Co-expression and significance of Dok2 and Ras p21 protein activator 1 in breast cancer

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Abstract. Docking protein 2 (Dok2) and Ras p21 protein activator 1 (RASA1) are tumor suppressors which have been identified in numerous solid tumors; however, the association between their expression in breast cancer and patient prognosis remains unclear. A total of 285 consecutive patients diagnosed histopathologically with breast cancer who underwent surgery at Jingzhou Central Hospital were selected for the present study. Dok2 and RASA1 protein were explored using histopathology and western blotting techniques, and the association of patient prognosis with clinicopathological parameters was investigated using univariate and multivariate analyses. Weak expression of Dok2/RASA1 was associated with poorly differentiated breast adenocarcinomas; negatively expressed Dok2 and RASA1 were associated with increased tumor size, a higher proportion of axillary lymph node metastasis and later clinical staging. Additionally, Dok2 and RASA1 expression were associated with disease-free survival of patients with breast cancer. As indicated by Cox's regression analysis, Dok2 and RASA1 expression and the high proportion of axillary lymph node metastasis served as significant independent predictors for the recurrence of breast cancer. The results of the present study suggested that combined Dok2 and RASA1 negative expression may serve as an independent prognostic factor for patients following breast cancer surgery.

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Introduction

The American Cancer Society stated at the 2014 American Society of Clinical Oncology annual meeting that breast, lung and colon cancer were the most common types of cancer observed in females; breast cancer exhibited the highest incidence (29%) and second highest mortality rate (15%) (1). It is reported that China exhibits one of the fastest growing incidences of breast cancer; increasing in recent years at 3% annually, breast cancer has become the leading cause of mortality in urban females in China (2). Despite marked progress in long-term survival, early diagnosis and treatment of breast cancer, the prognosis of patients with advanced cancer remains poor and heterogeneous (3). The earlier the diagnosis, the better the prognosis for the patient with breast cancer. Although there have been numerous biological markers identified to assist breast cancer diagnosis including Her2/neu, estrogen receptor (ER) and progesterone receptor (PR) (4-6), the identification of further biological markers is required urgently.

Docking protein 2 (Dok2) is a member of the DOK adaptor protein family that functions in feedback loops to modulate tyrosine kinase signaling, involving a number of tyrosine kinase receptors including epidermal growth factor receptor, platelet-derived growth factor receptor, c-Kit, Tie2 and human epidermal growth factor receptor 2 (Her2)/neu (7,8). A previous study demonstrated the clinical significance of Dok2 in the prognostic evaluation of patients with gastric cancer (9). A previous study demonstrated that Dok2 may potentially be used as a marker of poor prognosis in patients with colorectal cancer following curative resection (10).

Ras p21 protein activator 1 (RASA1) is a mediator between Ras-GTP and Ras-GDP and may decrease cellular proliferation through the Ras/rapidly accelerated fibrosarcoma/mitogen-activated protein kinase kinase/extracellular-signal-regulated kinase pathway (11,12). Previous studies have identified that RASA1 may be a potential tumor suppressor (13,14).

The aim of the present study was to assess whether Dok2 and RASA1 are dysregulated in breast cancer using analytical clinicopathological features and their potential value in the prognosis of patients with breast cancer. The results of the present study demonstrated that downregulation of Dok2 and RASA1 in the tissues was associated with clinicopathological features, suggesting that they may serve as independent prognostic factors for patients following surgery.

Materials and methods

Patients. Between October 2008 and March 2013, a total of 285 patients, histopathologically diagnosed with breast cancer, underwent surgery at Jingzhou Central Hospital (Jingzhou, China). Following surgery, patients were followed up every 3 months and administered appropriate clinical examinations. A total of 4 frozen samples (N1-N4) selected from the 285 patients were analyzed using western blotting. The average patient age was 54.8 (range, 25-87 years). The Ethics Committee of Yangtze University approved the present study protocol and all patients provided written informed consent.

Immunohistochemical staining. Dok2 and RASA1 were detected using immunohistochemical staining as described previously (10). The 3.0 μm breast cancer tissue and normal breast mucosa sections were heated at 12˚C for 20 min in EDTA-Tris buffer, pH 9.0, for antigen retrieval following deparaffinization in xylene and dehydration in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections with 30 ml/l H_2O_2 for 20 min. Following incubation with a primary mouse anti-Dok2 (dilution 1:200, sc-17830; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or a mouse anti-RASA1 (dilution 1:200, ab-40677; Abcam, Cambridge, UK) monoclonal antibody at 4˚C overnight, staining was performed using the labeled streptavidin-biotin method. Negative controls of immunohistochemical reactions were established through omission of the primary antibody. Lymphocytes were used as positive control. Dok2 and RASA1 staining was judged to be positive when the cancer cells in the section demonstrated immunoreactivity to Dok2 and RASA1. All slides were assessed independently by two pathologists and any disagreements were resolved by consensus. Pathologists were blinded to the clinicopathological data.

Western blot analysis. Proteins of tissues were resolved by SDS-PAGE (10% gels) and transferred onto a polyvinylidene membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 3% fat-free milk dissolved in PBS-T, and incubated with antibodies against RASA1 (1:500 dilution, ab-40677; Abcam), Dok2 (1:500 dilution, sc-17830; Santa Cruz Biotechnology) and β-actin (1:1,000 dilution, sc-47778; Santa Cruz Biotechnology) overnight at 4˚C. Next, an appropriate secondary antibody (dilution 1:5,000, cat. nos. BA1075 and BA1055, anti-mouse or anti-rabbit IgG, respectively; Wuhan Boster Biological Technology, Ltd., Wuhan, China) was applied for 1 h at room temperature. Immunoreactivity was detected using an enhanced chemiluminescent kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and analyzed with a GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Table I. Association between Dok2 expression and various clinicopathological parameters.

	Dok2 expression			
Parameter	Positive	Negative	χ^2	P-value
All cases	94	191		
Age, years			0.093	0.76
≤55	52	102		
>55	42	89		
Tumor size, cm			6.131	0.013
≤2	56	84		
>2	38	107		
LN metastasis			8.424	0.015
No	56	79		
Yes	36	105		
Unknown	2	7		
Histological grade			7.804	0.020
≤II	57	83		
>II	33	100		
Unknown	4	8		
Clinical stage			9.106	0.011
I	50	66		
II	27	79		
III	17	46		
ER			9.016	0.011
Negative	57	82		
Positive	32	101		
Unknown	5	8		
HER-2			5.512	0.064
Negative	33	75		
Positive	51	109		
Unknown	10	7		
Tumor type			0.085	0.771
IDC	80	160		
Non- IDC	14	31		
Molecular subtype			5.282	0.022
Triple negative	17	59		
Other	77	132		

Non-IDC is invasive lobular carcinoma, mucinous or colloid carcinoma, medullary carcinoma, metaplastic carcinoma. LN, lymph node; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; Dok2, docking protein 2; IDC, invasive ductal carcinoma.

Statistical analysis. Associations between Dok2 and RASA1 expression and various clinicopathological parameters were evaluated using the χ^2 and Fisher's exact probability test. Prognostic variables were assessed using a log-rank test and disease-free survival rate (DFS) was analyzed using the Kaplan-Meier estimator method. In the multivariate analysis, a Cox's proportional hazard model was employed. $P<0.05$ was considered to indicate a statistically significant difference. The

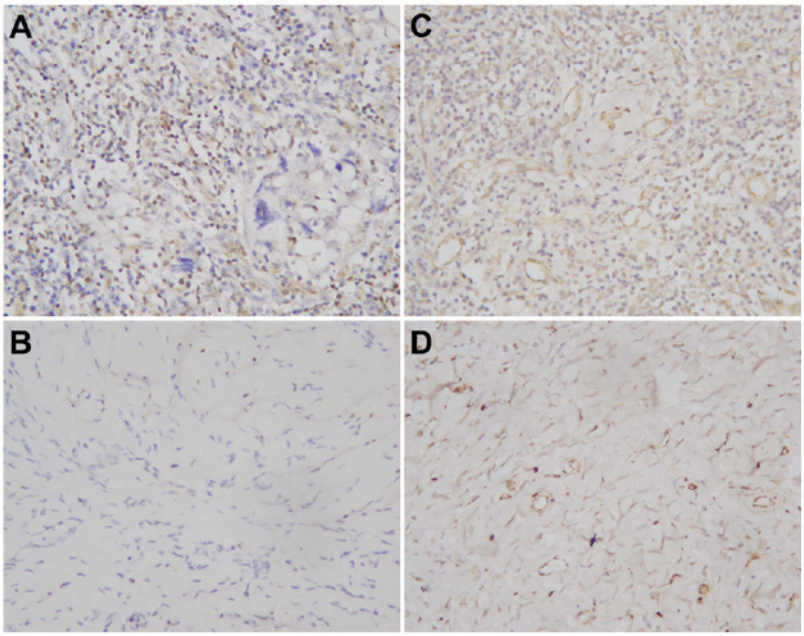

Figure 1. Immunostaining for Dok2 and RASA1 in breast cancer tissues (magnification, x200). (A) Dok2 immunoreactivity in moderately differentiated breast adenocarcinoma. (B) Dok2 immunoreactivity in poorly differentiated breast adenocarcinoma. (C) RASA1 immunoreactivity in moderately differentiated breast adenocarcinoma. (D) RASA1 immunoreactivity in poorly differentiated breast adenocarcinoma. RASA1, Ras p21 protein activator 1; Dok2, docking protein 2.

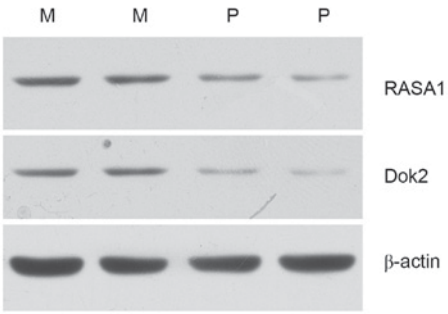

Figure 2. Western blot analysis for RASA1 and Dok2 expression in a total of 4 samples (2 M and 2 P). β-actin was used as a reference. M, moderately differentiated adenocarcinoma; P, poorly differentiated adenocarcinoma; RASA1, Ras p21 protein activator 1; Dok2, docking protein 2.

statistical analyses were performed using SPSS (version 22.0; IBM Corp., Armonk, NY, USA).

Results

Immunohistochemical tissue staining for Dok2 and RASA1. Dok2 and RASA1 staining was primarily observed in the nuclei and cytoplasm of the breast tumor cells. Additionally, 94 (33.0%) patients exhibited positive levels of Dok2, with decreased Dok2 immunostaining intensity observed in the breast cancer tissue samples diagnosed as poorly differentiated adenocarcinoma compared with the remaining moderately differentiated adenocarcinoma samples (Fig. 1A and B). RASA1 demonstrated comparable staining characteristics, with 89 (31.2%) of breast tumor samples exhibiting positive levels, while presenting as markedly weaker in poorly differentiated adenocarcinoma compared with moderately differentiated adenocarcinoma (Fig. 1C and D).

Expression of Dok2 and RASA1 protein in breast cancer determined using western blot analysis. The results of the western blot analysis were consistent with the results of the immunohistochemical staining. Dok2 and RASA1 expression was markedly decreased in two poorly differentiated adenocarcinoma samples compared with two moderately differentiated adenocarcinoma samples (Fig. 2).

Association between Dok2 expression and clinicopathological parameters. All breast cancer samples were grouped as either Dok2-positive or -negative. Notably, patients with Dok2-negative breast cancer exhibited poor histological differentiation and increased tumor size. The positive group exhibited an increased proportion of axillary lymph node metastasis, later clinical staging and was associated with the expression of ER. No significant differences in other clinical characteristics including age, pathological type and expression of HER-2 were identified (Fisher's exact test, P>0.05; Table I).

Association between RASA1 expression and clinicopathological parameters. The samples were grouped as RASA1-positive or -negative. Notably, the patients with RASA1-negative breast cancer exhibited poor histological differentiation and increased tumor size. The RASA1-positive group exhibited an increased proportion of axillary lymph node metastasis, later clinical staging and was associated with the expression of ER. No significant differences in other clinical characteristics including age, pathological type and expression of HER-2 were identified (Fisher's exact test, P>0.05; Table II).

Association between Dok2/RASA1 expression and clinical outcome. Disease relapse following surgery was diagnosed in 84/285 patients (29.5%), with a median time to relapse of

Table II. Association between RASA1 expression and various clinicopathological parameters.

	RASA1 expression			
Parameter	Positive	Negative	χ^2	P-value
All cases	89	196		
Age, years			0.288	0.592
≤55	46	108		
>55	43	88		
Tumor size, cm			5.496	0.019
≤2	56	94		
>2	33	102		
LN metastasis			8.092	0.017
No	53	82		
Yes	33	102		
Unknown	3	9		
Histological grade			8.334	0.016
≤II	55	85		
>II	31	102		
Unknown	3	9		
Clinical stage			8.023	0.018
I	44	72		
II	34	75		
III	11	52		
ER			9.088	0.011
Negative	53	86		
Positive	30	103		
Unknown	6	7		
HER-2			3.666	0.160
Negative	28	80		
Positive	53	107		
Unknown	8	9		
Tumor type			0.136	0.712
IDC	76	164		
Non-IDC	13	32		
Molecular subtype			4.996	0.025
Triple negative	16	60		
Other	73	136		
Dok2			8.377	0.004
Negative	49	142		
Positive	40	54		

Non-IDC is invasive lobular carcinoma, mucinous or colloid carcinoma, medullary carcinoma, metaplastic carcinoma. RASA1, Ras p21 protein activator 1; LN, lymph node; ER, estrogen receptor; HER-2, human epidermal growth factor receptor 2; IDC, invasive ductal carcinoma; Dok2, docking protein 2.

19.2 months. DFS was decreased in patients with Dok2-negative tumors compared with Dok2-positive (P=0.007, log-rank test; Fig. 3A). Additionally, the group without detectable RASA1 expression was markedly associated with decreased DFS among 196 patients (P=0.026, log-rank test; Fig. 3B).

Table III. Multivariate independent prognostic factor analyses of overall survival in 285 patients with breast cancer.

Parameters	HR	95% CI	P-value
Tumor size (≤2 cm/>2 cm)	0.915	0.645-1.328	0.725
LN metastasis (no/yes)	1.233	0.815-1.789	0.005
Histological grade (≤II/>II)	1.456	0.976-2.024	0.023
ER (-/+)	0.768	0.489-1.115	0.185
Dok2 (-/+)	0.454	0.297-0.735	0.001
RASA1 (-/+)	0.625	0.484-1.016	0.018

HR, hazard ratio; CI, confidence interval; LN, lymph node; ER, estrogen receptor; Dok2, docking protein 2; RASA1, Ras p21 protein activator 1.

Comparing the association between Dok2 or RASA1 expression with patient outcome, DOK2 and RASA1 negative expression was associated with the poorer outcome [Dok2 (-) RASA1 (-) 78.0%, Dok2 (+) RASA1 (+) 22.0%, P<0.001, log-rank test] (Fig. 3C). These results indicated a statistically significant association between Dok2/RASA1 downregulation and poorer survival rate.

Following the multivariate Cox's proportional hazard model results, it was identified that decreased Dok2 (HR, 0.454; 95% CI, 0.297-0.735; P=0.001) and RASA1 (HR, 0.825; 95% CI, 0.584-1.216; P=0.018) expression were independent prognostic factors for DFS in patients with breast cancer. In addition, the proportion of axillary lymph node metastases and histological grade were associated with the prognosis of breast cancer in which the high node metastasis was the most effective in DFS (HR, 1.233; 95% CI, 0.815-0.1.789; P=0.005). Although the ER and tumor size were associated with decreased Dok2 and RASA1 expression, the multivariate analysis indicated that neither were independent prognostic factors in breast cancer (Table III).

Discussion

Breast cancer is the most common type of cancer and the second leading cause of cancer-associated mortality among females in Asia, accounting for 39% of all breast cancers diagnosed worldwide (15). Although marked progress has been made in treatment strategy, the survival rate of patients with late-stage breast cancer remains poor. Therefore, research into appropriate tumor markers for early diagnosis of breast cancer is urgently required.

The tumor suppressor gene Dok2 has been identified in lung cancer (16), acute leukemias (17), chronic myelomonocytic leukemia (18), and gastric and colorectal cancers (19). Additionally, Dok2 acts as a marker of poor prognosis in patients with colorectal cancer and gastric adenocarcinoma following curative resection (9,10). Dok2 inhibits epidermal growth factor receptor-mutated lung adenocarcinoma in mouse models (20). Loss of Dok2 induces chemotherapy resistance by decreasing the level of apoptosis in response to treatment (21). Although Dok2 was identified as a cancer marker using the plasma antibody test in breast cancer (22), its expression in

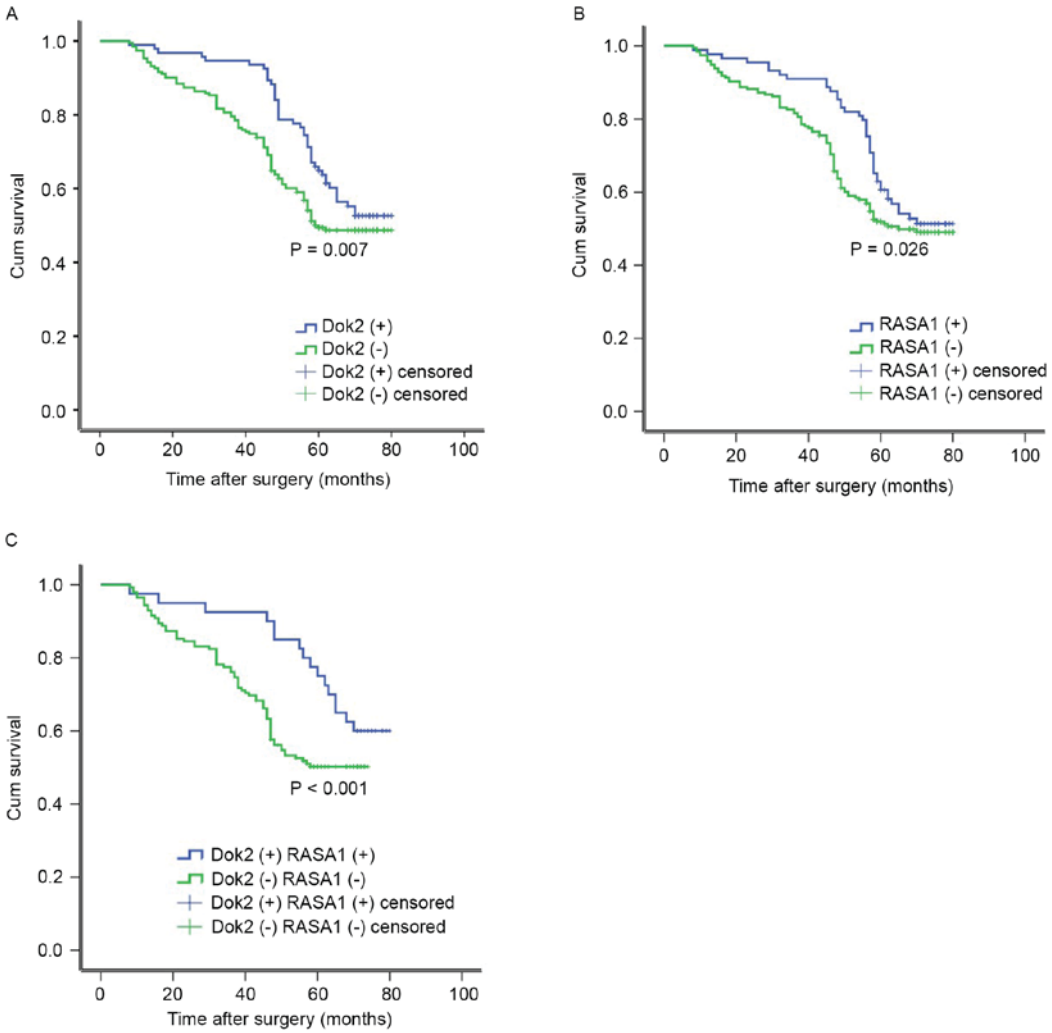

Figure 3. Kaplan-Meier estimator analyses of disease free survival according to: (A) Dok2 expression (P=0.007, log-rank test); (B) RASA1 expression (P=0.026, log-rank test); (C) Dok2 and RASA1 expression (P<0.001, log-rank test). RASA1, Ras p21 protein activator 1; Dok2, docking protein 2; cum, cumulative.

breast cancer and its association with clinicopathological features require investigation.

Ras, a small GTP-binding protein that is frequently mutated in human cancers, is regulated by Ras GTPase-activating proteins (RasGAPs); inactivation of RasGAPs may increase the risk of tumor development (23). RASA1 (a GTPase-activating protein), also called p120RasGAP, was the first RasGAP protein to be identified. In addition to numerous biological roles including actin filament polymerization, vascular development, cellular apoptosis and cell motility (24,25), the role of RASA1 as a tumor suppressor has gained increased attention and research time. RASA1 was first identified as a tumor suppressor in the acute myelogenous tumor line HL-60 following microarray-based comparative genome hybridization studies in 2003 (26) prior to being observed in breast cancer (12,14,27), liver cancer (28,29), colorectal cancer (11,13,30-32), lung cancer (33,34), prostate cancer (35,36), cutaneous squamous cell carcinoma (37), gastric cancer (38), acute lymphoblastic leukemia (39), spinal cancer (40), papillary thyroid carcinoma (41), gastroenteropancreatic neuroendocrine (42) and pancreatic cancer (43) in succession. Dok2 may upregulate RASA1 expression and the two were associated with the tumor gene Ras (44).

The present study investigated the association between Dok2/RASA1 expression and the clinicopathological features of breast cancer. Using immunohistochemistry and western blot analysis, it was revealed that weak expression of Dok2/RASA1 was associated with poorly differentiated breast adenocarcinomas. Further results indicated that negative expression of Dok2/RASA1 was associated with increased tumor size, increased rate of lymph node metastasis and later clinical staging. Absence of Dok2 or RASA1 may lead to Ras/extracellular-signal-regulated kinase signaling cascade activation, resulting in abnormal cell cycle processes (45,46). Additionally, the negative expression of RASA1 was associated with negative Dok2 expression (χ^2=8.377, P=0.004), indicating that RASA1 may regulate Dok2 expression (44); however, further studies are required to support this. Dok2 and RASA1 are both tumor suppressors and, combined, their detection may improve diagnosis sensitivity in breast cancer.

Survival analysis indicated that Dok2 and RASA1 may be independent prognostic factors for DFS in patients with breast cancer, and combined negative Dok2/RASA1 expression was the most promising unfavorable prognostic factor in DFS, offering therapeutic potential for diagnosis. Cox's regression analysis was applied to identify significant prognostic factors

alongside Kaplan-Meier estimator analysis. Results of the present study revealed that downregulation of Dok2 and RASA1 are associated with poor outcome and relapse of breast cancer; the DFS hazard ratio for Dok2 was 0.454 ($P<0.01$) and the DFS hazard ratio for RASA1 was 0.625 ($P<0.05$), indicating that patients with Dok2- or RASA1-positive cancer have a 54.6 and 37.5% decreased risk of relapse compared with patients negative for Dok2 or RASA1. The results of the present study also revealed that lymph node metastasis and histological grade may be the significant prognostic factors; however, no significant association with ER was identified (47).

In conclusion, the results of the present study demonstrated that combined downregulation of Dok2 and RASA1 is associated with breast cancer progression, recurrence and poor survival rate. Therefore, Dok2/RASA1 combined detection may be an effective predictor of prognosis and a novel therapeutic target for patients with breast cancer.

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