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Combination of cytoplasmic and nuclear girdin expression is an independent prognosis factor of breast cancer

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ABSTRACT: Girdin is an actin-binding protein playing key roles in the development of various carcinomas. Although online tools have predicted nuclear localization of girdin with a high probability, convincing proof has rarely been provided until now. The purpose of this study was to discover girdin's precise subcellular distribution and the potential prognostic value corresponding to its localization. The subcellular distribution of girdin was detected in a human breast cancer cell line and in >800 samples of human breast tissue by clinical pathologic analysis. In this study, we discovered for the first time that girdin could attach to chromatin and interact with topoisomerase-IIα in nucleus. Cytoplasmic and nuclear girdin exhibited different roles in prognosis of breast cancer: cytoplasmic girdin expression was an independent prognostic factor for progression-free survival (PFS), whereas nuclear girdin expression was an independent prognostic factor for overall survival (OS). More important, combination cytoplasmic and nuclear girdin was an independent prognosis factor of both OS and PFS. In conclusion, our research results strongly recommend combination analysis of cytoplasmic and nuclear girdin for a precise prognostic prediction in breast cancer.—Zhang, H., Yu, F., Qin, F., Shao, Y., Chong, W., Guo, Z., Liu, X., Fu, L., Gu, F., Ma, Y. Combination of cytoplasmic and nuclear girdin expression is an independent prognosis factor of breast cancer. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: subcellular distribution · topoisomerase-IIα · survival · recurrence · metastasis

Breast cancer is a heterogeneous disease, characterized by distinct morphology, biologic behavior, and clinical implications (1, 2). Unfortunately, such interindividual variability is only partially explained by traditional clinicopathological parameters and certain well-known molecular biomarkers such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER)-2, and so on, which are not enough to meet the needs of precision medicine (3). Therefore, identification of novel biomarkers for accurate prognostication and stratification is urgently needed to move toward the goal of precision cancer care.

As an actin-binding protein, girdin plays key roles in various cancer development processes (4–9). It has been shown to be a signal transducer that modulates multiple signaling pathways (6, 10–16). Recently, girdin was reported to serve as a useful prognosticator adjunct to traditional staging strategies in colon cancer, and its high expression predicts a worse outcome in esophageal squamous cell carcinoma (17–19). However, the clinical implication of girdin in breast cancer has been investigated in a few studies involving small cohorts of patients. Those studies were mainly focused on its cytoplasmic expression and took no account of its subcellular nuclear distribution (20–25).

Several online tools, such as WoLF PSORT (26) and YLoc (*https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2896088/*; National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, USA) have predicted nuclear localization of girdin with a high probability.

ABBREVIATIONS: CC, coiled–coil; CI, confidence interval; CT, C terminus; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; GFP, green fluorescent protein; HA, hemagglutinin; HER, human epidermal growth factor receptor; HR, hazard ratio; IDC, invasive ductal carcinoma; IHC, immunohistochemistry; IP, immunoprecipitation; LMB, leptomycin B; NT, N terminus; overall survival; PFS, progression-free survival; PR, progesterone receptor; S-P, streptavidin-peroxidase; SRB, sulforhodamine B; topo-IIα, topoisomerase-IIα

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PSORT II Prediction (*https://www.genscript.com/psort.html/* GenScript, Piscataway, NJ, USA) and the NetNES 1.1 Server (27) also predicted nuclear localization signals and nuclear export signals in girdin, respectively. However, more solid and convincing evidence for girdin's nuclear expression is needed. Meanwhile, the clinical effect of girdin in relation to its subcellular distribution, including nuclear localization, must be identified.

In our present study, we provided more precise evidence to confirm the subcellular distribution of girdin and investigated its clinical importance in a large cohort of patients including 515 with invasive ductal carcinoma (IDC), 254 with benign lesions, and 114 with ductal carcinoma *in situ* (DCIS). First, we provided definite proof of nuclear localization of girdin and found it could attach to chromatin and interact with topoisomerase (topo)-IIα in nucleus. Second, we demonstrated that cytoplasmic and nuclear girdin have different roles in prognosis of breast cancer. Third, we proved for the first time that combination analysis of cytoplasmic and nuclear girdin expression

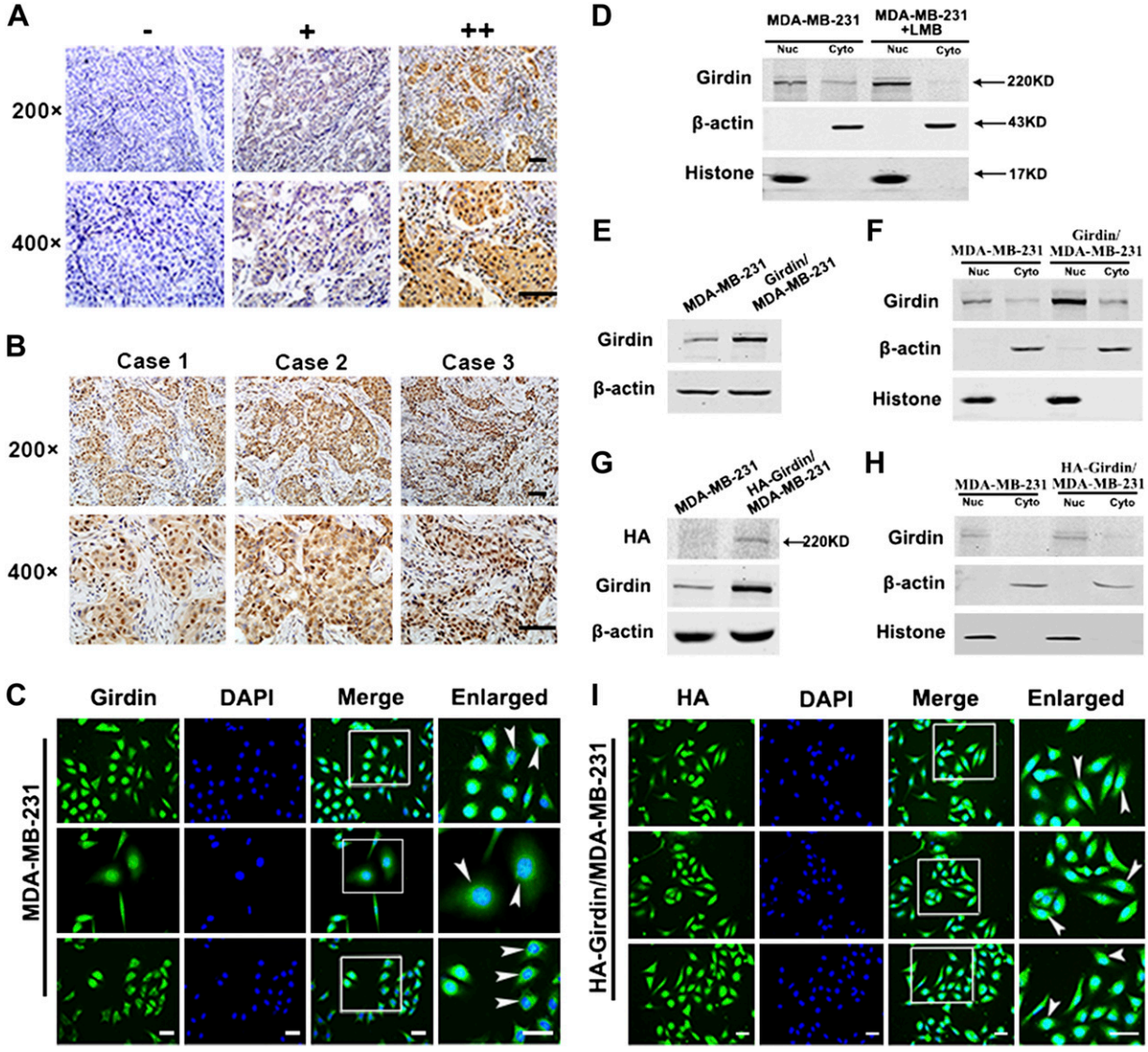

Figure 1. Girdin expression in human breast tissue and its subcellular distribution. *A*) Staining intensity of girdin in IDC specimens: (−), no or low staining; (+), moderate staining; and (++), intense staining. *B*) Representative images of nuclear girdin expression in IDC specimens. Original magnification, ×200 and 400. *C*) Endogenous girdin expression (green) in MDA-MB-231 cells was analyzed by immunofluorescence. DAPI was used to stain the nuclei. Arrowheads: nuclear colocalization. *D*) Western blot analysis of endogenous girdin expression in cytoplasm and nuclei of MDA-MB-231 cells. β-Actin and histone were used as specific markers for cytoplasm and nuclei, respectively. *E*) Western blot of girdin expression in girdin/MDA-MB-231 cells. β-Actin was used as a loading control. *F*) Comparison of nuclear girdin expression in MDA-MB-231 cells and girdin/MDA-MB-231 cells in Western blot. *G*) Western blot of girdin expression in HA-girdin/MDA-MB-231 cells. *H*) Comparison of nuclear girdin expression in MDA-MB-231 and HA-girdin/MDA-MB-231 cells. *I*) Exogenous HA-labeled girdin protein was detected by immunofluorescence with anti-HA antibody in HA-girdin/MDA-MB-231 cells. Arrowheads: cells with nuclear colocalization. *J*) Western blot of girdin expression in vector/MDA-MB-231and 3× flag-girdin/MDA-MB-231 cells. Girdin was detected by anti-flag and anti-girdin antibodies. *K*) Comparison of nuclear girdin expression in vector/MDA-MB-231 and 3× flag-girdin/MDA-MB-231 cells. *L*) Girdin expression was detected by a biochemical fractionation scheme. Final fractions used for analysis were boxed (S2, S3, P1, and P3, top panel). Scale bars, 100 μm.

is an independent prognosis factor and a better survival predictor than either cytoplasmic or nuclear girdin alone.

MATERIALS AND METHODS

Patient selection

Paraffin-embedded specimens from 515 patients diagnosed with IDC from February 1, 2004 to April 30, 2009: 114 patients with DCIS and 254 patients with benign lesions (230 cases of fibroadenoma, 9 cases of benign phyllodes tumors, 2 cases of adenoma, and 13 cases of adenosis) were randomly selected and reviewed from the archives of the Department of Breast Cancer Pathology and Research Laboratory, Tianjin Medical University Cancer Institute and Hospital. Of the total cases, 117 were excluded. The histopathology and diagnosis in each case was confirmed independently by two pathologists according to the World Health Organization criteria for the classification of breast cancer. None of the patients had received neoadjuvant chemotherapy or preoperative radiation therapy. This study was approved by Institutional Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (bc2016029), and each participant signed an informed-consent document. All experiments were performed in accordance with relevant guidelines and regulations of Institutional Ethics Committee of Tianjin Medical University Cancer Institute and Hospital.

Patients' clinical information

All 515 patients with IDC were women aged from 27 to 80 yr (median age, 50 yr). A total of 398 cases were included for prognostic analysis, excluding those with no follow-up data (117 cases). During follow-up (median, 78 mo; range, 1–140 mo), 10 (2.5%) patients had recurrence, 68 (17.1%) developed distant metastases (45 cases with bone metastasis, 18 with lung metastasis, 11 with liver metastasis, 6 with brain metastasis, 4 with kidney metastasis, 4 with thyroid metastasis, 2 with uterus metastasis, 1 with ovarian metastasis, and 1 with intestine metastasis) and 49 (12.3%) patients died of breast cancer. Notably, multiple organic metastases were recorded for 17 patients. Eighty patients had disease progression (recurrence, distant metastasis, or death) within 5 yr and 245 patients were disease-free for >5 yr.

As proposed in the St. Gallen International Expert Consensus of 2011 (30), patients were classified into 4 molecular subtypes by immunohistochemical (IHC) surrogates. Among the 398 cases analyzed, 325 (81.7%) had breast cancer of luminal subtype (including 65 patients classified as luminal A, 256 patients classified as luminal B, and 4 patients classified as luminal subtype because of positive ER status, as well as unknown Ki-67/HER2 status), 45 (11.3%) patients were triple-negative subtype, and 28 (7%) patients were HER2 overexpression subtype. Among 45 patients with bone metastasis, 40 belonged to the luminal subtype group, 1 belonged to the HER2 overexpression group, and 4 belonged to the triple-negative group.

Immunohistochemistry staining

IHC for girdin was performed with standard techniques by the streptavidin-peroxidase (S-P) method. Antigen retrieval was performed at 121°C for 2 min 30 s. Sections were incubated with primary antibody against girdin overnight at 4°C and then were incubated with a second antibody. The enzyme substrate was 3, 3′-diaminobenzidine tetrahydrochloride.

Evaluation of immunostaining

Girdin expression in cytoplasm was evaluated according to the H score system (*http://www.e-immunohistochemistry.info/web/H-score.htm*), which was based on the staining intensity and the

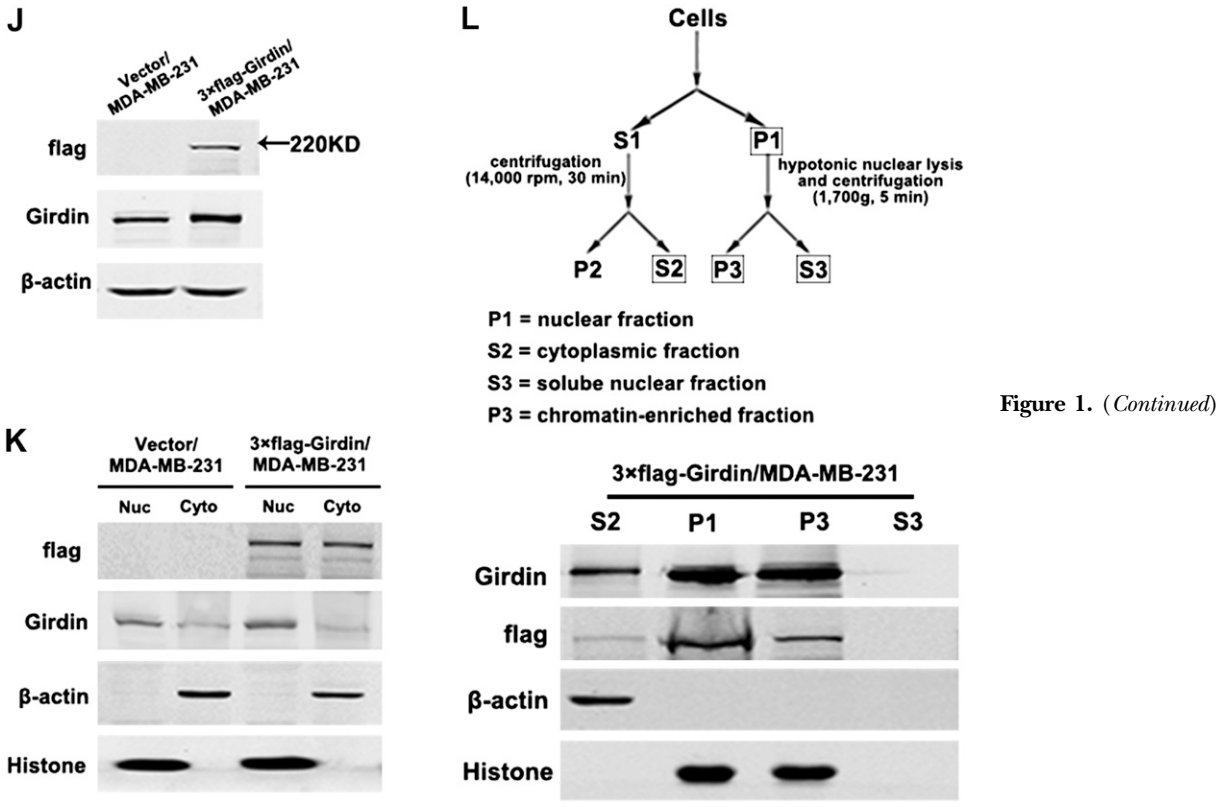

Figure 1. (*Continued*)

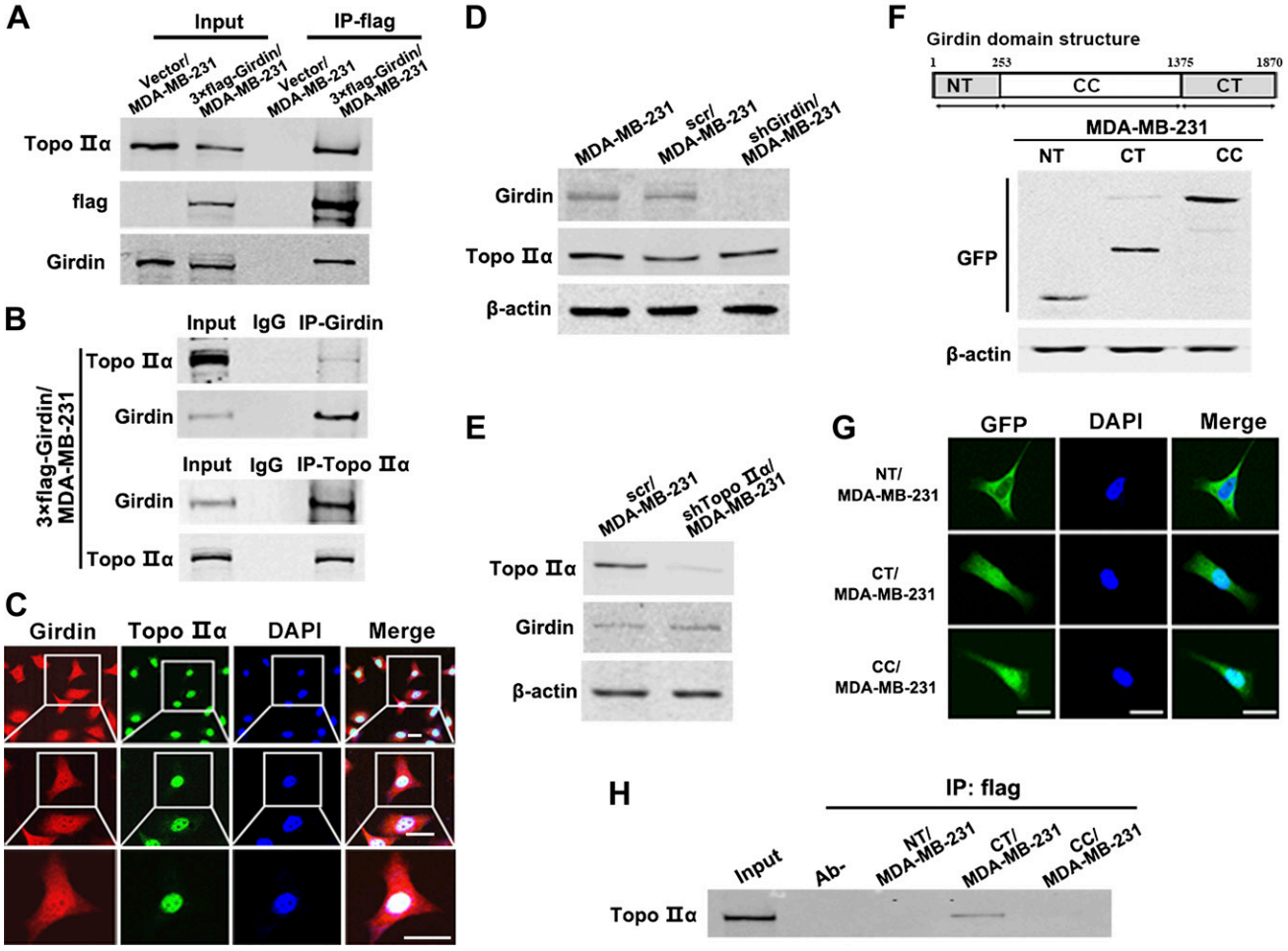

Figure 2. Girdin coimmunoprecipitated with topo-IIα. *A*) IP was performed by using an anti-flag M2 affinity gel. Expression of flag-tagged girdin and topo-IIα was determined by Western blot analysis. *B*) IP was performed by using antibodies against girdin, topo-IIα, or control IgG. Expression of girdin and topo-IIα was determined by Western blot analysis. *C*) Representative immunofluorescent images of colocalization of girdin and topo-IIα, in which the colocalization is white in the merged images. *D*) Girdin was knocked down in MDA-MB-231 cells, and expression of girdin and topo-IIα was detected by Western blot analysis. *E*) Topo-IIα was knocked down in MDA-MB-231 cells, expression of topo-IIα and girdin was detected by Western blot analysis. *F*) Various fragments of girdin labeled with GFP-3× flag were transfected into MDA-MB-231 cells. Top: domain structure of human girdin. Bottom: exogenous fragments monitored by anti-GFP antibody in Western blot analysis. *G*) Expression of exogenous fragments detected by immunofluorescence in MDA-MB-231 cells. *H*) Cell lysis of NT/MDA-MB-231, CT/MDA-MB-231, and CC/MDA-MB-231 was applied, and IP was performed on an anti-flag M2 affinity gel. Expression of topo-IIα was determined by Western blot analysis. Scale bars, 25 μm.

percentage of cells stained positively. Staining intensity was measured and scored as follows: 0 (−), no or low staining; 1 (+), moderate staining; and 2 (++), intense staining. Percentage of cells stained positively was scored as 0–100. Therefore, a total H score of cytoplasmic girdin ranged from 0 to 200 by multiplying the intensity and the percentage scores.

Because nuclear staining was present in a uniform intensity but to different extent, nuclear girdin expression was assessed by

TABLE 1. *Cytoplasmic/nuclear girdin expression in different breast tissues*

Histologic type	Cases	Girdin (cytoplasm, mean score), n^a		χ^2	P	Girdin (nucleus, mean score), n^b		χ^2	P
		Low	High			Low	High		
Benign lesions	254	192 (38.13)	62 (111.10)	53.595	<0.001*	221 (0.36)	33 (12.42)	29.922	<0.001*
DCIS	114	88 (55.68)	26 (99.23)			97 (0.52)	17 (13.24)		
IDC	515	317 (52.82)	198 (119.92)			366 (0.21)	149 (34.03)		

[a]The sequence of 3 group scores: benign lesions <DCIS (mean rank difference = 75.302; $P = 0.026$); benign lesions <IDC (mean rank difference = 141.935; $P < 0.001$); DCIS < IDC (mean rank difference = 66.633, $P = 0.034$). [b]The sequence of 3 groups scores: benign lesions *vs.* DCIS (mean rank difference = 18.503; $P = 1.000$); benign lesions <IDC (mean rank difference = 80.434; $P < 0.001$); DCIS < IDC (mean rank difference = 61.931; $P = 0.009$, Dunn's multiple comparisons test). *$P < 0.05$, calculated by Kruskal-Wallis test.

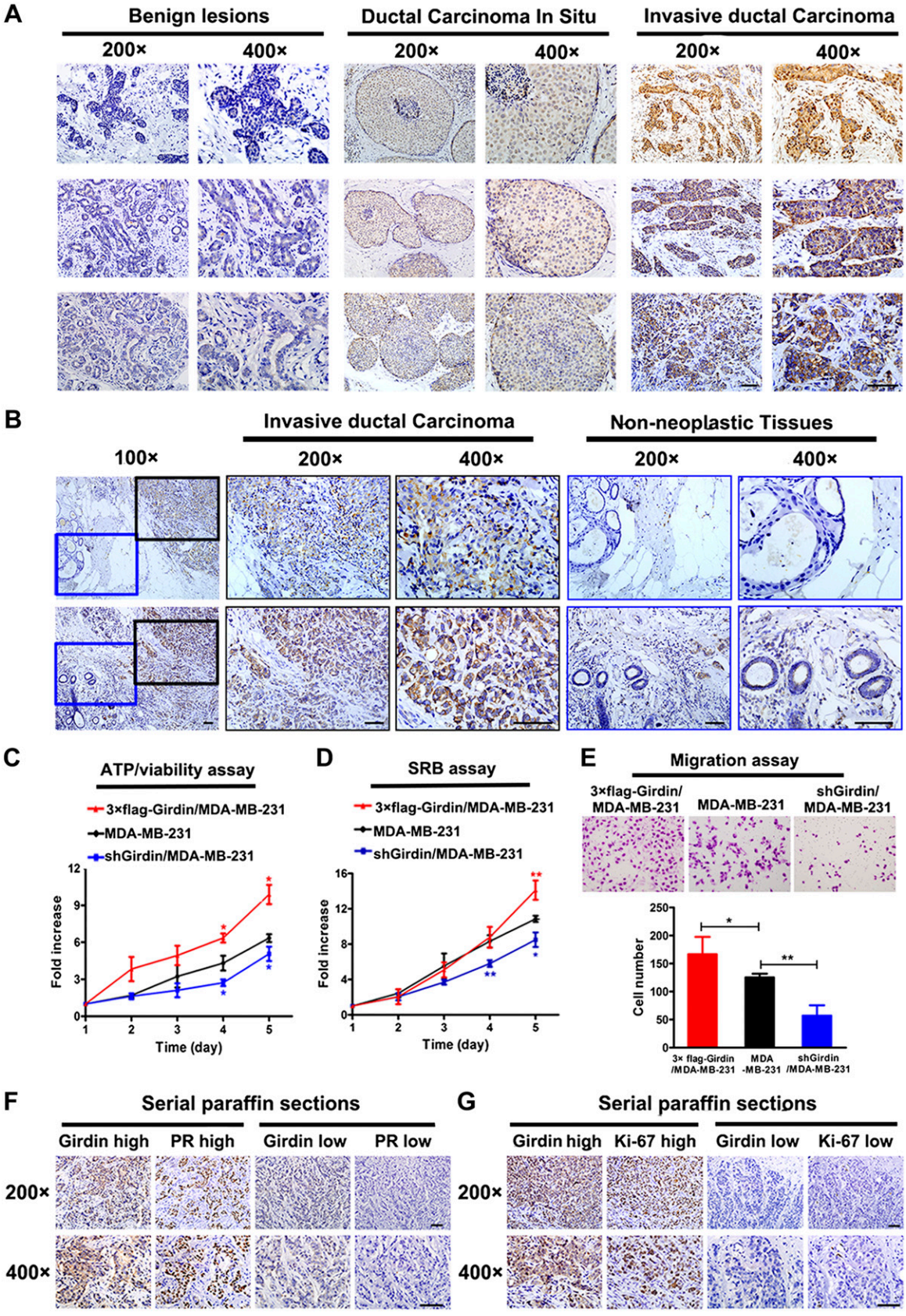

Figure 3. Cytoplasmic girdin expression correlated positively with breast cancer progression. *A*) Representative images of girdin expression in breast specimens of benign lesions, DCIS and IDC. *B*) Girdin expression was elevated in tumor sections compared to their nonneoplastic tissues adjacent to the tumor. Black rectangles: tumor sections; blue rectangles: nonneoplastic sections adjacent to tumor. Representative images of two specimens were shown. *C*) Proliferation ability was examined by ATP/viability

(*continued on next page*)

TABLE 2. *Relationship between clinicopathological characteristics and cytoplasmic/nuclear girdin expression in IDC patients*

Histologic type	Cases	Girdin (cytoplasm), *n*		r_s	*P*	Girdin (nucleus), *n*		r_s	*P*
		Low	High			Low	High		
Age				0.016	0.772			−0.005	0.917
<50	247	154	93			175	72		
≥50	268	163	105			191	77		
Tumor size[a]									
≤2 cm	133	79	54	−0.026	0.574	91	42	−0.042	0.365
>2 cm	336	209	127			244	92		
Histologic grade[a]				0.066	0.137			0.008	0.850
I	32	21	11			23	9		
II	385	242	143			270	115		
III	66	35	31			46	20		
Lymph node status[a]				0.028	0.530			0.023	0.600
Negative	203	129	74			147	56		
Positive	306	186	120			215	91		
ER status[a]				0.035	0.424			0.106	0.017*
Negative	166	106	60			129	37		
Positive	344	207	137			232	112		
PR status[a]				0.093	0.036*			0.054	0.226
Negative	143	98	45			107	36		
Positive	366	214	152			254	112		
HER2 status[a]				0.066	0.136			−0.042	0.348
− ~ +	382	242	140			266	116		
++ ~ +++	127	71	56			94	33		
Ki-67 status[a]				0.103	0.021*			0.047	0.296
Negative	130	91	39			97	33		
Positive	374	219	155			261	113		
Recurrence or metastasis[a]				0.140	0.006*			0.007	0.890
No	317	212	105			222	95		
Yes	78	39	39			54	24		

$n = 515$. [a]Some data are missing. *$P < 0.05$, Spearman's rank correlation test.

the percentage of positively nucleic-stained cells and scored on a scale of 0 to 100.

A cytoplasmic girdin level score of 90–200 was defined as C-high and a score of 0–89 as C-low; a nuclear girdin level score of 10–100was defined as N-high and a score of 0–9 as N-low.

Immunohistochemistry for ER and PR was re-evaluated using the 2010 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guideline. Cases were scored positive for ER and PR if nuclear immunoreactivity was present in more than 1% of tumor cells (28). Immunohistochemistry for HER2 was re-evaluated using the 2014 ASCO/CAP updated guideline (29). Ki-67 expression was re-evaluated by criteria set at the St. Gallen International Breast Adenocarcinoma Conference of 2011 (30).

Cell culture and reagents

MDA-MB-231 cells were cultured in DMEM supplemented with 10% fetal bovine serum in a 5% CO_2 incubator at 37°C. Cells were tested and authenticated in Beijing Microread Genetics (Beijing, China) by short tandem repeat profiling. Main antibodies used in this study included polyclonal rabbit anti-girdin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-topo-IIα (Santa Cruz Biotechnology), and anti-histone h3.1 (Ray Antibody Biotech, China). Leptomycin B (LMB), a nuclear export inhibitor, was obtained from Beyotime Biotechnology (Beijing, China).

Plasmid construction and transfection

A clone of *Homo sapiens* full-length girdin was obtained from OriGene Technologies (Rockville, MD, USA). Full-length girdin was amplified by PCR with primers for human girdin: forward, 5′-CGGGATCCATGGAGAACGAAATTTTTACT-3′, and reverse, 5′-ATAAGAATGCGGCCGCTTAGGAGCTTTGTTGCTCCCTAGACCT-3′. Nonlabeled, HA-labeled, and 3× flag-tagged girdin were inserted into pCDH-CMV-MCS-EF1-Puro lentiviral vector, respectively. Green fluorescent protein (GFP)-3× flag-tagged fragments of girdin, including NT (amino terminal domain, aa 1–253), CC (coiled–coil domain, aa 254–1374), and CT (carboxyl terminal domain, aa 1375–1870) were constructed (22, 31, 32). The girdin-specific shRNA sequence (CCGGGAAGGAGAGGCAACTGGATCTCGAGATCCAGTTGCCTCTCCTTCTTTTTG), topo-IIα-specific

assay and MDA-MB-231 cells were used as a control group. *D*) Proliferation was examined by an SRB assay. *E*) Migration assay results. Original magnification, ×200. *F*) Expression of girdin and PR was detected in serial paraffin-embedded sections with IHC analysis. Bottom: the amplification of the top panel. *G*) Expression of girdin and Ki-67 was detected in serial paraffin-embedded sections by IHC analysis. Bottom: enlargement of the top panel. All experiments were performed 3 times independently. *$P <$ 0.05; **$P < 0.01$, Student *t* test. Scale bars, 100 μm.

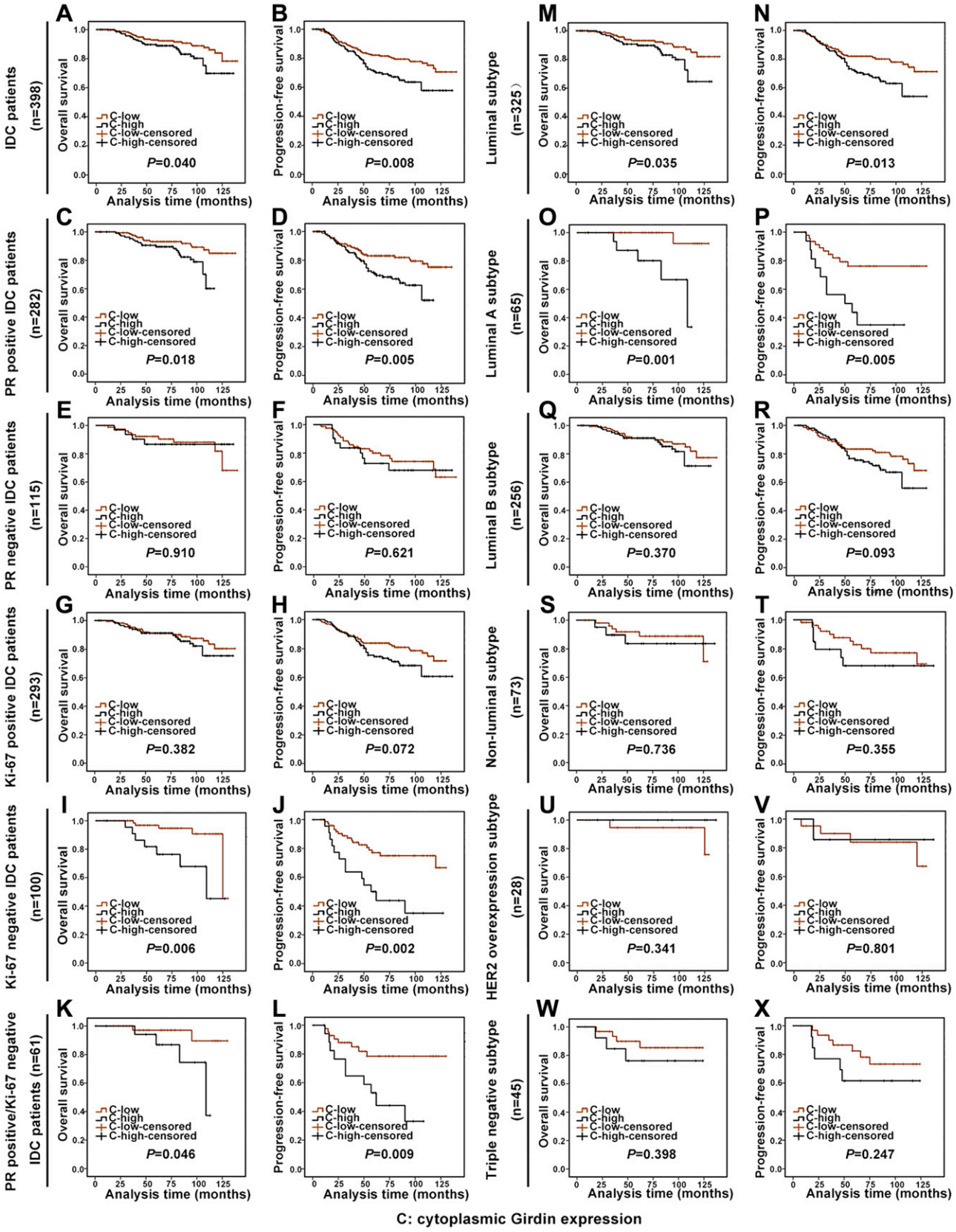

Figure 4. Effect of cytoplasmic girdin on prognosis of breast cancer in different subgroups. High cytoplasmic girdin expression indicated a shorter OS and PFS in IDC (*A*, *B*) and PR^+ (*C*, *D*) patients. Cytoplasmic girdin had no effect on prognosis in PR^- (*E*, *F*) or Ki-67^- (*G*, *H*) patients. High cytoplasmic girdin expression indicated a shorter OS and PFS in Ki-67^- (*I*, *J*) and PR^+/Ki-67^- (*K*, *L*) patients. High cytoplasmic girdin expression indicated a shorter OS and PFS in patients a luminal subtype (*M*, *N*) and in those with the luminal A subtype (*O*, *P*). Cytoplasmic girdin had no effect on prognosis in patients with the luminal B (*Q*, *R*), nonluminal (*S*, *T*), HER2 overexpression (*U*, *V*), or triple-negative (*W*, *X*) subtype. C, cytoplasmic girdin expression.

TABLE 3. *Cytoplasmic and nuclear girdin expression exhibited different roles in prognosis of IDC patients*

Variable	OS						PFS					
	Univariate		Multivariate				Univariate		Multivariate			
	HR	*P*	HR	*P*	HR	*P*	HR	*P*	HR	*P*	HR	*P*
Age	1.222 (0.693–2.153)	0.488					1.482 (0.988–2.221)	0.057	1.395 (0.917–2.123)	0.120	1.466 (0.966–2.224)	0.073
Histologic grade	1.194 (0.600–2.376)	0.614					1.078 (0.659–1.764)	0.764				
Tumor size	1.554 (0.772–3.127)	0.217					1.610 (0.982–2.639)	0.059	1.489 (0.903–2.455)	0.119	1.489 (0.903–2.455)	0.119
Lymph node status	3.089 (1.498–6.368)	0.002	3.041 (1.475–6.272)	0.003*	3.141 (1.523–6.477)	0.002*	3.168 (1.536–6.533)	0.002*	1.583 (1.033–2.423)	0.035	1.645 (1.054–2.566)	0.028*	1.707 (1.093–2.664)	0.019*
ER status	0.772 (0.433–1.375)	0.379							0.860 (0.567–1.304)	0.478				
PR status	1.032 (0.553–1.927)	0.921							0.966 (0.626–1.493)	0.878				
HER2 status	1.142 (0.612–2.129)	0.677							0.977 (0.616–1.548)	0.921				
Ki-67 status	0.945 (0.492–1.815)	0.866							0.679 (0.442–1.045)	0.078	0.553 (0.349–0.874)	0.011*	0.571 (0.363–0.900)	0.016*
Girdin status (cytoplasm)	1.798 (1.019–3.172)	0.043	1.756 (0.997–.092)	0.051					1.711 (1.147–2.553)	0.008	1.714 (1.129–2.604)	0.011*		
Girdin status (nucleus)	1.789 (1.030–3.258)	0.049			1.831 (1.030–3.258)	0.039*			1.170 (0.769–1.780)	0.462				
Girdin status (cytoplasm/nucleus)							2.601 (1.439–4.701)	0.002*					1.733 (1.082–2.775)	0.022*

Values in parentheses indicate 95% CI. $n = 398$. *$P < 0.05$.

shRNA sequence (CCGGCAAGAAGTGTTCAGCTGTACT-CGAGTACAGCTGAACACTTCTTGTTTTTG), and scrambled sequence were synthesized and cloned into pLKO.1 pure vector. Lentivirus was produced by cotransfection of lentiviral plasmid, with plasmids ΔR and pVSVg packed into HEK-293T cells. Stable lentivirus-infected cells were selected with puromycin and verified by Western blot analysis.

Immunofluorescence analysis

Cells were fixed with 4% paraformaldehyde first and then were permeabilized with 0.2% Triton X-100. Primary antibody was used at 4°C, and secondary antibodies were used at room temperature. Cell nuclei were stained with DAPI, and the cells were examined by fluorescence microscopy.

Western blot analysis

In brief, the cells were lysed in 1× SDS lysis buffer first. Equal amounts of protein were loaded and separated by SDS-PAGE and then were transferred onto nitrocellulose membranes. The membranes were incubated overnight at 4°C with the primary antibody and were then treated with secondary antibodies. Infrared signals were examined by using the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA).

Preparation of cytosol/nuclear extract

Cytoplasmic and nuclear extracts were prepared according to the manufacturer's instructions for the Nuc-Cyto-Mem Preparation Kit (P1201; Applygen Technologies, Beijing, China). In brief, cells were lysed by Dounce homogenization with prechilled buffer cytosol extraction reagent (CER) on ice. Subsequently, the whole-cell lysate was centrifuged at 800 *g* for 5 min at 4°C. The pellet (nuclear component) was washed with the ice-cold buffer nuclear extraction reagent (NER), clarified by low-speed centrifugation, and collected as nuclei (Nuc). The supernatant of whole-cell lysate was incubated with the ice-cold buffer membrane extraction reagent (MER) on ice before centrifugation at 14,000 rpm. The supernatant was incubated with 30% (v/v) trichloroacetic acid overnight at −20°C, followed by centrifugation at 5000 *g*. Then the pellet was collected as cytoplasmic fraction (Cyto). The isolated protein fractions were analyzed by Western blot. To evaluate the purity of protein fractions and exclude the possibility of contamination by other protein fractions, β-actin and histone were used as specific markers for cytoplasmic and nuclear fractions, respectively.

Small-scale biochemical fractionation

The biochemical fractionation was performed essentially as described (33–37). In brief, cells were collected, washed with PBS, then resuspended at the concentration in buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, and protease inhibitor cocktail). Triton X-100 was added (final concentration, 0.1%), and the cells were incubated for 10 min on ice. Nuclei (fraction P1) were collected in the pellet by low-speed centrifugation (5 min, 1300 *g*, 4°C). The supernatant (fraction S1) was clarified by high-speed centrifugation (30 min, 14,000 rpm, 4°C), and supernatant (fraction S2) was collected. The P1 nuclei were washed once in buffer A before they were lysed for 30 min in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitor cocktail), then insoluble chromatin (fraction P3) and soluble fraction (fraction S3) were separated by low-speed centrifugation (5 min, 1700 *g*, 4°C). The P3

TABLE 4. *Cytoplasmic girdin was an independent prognosis factor of PR positive breast cancer patients*

Variable	OS				PFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	*P*	HR	*P*	HR	*P*	HR	*P*
Age	1.145(0.590–2.223)	0.688			1.489(0.925–2.396)	0.102		
Histologic grade	1.077(0.472–2.457)	0.860			1.097(0.613–1.962)	0.755		
Tumor size	1.472(0.666–3.252)	0.339			1.770(0.982–3.191)	0.057	1.677(0.925–3.039)	0.088
Lymph node status	5.158(1.820–14.615)	0.002	5.294(1.867–15.012)	0.002*	1.997(1.166–3.420)	0.012	1.967(1.129–3.428)	0.017*
ER status	0.592(0.284–1.234)	0.162			0.662(0.383–1.145)	0.140		
HER2 status	1.867(0.913–3.817)	0.087			1.153(0.650–2.044)	0.626		
Ki-67 status	1.231(0.510–2.794)	0.644			0.663(0.387–1.136)	0.135		
Girdin status (cytoplasm)	2.248(1.129–4.477)	0.021	2.321(1.173–4.595)	0.016*	1.952(1.207–3.158)	0.006	1.832(1.126–2.980)	0.015*

Values in parentheses indicate 95% CI. $n = 282$. *$P < 0.05$.

fraction was washed with buffer B before resuspension in 1× SDS sample buffer. The samples were boiled and loaded for Western blot analysis.

Coimmunoprecipitation

Cells were washed 3 times with ice-cold PBS and then resuspended with coimmunoprecipitation (Co-IP) lysis buffer. Cell lysates were gently rotated at 4°C overnight followed by centrifugation at 12,000 *g* for 10 min, and the pellet was discarded. The supernatant was immunopurified with anti-flag M2 affinity gel and eluted with flag peptides. Finally, the eluate was subjected to Western blot to examine the expression of topo-IIα (see Fig. 2A, H).

For Fig. 2B, cell lysates were gently rotated at 4°C overnight followed by centrifugation, and the pellet was discarded. Nonspecific protein was removed by adding Protein A, the mixture was centrifuged, and the supernatant was divided into 3 groups by using antibodies against girdin, topo-IIα, and control IgG. Finally, the precipitates were subjected to Western blot to examine the expression of girdin, flag, and topo-IIα.

Cell ATP/viability assay and sulforhodamine B assay

Cells were plated in 24-well plates with 6 replicates for 5 d. ATP levels were measured using the CellTiter-Glo luminescent cell viability assay kit (catalog number G7571; Promega, Madison, WI, USA). For the sulforhodamine B (SRB) assay, the cells were fixed with 10% trichloroacetic acid and then were washed. SRB (0.4%) in 1% acetic acid was used to stain the cells. Tris-base (10 mM) was added to dissolve the SRB and absorbance was measured with a plate reader at 546 nm.

Migration assay

A migration assay was performed with 24-well Transwell migration chambers (Corning, New York, NY, USA) with polyethylene membranes (8 μm pore size). The upper chambers were seeded with 3.0×10^4 cells/well in 200 μl of serum-free DMEM. Serum-free DMEM (600 ml) with 5% FBS was added to the lower chambers. The cells were allowed to migrate for 24 h at 37°C. Afterward, cells on the upper layer of the membrane were scraped, and cells on the lower layer were stained with Giemsa solution and photographed under a microscope. The number of cells was quantified in randomly selected fields.

Statistical analysis

Overall survival (OS) was measured from the date of diagnosis to the date of death or last follow-up. Progression-free survival (PFS) was defined as time from surgery to either first disease progression (recurrence or distant metastasis) or cancer-specific death. Kruskal-Wallis test, Dunn's multiple comparisons test, and χ^2 test were performed for group comparisons. The nonparametric Spearman's correlation analysis was used to assess the association between 2 variables. Survival outcomes were estimated using the Kaplan-Meier method and were compared between the groups by using log-rank statistics. Univariate and multivariate Cox proportional hazards models were used to determine the associations of the clinical-pathologic parameters with survival outcomes. All reported *P* values were 2-sided and differences reaching $P < 0.05$ were regarded as statistically significant. In multivariate analysis; a $P < 0.08$ in the univariate

TABLE 5. *Cytoplasmic girdin was an independent prognosis factor of luminal subtype breast cancer patients*

Variable	OS				PFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR	*P*	HR	*P*	HR	*P*	HR	*P*
Age	1.214 (0.651–2.265)	0.541			1.540 (0.989–2.400)	0.056	1.379 (0.877–2.168)	0.164
Histologic grade	1.089 (0.500–2.372)	0.829			1.167 (0.681–2.001)	0.574		
Tumor size	1.727 (0.794–3.759)	0.168			1.807 (1.044–3.129)	0.035	1.720 (0.986–3.001)	0.056
Lymph node status	3.603 (1.512–8.587)	0.004	3.619 (1.519–8.627)	0.004*	1.599 (0.992–2.577)	0.054	1.699 (1.034–2.789)	0.036*
ER status	0.607 (0.297–1.243)	0.607			0.692 (0.405–1.182)	0.178		
PR status	0.947 (0.370–2.422)	0.910			0.811 (0.439–1.498)	0.503		
HER2 status	1.589 (0.807–3.128)	0.180			1.114 (0.666–1.863)	0.682		
Ki-67 status	1.260 (0.556–2.856)	0.579			0.636 (0.392–1.033)	0.067	0.496 (0.298–0.827)	0.007*
Girdin status (cytoplasm)	1.963 (1.037–3.175)	0.038	1.970 (1.047–3.707)	0.036*	1.742 (1.119–2.710)	0.014	1.780 (1.125–2.818)	0.014*

Values in parentheses indicate 95% CI. $n = 325$. *$P < 0.05$.

analysis was considered eligible for multivariate variables. All statistical analyses were done with SPSS software package (v17.0; SPSS, Chicago, IL, USA).

RESULTS

Expression pattern of girdin in breast tissues and its subcellular location

First, we detected the expression of girdin by IHC analysis. Girdin localized in both cytoplasm and nucleus. The intensity of cytoplasmic girdin staining is shown in representative images in **Fig. 1*A***, and 3 representative images of nuclear girdin staining are shown in Fig. 1*B*. Cytoplasmic girdin expression was observed in 99.6% of IDC cases (513/515), and 2 cases showed no girdin expression. Nuclear girdin expression was observed in 33.8% (such cases all had cytoplasmic staining) of cases (174/515). There were 339 cases that showed cytoplasmic staining of girdin without nuclear staining.

Subcellular localization of girdin was then confirmed in MDA-MB-231 breast cancer cells. Thirty-one percent of cells with nuclear expression were observed in immunofluorescence analysis; the colocalization of nuclei and girdin appeared as white dots in merged images (Fig. 1*C* and Supplemental Fig. 1) and was further validated with LMB (a nuclear export inhibitor) treatment in Western blot analysis (Fig. 1*D*). Then, 3 cell clones overexpressing nonlabeled girdin, hemagglutinin (HA)-labeled girdin and 3× flag-labeled girdin, respectively, were applied to indicate the nuclear localization in a nuclear/cytosol fractionation assay (Fig. 1*E–K*), and there were ~97.7% of cells with nuclear expression in HA-girdin/MDA-MB-231 cells clone (Supplemental Fig. 1). In the following, a small-scale biochemical fractionation was used. The 3× flag-girdin/MDA-MB-231 cell lysates prepared with a nonionic detergent were divided by sequential centrifugation into 4 fractions (named as P1, S2, S3, and P3), and girdin was discovered to attach to chromatin or some other insoluble structure in the nucleus (Fig. 1*L*). To investigate and predict the interacting proteins of girdin in the nucleus, we turned to the STRING database (*String-db.org*) and found that topo-IIα was one of the candidates. Then, we performed various immunoprecipitation experiments and revealed that girdin and topo-IIα could coimmunoprecipitate (**Fig. 2*A, B***). Furthermore, the immunofluorescence analysis confirmed their colocalization in the nucleus (Fig. 2*C*). Next, we knocked down expression of girdin and topo-IIα in MDA-MB-231 cells (Fig. 2*D, E*). We found that they did not affect the protein level of each other. Lentivirus-expressing GFP-3× flag-labeled girdin fragments were transfected into MDA-MB-231 cells and designated as NT/MDA-MB-231, CC/MDA-MB-231, and CT/MDA-MB-231 (Fig. 2*F*). GFP fluorescent signals of CC and CT were detected in both nucleus and cytoplasm, whereas the n terminal (NT) was primary localized in cytoplasm (Fig. 2*G*). The results suggest that CC and CT domains of girdin contain nuclear localization signals. Moreover, our results showed that it was the CT domain of girdin that could specifically bind with topo-IIα (Fig. 2*H*).

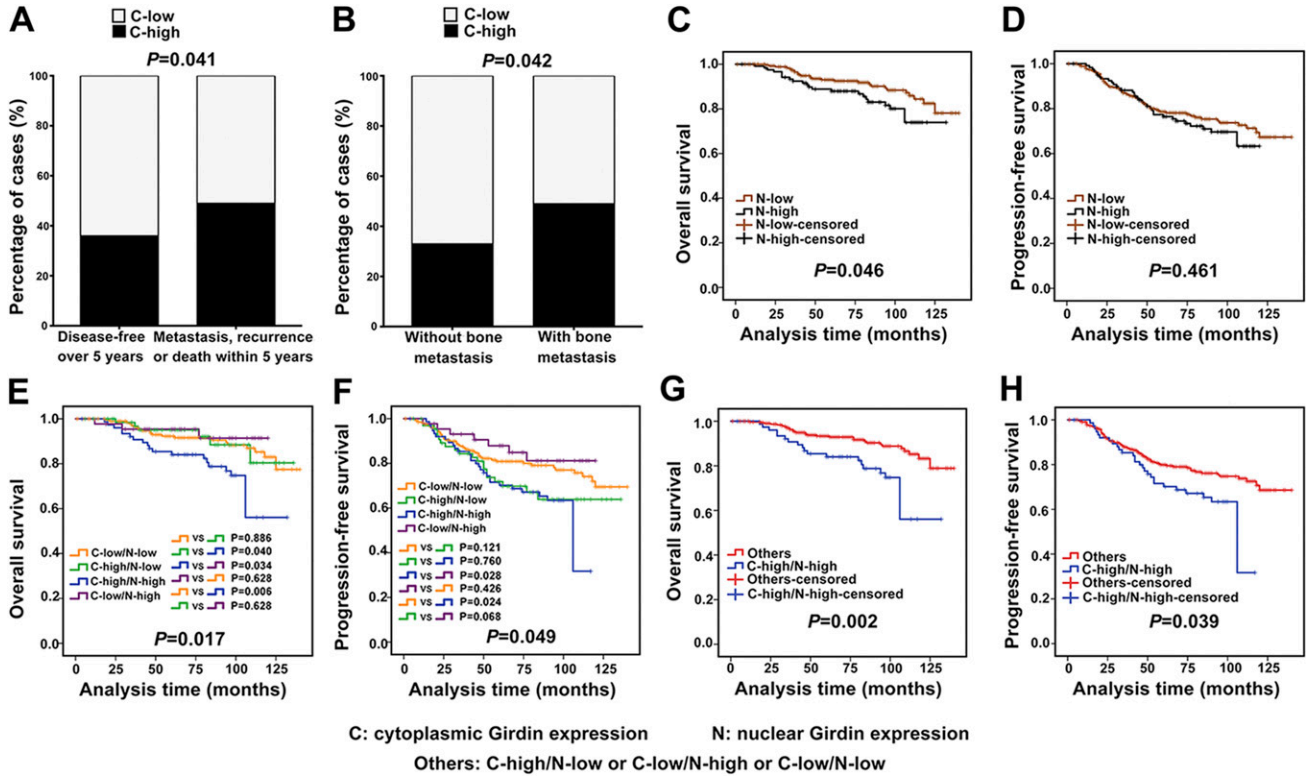

Figure 5. Effect of cytoplasmic and nuclear girdin on prognosis of breast cancer. *A*) Patients who developed metastasis, had a recurrence or died within 5 yr had a higher cytoplasmic girdin expression (48.8% *vs.* 35.9%, χ^2 test; $P = 0.041$). *B*) Patients with bone metastasis had a higher cytoplasmic girdin expression (48.9% *vs.* 33.4%, χ^2 test; $P = 0.042$). *C, D*) Patients with high nuclear girdin expression exhibited a shorter OS and a similar PFS compared with patients with low nuclear girdin expression. *E, F*) Kaplan-Meier analysis of survival of IDC patients with both cytoplasmic and nuclear girdin expression. *G, H*) Patients with C-high/N-high (simultaneous high cytoplasmic and nuclear girdin expression) showed a shorter OS and PFS than Others. C, cytoplasmic girdin expression; N, nuclear girdin expression; Others, C-high/N-low, C-low/N-high, or C-low/N-low.

Next, girdin expression in 114 cases of DCIS and 254 cases of benign lesions was evaluated. Score of cytoplasmic girdin in mammal epithelial cells was gradually up-regulated from benign lesions to DCIS ($P = 0.026$) and IDC ($P = 0.034$, **Table 1**); representative images are shown in **Fig. 3*A***. Although the nuclear girdin score in IDC was much higher than that of DCIS and benign lesions, no statistically significant difference was observed between benign lesions and DCIS (Table 1). Cytoplasmic and nuclear girdin in benign lesions, DCIS, and IDC are also shown in Supplemental Fig. 2. We noted a higher cytoplasmic girdin expression in IDC tissues than in their corresponding adjacent nonneoplastic tissues (Fig. 3*B*).

In the following *in vitro* experiments, we sought to validate the function of girdin in breast cancer. Girdin overexpression increased cell proliferation and migration, whereas down-regulation of girdin decreased proliferation and migration (Fig. 3*C*–*E*).

The correlation between girdin expression and clinicopathological characteristics is presented in **Table 2**. Cytoplasmic girdin correlated positively with PR status, Ki-67 status, and recurrence or metastasis. These results were further confirmed by IHC analysis of serial pathologic sections and representative images are shown in Fig. 3*F*, *G*.

Cytoplasmic and nuclear girdin expression exhibited different roles in prognosis prediction

Among 515 IDC patients, 398 cases with detailed follow-up data were included in prognosis analysis. Patients with

TABLE 6. *Relationship between cytoplasmic girdin expression and distant metastasis in IDC patients*

		Girdin (cytoplasm), *n* (%)			
Distant metastasis	Cases	Low	High	r_s	*P*
Bone metastasis				0.104	0.042*
No	338	225 (66.6)	113 (33.4)		
Yes	45	23 (51.1)	22 (48.9)		
Brain metastasis				0.039	0.447
No	377	245 (65.0)	132 (35.0)		
Yes	6	3 (50.0)	3 (50.0)		
Lung metastasis				0.094	0.065
No	365	240 (65.8)	125 (34.2)		
Yes	18	8 (44.4)	10 (55.6)		
Liver metastasis				0.069	0.175
No	372	243 (65.3)	129 (34.7)		
Yes	11	5 (45.5)	6 (54.5)		

*$P < 0.05$, Spearman's rank correlation test.

high cytoplasmic girdin expression (irrespective of high or low nuclear girdin expression) exhibited a shorter OS and PFS (**Fig. 4*A*, *B***). Cytoplasmic girdin expression was an independent risk factor for shortened PFS (**Table 3**). In addition, high cytoplasmic girdin expression led to a shorter OS and PFS in PR^+ or $Ki\text{-}67^-$ subgroups (Fig. 4*C–J*). In the PR^+ subgroup, cytoplasmic girdin was found to be an independent prognosis factor for both OS and PFS (**Table 4**). Patients with high cytoplasmic girdin expression exhibited a shorter OS and PFS in the $PR^+/Ki\text{-}67^-$ subgroup (Fig. 4*K*, *L*).

Cytoplasmic girdin expression did not show any effect on OS of $HER2^+$ ($P = 0.082$) or $HER2^-$ patients ($P = 0.211$; data not shown). Meanwhile, cytoplasmic girdin expression did not show any effect on OS of ER^+ ($P = 0.08$) or ER^- ($P = 0.216$; data not shown). High cytoplasmic girdin expression led to a shorter OS in the ER^+/PR^+ subgroup ($P = 0.036$, Supplemental Fig. 3*A*, *B*). Patients with high cytoplasmic girdin expression exhibited a shorter PFS in the ER^-/PR^+ subgroup ($P = 0.029$, Supplemental Fig. 3C, D). Meanwhile, in the ER^+/PR^- and ER^-/PR^- subgroups, cytoplasmic girdin expression did not affect prognosis (Supplemental Fig. 3*E–H*).

We next investigated the prognostic effect of cytoplasmic girdin in four molecular subtypes. High cytoplasmic girdin expression indicated a worse prognosis in the luminal, but not in the nonluminal, HER2 overexpression, or triple-negative subtype (Fig. 4*M–X*). It was shown to be an independent prognostic factor for the luminal subtype (**Table 5**). Moreover, cytoplasmic girdin influenced the prognosis of the luminal A, but not the luminal B, subtype (Fig. 4*O–R*).

In addition, patients who developed metastases, had a recurrence, or died within 5 yr had a higher cytoplasmic girdin expression, and patients with bone metastasis also had a higher cytoplasmic girdin expression (**Fig. 5*A*, *B***). Patients with high cytoplasmic girdin expression had earlier disease progression. We found that cytoplasmic girdin expression correlated positively with bone metastasis (**Table 6**).

We investigated the clinical effect of nuclear girdin expression (irrespective of high or low cytoplasmic girdin expression). A positive correlation between nuclear girdin expression and ER status was observed ($P = 0.017$; Table 2). Furthermore, patients with high nuclear girdin expression exhibited a shorter OS (Fig. 5*C*) and a similar PFS (Fig. 5*D*) compared with patients who had low nuclear girdin expression. Nuclear girdin expression was demonstrated to be an independent risk factor for OS (Table 3).

TABLE 7. *Relationship between clinicopathological characteristics and combined cytoplasmic/nuclear girdin expression status in IDC patients*

		Girdin (cytoplasm and nucleus), *n* (%)			
Histologic type	Cases	Others[a]	C-high/N-high	r_s	*P*
Age				−0.010	0.817
<50	247	198 (80.2)	49 (19.8)		
≥50	268	217 (81.0)	51 (19.0)		
Tumor size[b]				−0.058	0.206
≤2 cm	133	102 (76.7)	31 (23.3)		
>2 cm	336	275 (81.8)	61 (18.2)		
Histologic grade[b]				0.047	0.294
I	32	29 (90.6)	3 (9.4)		
II	385	306 (79.5)	79 (20.5)		
III	66	52 (78.8)	14 (21.2)		
Lymph node status[a]				0.021	0.633
Negative	203	166 (81.8)	37 (18.2)		
Positive	306	245 (80.1)	61 (19.9)		
ER status[b]				0.090	0.042*
Negative	166	142 (85.5)	24 (14.5)		
Positive	344	268 (77.9)	76 (22.1)		
PR status[b]				0.111	0.012*
Negative	143	125 (87.4)	18 (12.6)		
Positive	366	284 (77.6)	82 (22.4)		
HER2 status[b]				−0.011	0.807
− ~ +	382	306 (80.1)	76 (19.9)		
++ ~ +++	127	103 (81.1)	24 (18.9)		
Ki-67 status[b]				0.106	0.017*
Negative	130	114 (87.7)	16 (12.3)		
Positive	374	292 (78.1)	82 (21.9)		
Recurrence or metastasis[b]				0.081	0.110
No	317	261 (82.3)	56 (17.7)		
Yes	78	58 (74.4)	20 (25.6)		

$n = 515$. Others: C-high/N-low or C-low/N-low or C-low/N-high. [b]Some data are missing. *$P < 0.05$, Spearman's rank correlation test.

Combination analysis of cytoplasmic and nuclear girdin expression was an independent prognosis factor for both OS and PFS

Based on these results, we concluded that both cytoplasmic and nuclear girdin expression had impacts on patients' survival. Therefore, we divided the overall population into four subgroups according to the subcellular expression status of girdin in the following studies (C-low/N-low, n = 209; C-high/N-low, n = 68; C-high/N-high, n = 77; and C-low/N-high, n = 44). The C-high/N-high subgroup exhibited the worst OS compared with the other 3 subgroups. The C-high/N-low subgroup showed a much longer OS than the C-high/N-high subgroup. Similarly, the C-low/N-high subgroup patients exhibited a better OS than the C-high/N-high subgroup. These observations indicated that an accurate prediction of breast cancer prognosis was not only dependent on cytoplasmic girdin but also nuclear expression. In addition, as shown in Fig. 5*F*, the C-high/N-high subgroup exhibited a shorter PFS than the C-low/N-high subgroup, whereas no difference was observed in PFS between the C-high/N-high and C-high/N-low groups. This finding revealed that the prognostic ability of cytoplasmic girdin in PFS analysis was superior to nuclear girdin, which was in line with the results of Table 3.

Because patients with the C-high/N-high subtype showed the worst OS and no significant difference was observed among the other 3 subgroups, patients were redivided into two groups (C-high/N-high and others). We noticed that the C-high/N-high phenotype was positively associated with ER status, PR status, and Ki-67 status (**Table 7**). In addition, both OS and PFS of patients with C-high/N-high were shorter than that of patients of other subtypes (Fig. 5*G*, *H*).

Moreover, C-high/N-high patients had worse OS and PFS in the ER$^-$ subtype (**Fig. 6*A*–*D***), HER2$^+$ subtype (Fig. 6*E*, *F*) and Ki-67$^-$ subtype (Fig. 6*K*, *L*). C-high/N-high was associated with a shorter OS in the HER2$^-$ and Ki-67$^+$ subtype groups (Fig. 6*G*–*J*). C-high/N-high patients also had a much shorter OS in both luminal (especially luminal A subtype) and nonluminal (especially triple negative) subtypes (**Fig. 7**). Notably, because all patients with HER2 overexpression (n = 45) in our study belonged to the Other subgroup, a survival analysis of this subtype was not performed.

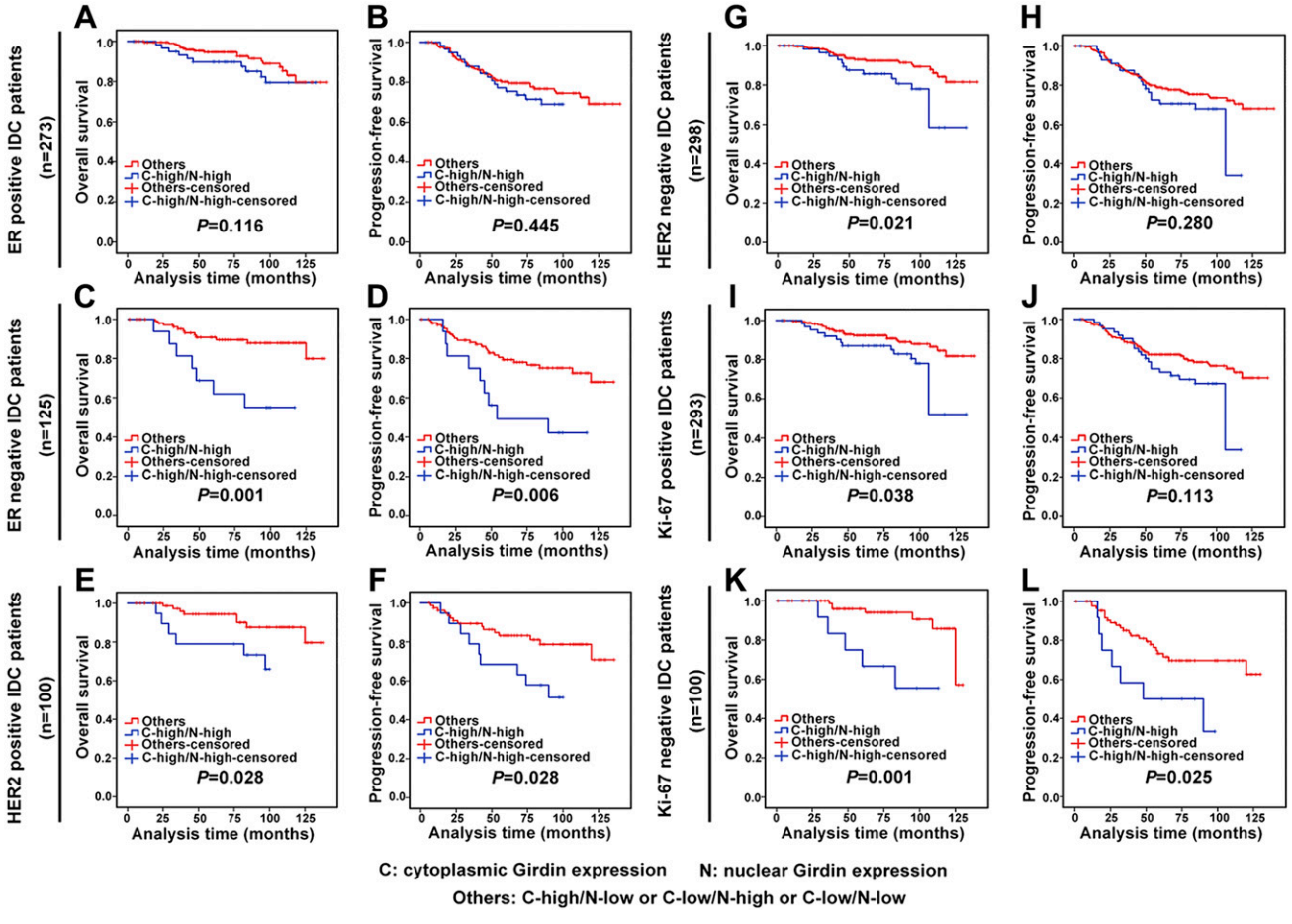

Figure 6. Prognosis analysis of patients with a combination of cytoplasmic and nuclear girdin expression in different subgroups. *A*, *B*) ER$^+$ patients with C-high/N-high exhibited a similar OS and PFS compared with others. Patients with C-high/N-high indicated a shorter OS and PFS in the ER$^-$ (*C*, *D*) and HER2$^+$ (*E*, *F*) subgroups. HER2$^-$ (*G*, *H*) and Ki-67$^+$ (*I*, *J*) patients with C-high/N-high exhibited a shorter OS and a similar PFS compared with other subtypes. *K*, *L*) Patients with C-high/N-high indicated a shorter OS and PFS in Ki-67$^-$ breast cancer. C, cytoplasmic girdin expression; N, nuclear girdin expression; Others, C-high/N-low, C-low/N-high, or C-low/N-low.

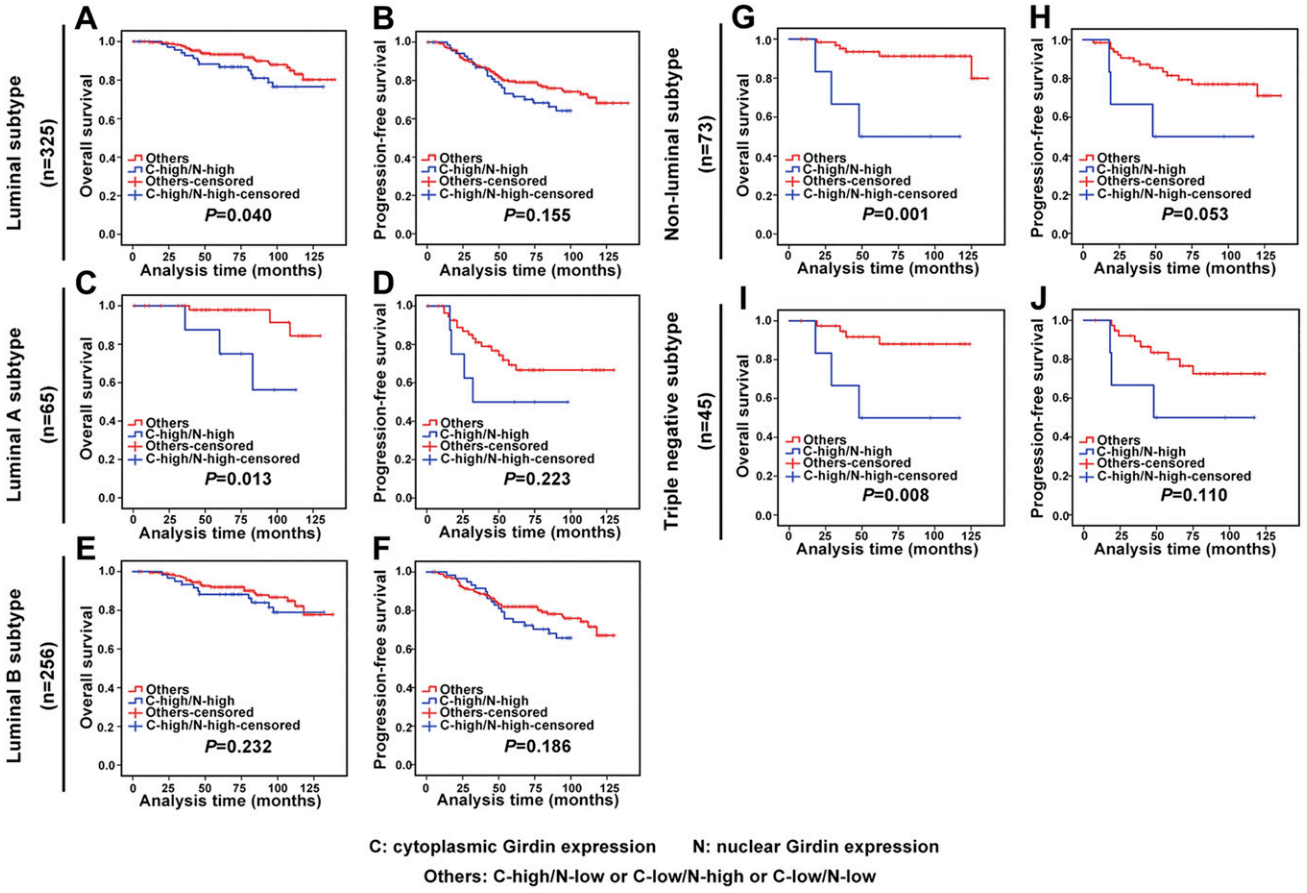

Figure 7. Prognosis analysis with combination of cytoplasmic and nuclear girdin expression in different molecular subtypes. Patients with luminal (*A*, *B*) or luminal A (*C*, *D*) breast cancer with C-high/N-high exhibited a shorter OS and a similar PFS compared with Others. *E*, *F*) In patients with the luminal B subtype, no difference was observed in OS or PFS between those with C-high/N-high and Others. Patients with nonluminal (*G*, *H*) or triple-negative (*I*, *J*) breast cancer with C-high/N-high exhibited a shorter OS and a similar PFS compared with Others . C, cytoplasmic girdin expression; N, nuclear girdin expression; Others, C-high/N-low, C-low/N-high, or C-low/N-low.

Most important, the prognostic value of combined cytoplasmic/nuclear girdin expression was evaluated by univariate and multivariate analysis. The results indicated that C-high/N-high was an independent risk factor for both OS and PFS in IDC patients (Table 2). All these findings indicate that combined analysis of cytoplasmic and nuclear girdin is a better prognostic tool than either alone. We noted that the histologic grade and expression of ER, PR, and HER2 had no effect on patients' survival (Supplemental Fig. 4).

DISCUSSION

Our study provided solid evidence that girdin attaches to chromatin and interacts with topo-IIα in the nucleus and that it has nuclear distribution in human breast cancer tissues, all of which confirms its nuclear localization. Topo-IIα is essential for survival of proliferating cells (38, 39). Our new finding suggests that girdin probably has a critical function in the nucleus with topo-IIα, indicating a new direction for research.

The clinical impact of girdin corresponded to its subcellular distribution. Our results demonstrated that nuclear girdin expression was an independent risk factor for OS, whereas cytoplasmic girdin was an independent risk factor for PFS. In fact, studies in animal models and cell lines have shown that the interaction between girdin and its proteins controls cell migration (25, 31, 40). Given that acquisition of the elevated ability to migrate/invade is a fundamental process of development and progression of cancer, it is rational that high cytoplasmic girdin expression is associated with breast cancer progression and serves as an independent prognosis factor for PFS. In addition, we speculated that girdin in the nucleus regulates the activity of cell proliferation– and apoptosis-related genes. This effect may partly explain the prognostic significance of nuclear girdin in OS, although the underlying mechanism is unknown. Our results were inconsistent with those in 2 previous studies. Dunkel *et al.* (41) proposed that both cytoplasmic and nuclear expression indicate a worse recurrence-free survival, whereas the results of Peng *et al.* (42) showed that neither cytoplasmic nor nuclear girdin exhibits an association with patient survival. Unfortunately, the samples enrolled in those two studies were limited, and the confidence intervals in the Cox proportional

regression analysis were both wide. In addition, their studies did not distinguish the individual prognostic impact of cytoplasmic and nuclear girdin.

Most important, the results of our combination analysis of cytoplasmic and nuclear girdin expression predicted patients' outcome more accurately than either alone based on the following evidence. First, the prognostic effect of cytoplasmic and nuclear girdin was complementary. OS of C-high/N-high patients was much shorter than that of C-high/N-low patients. Similarly, C-high/N-high patients had much shorter OS and PFS than did C-low/N-high patients. Second, C-high/N-high patients showed a worse outcome in distinct molecular subtypes, including luminal A and triple-negative subtypes, whereas the prognostic value of cytoplasmic girdin was limited to the luminal A subtype. Third, neither cytoplasmic nor nuclear girdin expression was related with prognosis in ER^- and $HER2^+$ patients (data not shown). However, C-high/N-high patients exerted a worse prognosis in those two subgroups. Fourth, the combination of cytoplasmic and nuclear girdin expression was an independent risk factor for both OS and PFS in our IDC cohort.

Although the present study showed that ER, PR, and HER2 had no effect on the survival of patients in our cohort, multiple studies have found that ER^+ patients have a better OS and PFS than do ER^- cases, and the triple-negative subtype has the worst outcomes (43, 44). Furthermore, $HER2^+$ patients have a better prognosis than $HER2^-$ patients because of targeted chemotherapy (45, 46). The possible reason for this inconsistency may be that our data collection period spanned the time when targeted chemotherapy was introduced and we had a limited number of patients. A large multicenter study would be preferable.

In conclusion, our study demonstrated for the first time that the prognostic effect of girdin in breast cancer depended on its subcellular localization. A combination of cytoplasmic and nuclear expression was necessary for a more precise prognosis. FJ

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AUTHER CONTRIBUTIONS

F. Gu, L. Fu, and Y. Ma conceived of and designed the study; H. Zhang, F. Yu, and F. Qin acquired the data; H. Zhang, F. Yu, and F. Qin performed the experiments; F. Yu and F. Qin analyzed the data; H. Zhang, Y. Shao, W. Chong, Z. Guo performed the statistical analyses; Y. Ma wrote the manuscript; H. Zhang and X. Liu prepared the tables and figures; and all authors reviewed the manuscript.

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