Title: Loss of FAT1 during the progression from DCIS to IDC and predict poor clinical outcome in breast cancer

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Abstract

FAT1 and β -catenin are important tumor regulatory factors. The aim of this study was to detect the possible disparity in their expression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC), and to explore its correlation with clinicopathological factors. We used immunohistochemistry to detect protein expression of FAT1 and β -catenin in breast cancer tissues from 113 cases of DCIS and 149 cases of IDC. As compared with DCIS, expression of FAT1 and β -catenin were significantly decreased in IDC ($P < 0.05$). In addition, our study also revealed a correlation between their expression and some clinicopathological factors. We found that FAT1 expression was associated with nuclear grade and comedonecrosis ($P < 0.05$) in DCIS, whereas FAT1 expression showed significant variation with histological grade and LN status ($P < 0.05$) in IDC. Similar associations were observed in the β -catenin subgroup. Furthermore, expression of FAT1 and β -catenin were correlated with each other in DCIS and IDC ($P < 0.05$). FAT1(-), β -catenin(-) or FAT1(-)/ β -catenin(-) may indicate worse DFS and OS in breast cancer ($P < 0.05$). This study suggests that loss of FAT1 and β -catenin are associated with breast cancer progression, aggressive behavior and poor prognosis. FAT1 alone or together with β -catenin might be a valuable biomarker in predicting the prognosis of patients with breast cancer.

Keywords breast cancer, ductal carcinoma in situ, invasive ductal carcinoma, FAT1, β -catenin

1. Introduction

The human FAT gene family consists of the FAT1, FAT2, FAT3 and FAT4 genes. Dunne et al reported complete coding sequence of FAT1 in 1995 (Dunne et al., 1995). Human FAT1 gene encodes

large protein with extracellular Cadherin repeats and a Laminin-G-like domain followed by 4 EGF-like domains. Ena/VAPS-binding to FAT1 induces actin polymerization at lamellipodia and filopodia to promote cell migration (Moeller et al., 2004), while Scribble-binding to FAT1 induces phosphorylation and functional inhibition of YAP1 to suppress cell growth (Skouloudaki et al., 2009). In cancer, FAT1 appears to act as tumor oncogenic or suppressive in a context-dependent manner (Katoh, 2012). Functional in vitro studies revealed that FAT1 acts as a regulator of oncogenic pathways in glioma cell lines (Dikshit et al., 2013). Neumann et al revealed an aberrant expression predominantly in mature BCP-ALL and thymic T-ALL and a high rate of FAT1 mutations in T-ALL (Neumann et al., 2014). Valletta et al showed a significant correlation of FAT1 expression with tumor stage and proliferation. Suppression of FAT1 expression by short hairpin RNA impaired proliferation and migration as well as apoptosis resistance of hepatocellular carcinoma (HCC) cells in vitro (Valletta et al., 2014). In contrast, frequent loss of heterozygosity of FAT1 has been described in astrocytic tumors (Chosdol et al., 2009). In cholangiocarcinoma, immunohistochemistry demonstrated reduced FAT1 expression at the normal membranous location (Settakorn et al., 2005). FAT1 is preferentially downregulated in invasive breast cancer (Kwaepila et al., 2006).

β -catenin plays important roles in mammary development and tumorigenesis through control of cell adhesion, signal transduction and gene transactivation (Incassati et al., 2010). At the plasma membrane, β -catenin functions as a cellular junction protein and regulates mammary epithelial integrity via interaction with cadherins. In the nucleus, it binds Lef/T-cell factor (TCF) and acts as a transcription activator to stimulate expression of target genes like c-Myc and cyclin D1. Morris et al. (Morris et al., 2013) found genetic, functional and mechanistic data that identify the protocadherin FAT1 gene at 4q35 as a tumor suppressor that, when inactivated, leads to aberrant Wnt/ β -catenin signaling in

multiple types of cancer.

A most recent review by Sadeqzadeh et al. (Sadeqzadeh et al., 2014) highlights that we are just beginning to understand the molecular interactions of FAT1, which vary in different tissues and in health and disease. Notably, the biological and clinicopathological value, and prognostic significance of FAT1 protein expression in breast cancer remain lacking, and the relationship of FAT1 and β -catenin has also been rarely investigated in breast tissue. Thus, we conducted this study to correlate the expressions of FAT1 and β -catenin expression in 113 cases of ductal carcinoma in situ (DCIS) and 149 cases of invasive ductal carcinoma (IDC) representing the progression of breast cancer. In addition, their expression were evaluated in a large, well-characterized and clinically annotated series of primary invasive breast carcinomas with a long-term follow-up to assess their biological, clinicopathological and prognostic significance.

2. Materials and methods

2.1. Case selection and clinical information

For immunohistochemistry, 113 cases of DCIS and 149 cases of formalin-fixed and paraffin-embedded surgical samples of breast IDC were collected randomly from 1 January 2004 to 31 December 2006 from the Tianjin Medical University Cancer Institute and Hospital. This was a well-characterized series of patients who had undergone long-term follow-up organized by a single institution. The diagnosis and histopathology results were reviewed independently by three pathologists (L.W., Y.N., and S.L.), using the World Health Organization criteria (Lakhani et al., 2012). All patients were women, ranging in age from 26 to 76 years (mean 50 years); other clinicopathological information is shown in Table 1. None had received neoadjuvant radiation or chemotherapy. The study protocol was approved by the

Human Ethical Committee of Tianjin Medical University Cancer Institute and Hospital. Informed consent was obtained from all patients before their surgery and before examination of the specimens.

2.2. Immunohistochemistry

Immunohistochemistry was performed by using the labelled streptavidin-biotin technique, with antibodies against FAT1, β -catenin, oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67 in all cases. Briefly, tissue sections were deparaffinized and rehydrated with xylene and a series of grades of alcohol. Antigen retrieval was carried out in citrate buffer (pH 6.0) for 2 min in an autoclave, and this was followed by cooling at room temperature for 45 min. After inactivation of endogenous peroxidase with 3% H₂O₂, sections were blocked with 10% normal goat serum for 30 min and then incubated with either anti-FAT1 antibody (1:100 dilution; Sigma) or anti- β -catenin antibody (1:100; Invitrogen) at 4°C overnight, or with either anti-ER antibody (1:200; ZETA), anti-PR antibody (1:200; ZETA), anti-HER-2/*neu* antibody (1:100; Invitrogen) or anti-Ki67 antibody (1:100; Invitrogen) at 37°C for 2 h. Normal mouse serum served as a negative control. After incubation with biotin-conjugated secondary antibody for 20 min at 37°C, and streptavidin-horseradish peroxidase for 20 min at 37°C, colour was developed by incubation with 3,3'-diaminobenzidine tetra-hydrochloride (DAB). The sections were counterstained with haematoxylin. All steps were preceded by rinsing with phosphate-buffered saline (PBS; pH 7.6).

2.3. Staining evaluation

The immunostaining was scored by three different pathologists, who were blinded to patients' clinicopathologic characteristics and outcomes. For each antibody, the location of immunoreactivity, percentage of stained cells, and intensity were determined. The evaluation of each protein expression was determined from the mean of the individual cases. FAT1 was expressed in the cytomembrane

and/or cytoplasm and β -catenin was expressed in the cytomembrane and/or cytoplasm in tumour cells. The staining intensity was categorized into four grades (0 to 3): (0, no reactivity; 1, weak reactivity; 2, moderate reactivity; 3, strong reactivity) and percentage of positive cells (0, 0% of cells reactive; 1, <25% of cells reactive; 2, 25–49% of cells reactive; 3, 50–74% of cells reactive; 4, $\geq$ 75% of cells reactive). Therefore, the sum of the grades of staining intensity and percentage of positive cells resulted in an overall score range of 0–7 for FAT1 and β -catenin (0–2, negative vs. 3–7, positive). ER and PR were considered positive if nuclear staining was present in more than 1 % of the tumor cells, HER2+ was defined as the whole membrane strong staining in $>10\%$ of the tumor cells, and Ki67 status was expressed in terms of percentage of positive cells, with a threshold of 20% of positive cells.

2.4. Statistical analysis

All statistical analyses were carried out using SPSS soft-ware (Version 16.0 for Windows). χ^2 test were performed for group comparisons, and correlations between two variables were evaluated with Spearman's rank correlation test. OS and DFS were conducted using the Kaplan-Meier curves. Prognostic values of variables were estimated by univariate/multivariate Cox proportional hazard ratio models. $P < 0.05$ was considered statistically significant.

3. Results

3.1. FAT1 and β -catenin are expressed in DCIS and IDC

Immunostaining for FAT1 and β -catenin was observed as uniform linear membranous staining in both DCIS and IDC (Fig. 1 a-d). Specifically, positive immunostaining for FAT1 and β -catenin was observed in 51(45.1%) and 65 (57.5%) in DCIS and 45 (30.2%) and 67 (45%) in IDC, respectively (Table 2). Pearson's chi-square tests revealed that the positive proportion rate was significantly higher

in DCIS than in IDC for both FAT1 and β -catenin ($P = 0.013$ and 0.044 , respectively).

Table 1 Patients' demographics and clinicopathologic characteristics in DCIS and IDC.

Characteristic	DCIS(n=113)		IDC(n=149)	
	No.	%	No.	%
Age at diagnosis (years)				
≤ 50	61	54	87	58.4
> 50	52	46	62	41.6
Menopausal status				
Premenopausal	60	53.1	92	61.7
Postmenopausal	53	46.9	57	38.3
Family history				
No	101	89.4	136	91.3
Yes	12	10.6	13	8.7
Tumor size (cm)				
≤ 2	46	40.7	74	49.7
2–5	60	53.1	66	44.3
> 5	7	6.2	9	6
Nuclear grade				
1	21	18.6		
2	48	42.5		
3	44	38.9		
Histological grade				
1			30	20.1
2			61	40.9
3			58	38.9
Lymph node status				
0	113	100	68	45.6
1–3	0	0	41	27.5
4–9	0	0	23	15.4
≥ 10	0	0	17	11.4
ER				
Negative	42	37.2	44	29.5
Positive	71	62.8	105	70.5
PR				
Negative	52	46	67	45
Positive	61	54	82	55
Her-2				
Negative	74	65.5	108	72.5
Positive	39	34.5	41	27.5
Ki67				
< 20	53	46.9	74	49.7

≥20	60	53.1	75	50.3
Chemotherapy				
No	113	100	68	45.6
Yes	0	0	81	54.4
Radiotherapy				
No	57	50.4	64	43
Yes	56	49.6	85	57
Endocrine therapy				
No	46	40.7	54	36.2
Yes	67	59.3	95	63.8

Figure 1 Immunohistochemical staining of FAT1 and β-catenin in DCIS (**a** and **c**) and IDC (**b** and **d**).

Magnification A-D: 400×

Table 2 FAT1 and β-catenin expressions of DCIS compared with IDC.

Characteristic	DCIS(n=113)		IDC(n=149)		χ^2	<i>P</i> -values
	No.	%	No.	%		
FAT1						
Negative	62	54.9	104	69.8	6.171	0.013

Positive	51	45.1	45	30.2		
β-catenin						
Negative	48	42.5	82	55	4.053	0.044
Positive	65	57.5	67	45		

3.2. Associations between FAT1 and β-catenin expressions and clinicopathological parameters

Table 3 shows associations between FAT1 expression and clinicopathological variables. Loss of FAT1 expression in DCIS was associated with biological parameters characteristic of aggressive behavior and poor outcome including higher nuclear grade and comedonecrosis ($P < 0.05$). Loss of FAT1 expression in IDC was also associated with biological parameters characteristic of aggressive behavior and poor outcome including higher histological grade and LN status ($P < 0.05$, Table 4). No significant association was found between positive staining of FAT1 and age, tumor size or hormone receptors ($P \geq 0.05$).

Similar associations were observed in the β-catenin subgroup.

Table 3 Correlations between FAT1 and β-catenin expressions and clinicopathological features in DCIS.

Characteristic	FAT1 expression n(%)			β-catenin expression n(%)		
	Negative	Positive	<i>P</i> -values	Negative	Positive	<i>P</i> -values
Age at diagnosis (years)						
≤50	31(50.8)	30(49.2)	0.349	25(41)	36(59)	0.728
> 50	31(59.6)	21(40.4)		23(44.2)	29(55.8)	
Tumor size (cm)						
≤2	24(52.2)	22(47.8)	0.237	18(39.1)	28(60.9)	0.655
2–5	32(53.3)	28(46.7)		26(43.3)	34(56.7)	
> 5	6(85.7)	1(14.3)		4(57.1)	3(42.9)	
Nuclear grade						
1	6(28.6)	15(71.4)	0.024	1(4.8)	20(95.2)	<0.001
2	28(58.3)	20(41.7)		22(45.8)	26(54.2)	
3	28(63.6)	16(36.4)		25(56.8)	19(43.2)	
Comedonecrosis						
Absence	19(40.4)	28(59.6)	0.009	13(27.7)	34(72.3)	0.007
Presence	43(65.2)	23(34.8)		35(53)	31(47)	
ER						

Negative	26(61.9)	16(38.1)	0.248	21(50)	21(50)	0.213
Positive	36(50.7)	35(49.3)		27(38)	44(62)	
PR						
Negative	33(63.5)	19(36.5)	0.09	27(51.9)	25(48.1)	0.061
Positive	29(47.5)	32(52.5)		21(34.4)	40(65.6)	
Her-2						
Negative	38(51.4)	36(48.6)	0.301	28(37.8)	46(62.2)	0.169
Positive	24(61.5)	15(38.5)		20(51.3)	19(48.7)	
Ki67						
<20	27(50.9)	26(49.1)	0.431	24(45.3)	29(54.7)	0.571
≥20	35(58.3)	25(41.7)		24(40)	36(60)	

Table 4 Correlations between FAT1 and β -catenin expressions and clinicopathological features in IDC.

Characteristic	FAT1 expression n(%)			β -catenin expression n(%)		
	Negative	Positive	<i>P</i> -values	Negative	Positive	<i>P</i> -values
Age at diagnosis (years)						
≤50	59(67.8)	28(32.2)	0.532	47(54)	40(46)	0.769
> 50	45(72.6)	17(27.4)		35(56.5)	27(43.5)	
Tumor size (cm)						
≤2	46(62.2)	28(37.8)	0.095	36(48.6)	38(51.4)	0.062
2–5	50(75.8)	16(24.2)		38(57.6)	28(42.4)	
> 5	8(88.9)	1(11.1)		8(88.9)	1(11.1)	
Histological grade						
1	12(40)	18(60)	<0.001	10(33.3)	20(66.7)	0.027
2	51(83.6)	10(16.4)		36(59)	25(41)	
3	41(70.7)	17(29.3)		36(62.1)	22(37.9)	
Lymph node status						
0	38(55.9)	30(44.1)	0.001	28(41.2)	40(58.8)	<0.001
1–3	29(70.7)	12(29.3)		20(48.8)	21(51.2)	
4–9	20(87)	3(13)		17(73.9)	6(26.1)	
≥10	17(100)	0(0)		17(100)	0(0)	
ER						
Negative	33(75)	11(25)	0.371	26(59.1)	18(40.9)	0.519
Positive	71(67.6)	34(32.4)		56(53.3)	49(46.7)	
PR						
Negative	47(70.1)	20(29.9)	0.933	37(55.2)	30(44.8)	0.966
Positive	57(69.5)	25(30.5)		45(54.9)	37(45.1)	
Her-2						
Negative	73(67.6)	35(32.4)	0.341	57(52.8)	51(47.2)	0.369
Positive	31(75.6)	10(24.4)		25(61)	16(39)	
Ki67						
<20	47(63.5)	27(36.5)	0.097	40(54.1)	34(45.9)	0.811
≥20	57(76)	18(24)		42(56)	33(44)	

3.3. Correlation between the expression of FAT1 and β -catenin in DCIS and IDC

The expression of β -catenin in DCIS was positively correlated with the expression of FAT1 ($P < 0.05$; Table 5).

The expression of β -catenin in IDC was positively correlated with the expression of FAT1 ($P < 0.05$; Table 5).

Table 5 Correlation between the expression of FAT1 and β -catenin in DCIS and IDC.

		FAT1(-)	FAT1(+)	χ^2	P	r	P
DCIS	β -catenin(-)	40	8	27.306	<0.001	0.492	<0.001
	β -catenin(+)	22	43				
IDC	β -catenin(-)	77	5	50.26	<0.001	0.581	<0.001
	β -catenin(+)	27	40				

3.4. Survival analysis

FAT1 expression was assessed using the Kaplan-Meier survival analysis. Loss of FAT1 expression exhibited a statistically significant reduction term for DFS in DCIS and IDC and OS in IDC ($P = 0.015$, 0.018 and 0.042, respectively) as compared to patients with positive FAT1 expression (Fig.2 a d g). These results suggest that FAT1 expression can serve as a marker of DFS and OS outcome in breast cancer patients.

β -catenin expression was assessed using the Kaplan-Meier survival analysis. Loss of β -catenin expression exhibited a statistically significant reduction term for DFS and OS ($P = 0.037$, 0.011 and 0.007, respectively) as compared to patients with positive β -catenin expression (Fig.2 b e h).

Next, we correlated FAT1 and β -catenin expression levels in breast cancer patients. This analysis revealed that the DFS in FAT1(-)/ β -catenin(-) patients was significantly worse ($P = 0.048$ and 0.034 , respectively) as compared to FAT1(+)/ β -catenin(+), FAT1(+)/ β -catenin(-) and FAT1(-)/ β -catenin(+) in DCIS and IDC. Furthermore, FAT1(-)/ β -catenin(-) patients showed significantly reduced OS ($P = 0.025$) as compared to FAT1(+)/ β -catenin(+), FAT1(+)/ β -catenin(-) and FAT1(-)/ β -catenin(+) patients (Fig. 2 c f i).

Figure 2 Kaplan-Meier curves of disease-free survival and overall survival according to FAT1 and

β -catenin expression in DCIS and IDC. *P*-value was calculated by the log-rank test.

The univariate analysis revealed that tumor size, histological grade, lymph node status, FAT1 expression, and β -catenin expression were significantly associated with both DFS and OS. The multivariate analysis revealed that lymph node status and FAT1 expression were independent significant prognostic factors for DFS and OS (Table 6). However, none of tumor size, histological grade, or β -catenin was significant prognostic factor for DFS or OS.

Table 6 Multivariate analysis of prognostic variables for overall survival and disease-free survival.

Variables	OS		DFS	
	<i>P</i> value	HR (95 % CI)	<i>P</i> value	HR (95 % CI)
Tumor size (cm) (≤ 2 vs. > 2)	0.827	1.149 (0.331-3.993)	0.272	1.765 (0.640-4.869)
Histological grade (1 vs. 2–3)	0.574	1.366 (0.461-4.046)	0.639	1.246 (0.497-3.124)
Lymph node (negative vs. positive)	0.037	4.732 (1.100-20.355)	0.025	3.706 (1.177-11.666)
FAT1 expression (negative vs. positive)	0.043	0.120 (0.015-0.935)	0.010	0.070 (0.009-0.534)
β -catenin expression (negative vs. positive)	0.155	0.171 (0.015-1.949)	0.153	0.187 (0.019-1.865)

4. Discussion

FAT1 is a member of cadherin superfamily (Katoh et al., 2006) and was first identified as a tumor suppressor in *Drosophila melanogaster*, acting via the Salvador–Warts–Hippo signaling pathway (Bennett et al., 2006; Reddy et al., 2008). The specific function of FAT1 in cancer development and progression is still under investigation. There are contrasting studies about the role of FAT1 in human cancers, pointing toward a dual role of FAT1 as both an oncogene as well as a tumor suppressor. Here, we found decreased FAT1 expression in IDC tissues compared with DCIS. Furthermore, loss of FAT1 expression correlated with higher grade and LN status in human breast cancer. These findings indicated

that FAT1 inhibit progression to an invasive phenotype in concordance with previous studies (Lee et al., 2012). In HCC and glioma cells, reduced migratory potential upon FAT1 suppression but surprisingly and in contrast to our findings in breast cancer suppressed proliferation (Valletta et al., 2014). In cholangiocarcinoma, FAT1 expression showed an inverse correlation with the proliferation index and loss of membranous FAT1 localization correlated with more aggressive tumor growth (Settakorn et al., 2005). FAT1 is overexpressed in pancreatic adenocarcinomas and ectodomain shedding is recapitulated in vivo leading to increased FAT1 serum levels in some pancreatic cancer patients (Wojtalewic et al., 2014). In addition to its varying effects on proliferation and migration in different cell types, FAT1 is engaged in several other biological functions such as polarity and adhesion or as a regulator of inflammatory pathways in cancer cells (Moeller et al., 2004).

FAT1 has previously been shown to be a potential prognostic marker in breast cancer; however, the associations with clinicopathologic variables and outcome measures reported in different studies are discrepant. We therefore evaluated FAT1 expression in a cohort of breast cancers with known patient clinicopathologic variables, outcome, and treatment. Loss of FAT1 expression was associated with higher nuclear grade, LN status and worse outcome. In addition, FAT1 was associated with absence of comedonecrosis and lower scores of histological grade, which is in accordance with previous studies (Catteau et al., 2012; Kwaepila et al., 2006). These findings may be explained by the tumor suppressor function of FAT1 in breast cancer.

β -catenin plays significant roles in the regulation of mammary development and tumorigenesis through its association with E-cadherin in cell adhesion, regulation of mammary stem cells and Wnt/ β -catenin signaling pathway. Most publications report a cytoplasmic or membranous pattern of β -catenin expression in breast cancer cells, whereas Prasad et al. (Prasad et al., 2007) have shown nuclear

localization. Previously, β -catenin cytoplasmic expression was reported to be associated with worse outcome (Dolled-Filhart et al., 2006; Fanelli et al., 2008), whereas Chung et al. (Chung et al., 2004) have identified no such relationship. Fanelli et al. have reported an association between cytoplasmic expression and ER-positive status, whereas others have found no association (Karayiannakis et al., 2001). Contradictory results are also reported for its associations with tumor size, grade, patient age and lymph node status.

Our results revealed that the expression levels of β -catenin were significantly decreased in breast cancer, which is similar to the findings in the previous study (Dolled-Filhart et al., 2006). Our finding was similar to Bankfalvi's study that reduced level of β -catenin was associated with positive lymph node status (Bankfalvi et al., 1999). Previous studies have found the prognostic values of β -catenin for stage III breast cancer patients (Gillett et al., 2001), and we found β -catenin expression can serve as a marker of DFS and OS outcome in breast cancer patients.

We also observed that positive staining of β -catenin was associated with the expression of FAT1. The following assumptions may explain our result. Morris et al. (Morris et al., 2013) found genetic, functional and mechanistic data that identify the protocadherin FAT1 gene at 4q35 as a tumor suppressor that, when inactivated, leads to aberrant Wnt/ β -catenin signaling in multiple types of cancer. Therefore, FAT1 and β -catenin are important tumor regulatory factors (Figure 3).

Figure 3 Loss of FAT1 and β -catenin are associated with breast cancer progression, aggressive behavior and poor prognosis.

We found patients with loss of FAT1 as well as β -catenin were associated with a poor outcome, meanwhile the DFS and OS of FAT1 positive/ β -catenin positive tumors in our study was significantly longer than that of FAT1 negative/ β -catenin negative tumors. Therefore, it might be helpful to integrate the FAT1 with β -catenin expression status to help predict the prognosis of breast cancer.

There are several limitations to this current study, including the small data set, few cases of patients, no validation in an independent series of cases, categorize the accumulation of FAT1 and β -catenin in breast cancers by immunohistochemical to positive and negative rather than scoring.

Loss of FAT1 and β -catenin in breast cancer indicate poor outcome of patients. FAT1 might be a

valuable biomarker in predicting the prognosis of patients with breast cancer. FAT1 alone or together with β -catenin might be helpful in identifying patients who might benefit from early systemic treatment. The potential mechanism between loss of FAT1 expression and Wnt/ β -catenin pathway activation and the mechanism by which the Wnt/ β -catenin pathway is activated in breast cancer is still unknown. It is possible that through further understanding of the role of Wnt/ β -catenin pathway activation, combination of FAT1 and β -catenin would be a potential therapeutic target for breast cancer.

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