RESEARCH COMMUNICATION

Up-regulation of Human Arrest-defective 1 Protein is Correlated with Metastatic Phenotype and Poor Prognosis in Breast Cancer

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Abstract

Background: Human arrest defective 1 protein (ARD1), as a N-terminal acetyltransferase, has been reported to play a crucial role in tumorigenesis, but the results are somewhat controversial. To explore the clinical and pathological significance of ARD1 in breast tumorigenesis, we analyzed ARD1 status in multiple types of breast disease. Methods: The expression of ARD1 protein was assessed by immunohistochemistry in 356 cases including 82 invasive ductal carcinomas (IDC), 159 fibroadenomas, 66 hyperplasia of mammary glands, 19 inflammatory breast disease, 30 breast cysts, and in 29 postoperative treatment patients. We assessed the relationship of ARD1 protein with clinical and pathological characteristics using $\chi 2$ test. Results: ARD1 protein was observed at 61.0% (50/82), 54.7% (87/159), 37.9% (25/66), 36.8% (7/19) in IDC, fibroadenoma, hyperplasia, and inflammation, respectively, and less than 30.0% for breast cyst. Thus, high ARD1 expression correlated with breast cancer (relative risk = 1.32, $P < 0.005$). Moreover, the level of ARD1 protein in carcinoma patients was distinctly related to lymph node metastasis and ER status, with 94.0% (47/50) as copmpared to 6.0% (3/50) in metastatic and non-metastatic ($P < 0.001$), and 84.0% (42/50) and 16.0% (8/50) for ER + and ER - ($P < 0.01$), respectively. In addition, the level of ARD1 appeared to have potential for evaluation of prognosis in breast cancer patients after postoperative therapy. Conclusions: These results suggest that ARD1 expression may be as a potential target for exploring the mechanism of breast cancer metastasic to lymph nodes and hormone-responsive regulation.

Keywords: Arrest defective 1 protein - estrogen receptor - breast cancer - prognosis

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Introduction

In China, the morbidity of breast cancer has increased progressively and will keep on in the future(Linos et al., 2008; Yang et al., 2003). To explore the novel and underlying mechanisms in breast cancer development will shed more light on diagnosis, treatment and prognosis. Human arrest-defective 1 protein (ARD1) was described as a N-terminal acetyltransferase to catalyze the α- and ε-acetylation of proteins (Arnesen et al., 2005a; Lim et al., 2006; Shin et al., 2009; Kuo et al., 2010b). Previous reports have shown that its role in tumorigenesis has been still controversial (Arnesen et al., 2006; Lim et al., 2006; Shin et al., 2009; Kuo et al., 2010b). As oncogenic function, ARD1 was reported to promote lung cancer cell proliferation by acetylating and activating β-catenin via Wnt/β-catenin pathway (Lim et al., 2006) and to induce apoptosis by RNAi-mediated knockdown of ARD1 in human cervical carcinoma HeLa cells (Arnesen et al., 2006). In contrast, ARD1 has also been shown to suppress tumor cell migration and cell invasion by acetylating and inactivating the myosin light chain kinase (MLCK) in human fibrosarcoma HT1080 cells (Shin et al., 2009) and to suppress tumorigenesis by mTOR-mediated pathway through acetylating and increasing tuberous sclerosis 2 (TSC2) stability in breast cancer (Kuo et al., 2010b). By immunohistochemical assay (IHC) using the self-prepared monoclonal antibodies, Ren et al reported that ARD1 level was significantly related to colorectal cancer, compared 41/50 of ARD1 positive in colorectal cancer tissues to 12/50 in matched normal tissues, and moreover, ARD1 was also high expression in six human colorectal cancer cell lines at both protein and mRNA levels(Ren et al., 2008). Our previous results showed that ARD1 protein was extensively expressed and remarkably higher in multiple types of cancer compared with non-cancerous tissues (Yu et al., 2009a). The results based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry demonstrated ARD1 was positively related to control cell proliferation in MCF-7 cells (Yu et al., 2009b).

In current study, by immunohistochemical staining, we

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further performed the level of ARD1 protein in multiple types of breast diseases and the correlation with clinical and pathological characteristics including histology, metastases, estrogen receptor (ER) or progestogen receptor (PR) status, and primarily, evaluated the role of ARD1 as a prognostic factor in breast cancer patients after postoperative therapy.

Materials and Methods

Patients and tissue samples

356 clinical breast specimens with informed consent were obtained from the patients who visited clinics and underwent partial or whole mastectomy and were histopathologically diagnosed by the professional pathologists of the Clinic Technological Section, First People's Hospital of Yunnan Province between August 2006 and January 2008, and 29 specimens were collected from the patients due to recurrence after primary surgery and treatment. All patients had no history of any other types of malignancies and had not previously accepted any kinds of therapies, including radio-, chemo-, or endocrine therapy except for the patients undergone the second surgical examination. The information about the privacy of patients was removed before performance. The whole procedure to treat the specimens conformed to the consensus statement on use of archival tissues (Clayton et al., 1995).

Tissue processing and immunohistochemistry

Rabbit sera against full-length of recombinant ARD1 was generated and reported previously(Yu et al., 2008), Mouse anti-ER and Mouse anti-PR antibodies were purchased from Maixin-Bio Company (Fuzhou, China). The detailed procedure for tissue processing was described previously (Yu et al., 2009b). Briefly, fresh surgically resected specimens were cut into 1 cm^3 blocks, fixed in formaldehyde for 2 days, dehydrated in graded ethanol solutions for 30 min, immersed into xylene for 30 min, and embedded in paraffin. Four micrometer thick paraffin sections were cut from tissue blocks and immunohistochemical stained with anti-ARD1, anti-ER and anti-PR antibodies diluted at 1:100, respectively, using SP kit (Maixin-Bio, Fuzhou, China) according to the manufacture's protocol. The ARD1 level was scored independently by two trained pathologists (Li and Yang) and the final scores were calibrated using a modified immunohistochemical scoring system as described previously (Bosman et al., 1992; Rau et al., 2003).

In brief, 500 cells from representative tumor fields were evaluated for staining intensity (SI, 0 = negative; 1 = weak, buff yellow; 2 = middle, brown yellow; 3 = strong, brown) and percentage of total immunoreactive cells (PS; 0~100%). For each section, ARD1 levels (L) were evaluated as the sum of each SI and corresponding PS. Finally, the ARD1 protein staining index was scored from 1 to 4: index 1, negative (L = 0); index 2, low positive ($0 \le L < 0.5$); index 3, positive ($0.5 \le L < 2$); and index 4, very highly positive ($2 \le L \le 3$). The association of staining patterns with clinicopathological features was assessed with $\chi 2$ test.

Identification of breast cancer metastatic to lymph nodes

The cellular morphology of the metastatic to lymph nodes was indentified by the trained and professional pathologists under microscope. Sections of tumor cells invaded into lymph nodes were regarded as metastases and the grades of metastases were assorted according to the lymph node ratio (LNR), which is the number of metastatic lymph nodes divided by the number of retrieved lymph nodes for each patient.

Followed-up examination of post-surgery patients

Of 29 patients with invasive ductal carcinoma (IDC) and positive ARD1 expression at primary diagnosis, undergone adjuvant treatment with either tamoxifen or chemotherapy, recurred and followed up a second surgical examination with the original site of primary surgery. The surgical tissues were evaluated by histopathological morphology and the level of ARD1 protein using immunohisochemical assay.

Statistical analyses

All statistical analysis were performed with $\chi 2$ test using SPSS software. A P value of < 0.05 was considered statistically significant.

Results

ARD1 expression in breast pathological tissues

To further understand the relationship of the level of ARD1 protein and breast disease progression on pathological specimens, we analyzed 356 paraffined breast tissue blocks from patients with invasive ductal carcinoma (IDC), fibroadenoma (FA), mammary gland hyperplasia (MGH), inflammatory breast disease and breast cyst by immunohistochemical assay. The results showed that the highest frequency of ARD1 staining occurred in IDC specimens, which up to 50/82 (61.0%), and secondly higher in FA, 87/159 (54.7%), and the frequency only up to 25/66 (37.9%) in MGH, 7/19 (36.8%) in flammatory breast diseases, 8/22 (26.7%) in breast cyst, respectively (Table 1). Most of ARD1 is localized in the cytoplasmic parts of breast glandular cells (Fig 1). Consequently, the occurrence rate of ARD1 in IDC was remarkably higher than those in other non-cancerous and hyperplasia tissues (relative risk = 1.32), ARD1 expression in IDC and FA was more likely available than other types of breast diseases ($P < 0.005$), but there was no significantly difference between IDC and FA ($P = 0.35$) (Table 1).

Correlation between ARD1 expression and clinical characteristics of breast cancer

Based on the above results, to explore the correlation between ARD1 expression and clinical characteristics of breast cancer, 82 women patients with IDC undergone axillary dissection were further independently and statistically analyzed by $\chi 2$ test. The clinical characteristics of women with IDC are presented in Table 2. Patients with ARD1 positive were significantly more likely than those with ARD1 negative to correlate with breast cancer metastatic to lymph nodes, comparing 47/50 (94.0%)

Table 1. ARD1 Status in Clinical and Pathological Breast Diseases

Breast disease	Cases	ARD1 - (%)	ARD1 + (%)				P value[a]
			Weak	Middle	Strong	Total	
IDC[b]	82	32 (39.0)	29 (35.4)	16 (19.5)	5 (6.1)	50 (61.0)	-
FA[c]	159	72 (45.3)	50 (31.4)	26 (16.4)	11 (6.9)	87 (54.7)	0.35
MGH[d]	66	41 (62.1)	10 (15.2)	11 (16.7)	4 (6.1)	25 (37.9)	< 0.005
Inflammation	19	12 (63.2)	3 (15.8)	3 (15.8)	1 (5.3)	7 (36.8)	< 0.002
Cyst	30	22 (73.3)	4 (13.3)	4 (13.3)	0 (0.0)	8 (26.7)	< 0.001
Total	356	179 (50.3)	96 (27)	60 (17)	21 (5.9)	177 (49.7)	-

[a]P value was calculated as IDC versus other groups, respectively; [b]IDC, invasive ductal carcinoma; [c]FA, fibroadenoma; [d]MGH, mammary gland hyperplasia.

Table 2. ARD1 Status in Invasive Ductal Breast Carcinoma Patients

Clinical features	ARD1 +	ARD1 -	P
Age (years)	50 (50±6.8)	32 (52±7.5)	0.47
Gender			ND[a]
Male	0 (0%)	0 (0%)	
Female	50 (100%)	32 (100%)	
Tumor size			0.75
≤ 2.0 cm	19 (38.0%)	11 (34.4%)	
2.0 cm < T ≤5.0 cm	22 (44.0%)	13 (40.6%)	
> 5.0 cm	9 (18.0%)	8 (25.0%)	
Lymph node metastases			<0.001
Negative	3 (6.0%)	18 (56.2%)	
Positive	47 (94.0%)	14 (43.8%)	
ER status[b]			<0.01
Negative	8 (16.0%)	13 (40.6%)	
Positive	42 (84.0%)	19 (59.4%)	
PR status[c]			0.20
Negative	15 (30.0%)	14 (43.8%)	
Positive	35 (70.0%)	18 (56.2%)	

Notes: [a]ND, not determined; [b]ER, estrogen receptor; [c]PR, progesterone receptor

Table 3. The Relationship Between ARD1 Status and Lymph Node Ratio

LNR[a]	ARD1 +	ARD1 -	Total
< 0.5	7 (36.8%)	12 (63.2%)	19
≥ 0.5	40 (95.2%)	2 (4.8%)	42

[a]LNR, lymph node ratio, which is the number of metastatic lymph nodes divided by the number of retrieved lymph nodes for each patient; P < 0.005

Table 4. ARD1 Status in Relapsing Patients with IDC and ARD1 Positive after Postoperative Treatment

Group patients	ARD1 -	ARD1 +	P
Adjuvant therapy			< 0.04
Tamoxifen	16	2	
Chemotherapy	6	5	
Pathological feature			< 0.001
Metastases	0	4	
MGH	2	1	
Normal	20	2	

patients to 14/32 (43.8%) ones (P < 0.001), and to correlate with estrogen receptor status, 42/50 (84.0%) to 19/32 (59.4%) (P < 0.01) (Table 2). However, in patients with ARD1 positive, neither age nor the size of tumor or PR status differed significantly from ARD1 negative ones (Table 2). In addition, the occurrence rate of ARD1 in patients containing more lymph node metastases, depending on the LNR, was notably than that in fewer ones, comparing 40/42 (95.2%) to 7/19 (36.8%), (Table 3).

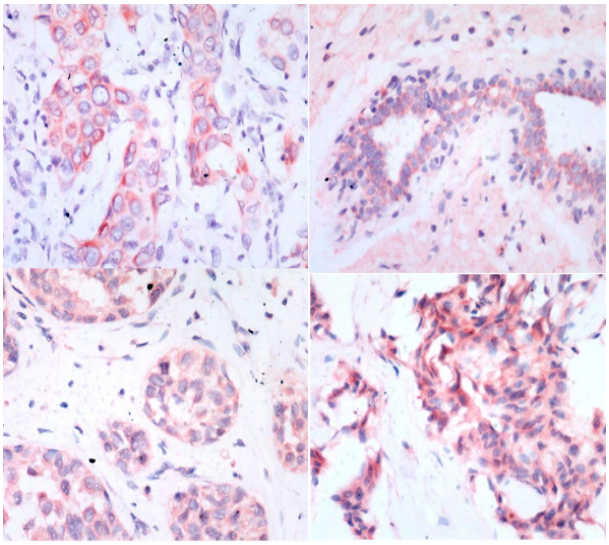

Figure 1. Immunohistochemical Staining of Arrest-defective 1 Protein (ARD1) in Invasive Ductal Carcinomas. Note signals only in ductal epidermal cells, not in surrounding cells (×200)

ARD1 expression and pathological examination after post-surgery and treatment

After postoperative treatment with tamoxifen or chemotherapy, a total of 29 patients with IDC and ARD1 positive expression at primary diagnosis recurred and underwent a second surgical examination and the sections were analyzed by immunohistochemical assay. Among 7 of ARD1 positive patients, the presence of ARD1 still remained in whole 4 cases of metastatic to lymph nodes, 1 of MGH, and 2 of disease-free survival, respectively (Table 4). Whereas, 20 out of 22 ARD1 negative ones were found to be disease-free survival and only 2 to be MGH (Table 4). The results initially and potentially indicated that ARD1 expression was closely correlated to a poor prognosis for patients with breast IDC (P < 0.001). Similarly, of the 29 cases in the study for whom adjuvant therapy, the level of ARD1 was significantly associated with those women who received tomoxifen therapy than those who received chemotherapy, comparing 2/18 to 5/11 cases (P < 0.04) (Table 4).

Discussion

ARD1 protein is widely expressed in mammalian

tissues (Bilton et al., 2005). With regard to the role of ARD1 in cell proliferation and tumorigenesis, previous studies generated controversial results. In lung cancer cells, ARD1 promotes cell proliferation by activating β-catenin (Lim et al., 2006), and by positively regulating DNA-methyltransferase 1 (DNMT1) enzymatic activity to activate the promoter of the tumor suppressor genes, such as E-cadherin (Lee et al., 2010). Lee et al. also reported that the mRNA and the protein level of ARD1 was higher in lung tumor specimens than the non-neoplastic tissues(Lee et al., 2010). In addition, two research teams demonstrated that ARD1 protein has a higher expression in colorectal cancer tissues (Ren et al., 2008) and in breast cancer tissues (Yu et al., 2009b), respectively. In contrast, Arnesen et al. showed that ARD1 protein is downregulated in thyroid neoplasm samples compared with non-neoplastic tissues (Arnesen et al., 2005b) and Kuo et al. manifested that ARD1 as a suppressor to inhibit breast cancer cell growth by α-acetylation of tuberous sclerosis 2 (TSC2) and regulation of mTOR signaling, and suggested that its role of tumor suppression in breast cancer contributes to the loss of heterozygosity (LOH) at the ARD1 locus on Xq28 (Kuo et al., 2010b). This difference of ARD1 in different tissues makes it difficult to explore and understand the specific function of ARD1 in mammalian cells and also exhibits that ARD1 protein is potential for multiple function of regulating as a N-acetyltransferase in vivo.

In current study, we showed that ARD1 expression correlates with the progression of breast diseases, higher in breast cancer tissues (61.0%) than in hyperplasia of mammary glands, inflammatory breast diseases and breast cyst. The occurrence rate of ARD1 expression in fibroadenoma (54.7%) is closed to cancer specimens, which is consistent with fibroadenoma as a long-term risk factor for breast cancer(Dupont et al., 1994) and will provide a clue to explain its mechanism. The explanation for the discrepancy of ARD1 expression in breast cancer with other groups will be expected to be involved in different human race, individual difference, and sample size. In addition, Kuo et al explained that this difference might contribute to the different source, the specificity, and quality of antibodies used for the IHC staining(Kuo et al., 2010a).

Some breast cancers are hormone responsive and depend on the regulation of ER and/or PR. Tamoxifen is an antagonist used for treating breast cancer, mediated to bind ER and inhibit the activation of estrogen, such as β-estradiol (Kim et al., 2007; Riggins et al., 2007). Breast cancers also were thought to the correlation with ER and high level of ER raises the risk of breast cancer (Friman et al., 2007; Willett et al., 1994). Previous research reported that tamoxifen reduced the ARD1 expression in breast cancer cell line ZR-75-1(Yu et al., 2008). This is consistent with the results that elevated ARD1 protein frequently occurred in ER-positive specimens, and similarly, we observed that tamoxifen therapy in patients with ARD1 and ER positive markedly decreased the level of ARD1 protein. Future investigation should elucidate the regulatory mechanisms of breast tumorigenesis from the relationship between ER and ARD1.

The lymph node metastases is one of the most important characteristics of malignancy. In this study, we showed that 47 / 61 of ARD1-positive patients had lymph node metastases, which was significantly higher than that in ARD1-negative ones (3 / 21) and the occurrence rate of metastases was positively correlated with elevated ARD1 expression, which indicating that breast cancer patients expressing ARD1 tends to metastasize. Similarly, in specimens of the post-surgery examined patients, ARD1-positive patients had higher occurrence rate of metastases than ARD1-negative ones. Taken together, these observations suggested that elevated ARD1 protein relates to poor prognosis in breast cancer patients, and thus, ARD1 may be as an alternative protein for the prediction of prognosis.

In conclusion, this research focused on the analysis of ARD1 expression in breast diseases suggest that ARD1 may have potential role in the predicting prognosis for patients with breast cancer and indicate that identification of ARD1 will trace the clinical outcomes and improve treatment strategies. ARD1 expression was related to poor prognosis of lymph node metastases and ARD1 could use as an alternative element for the elevation of prognosis in breast cancer.

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