA in TCGA database and overall survival of breast cancer patients.