Report

Daintain/AIF-1 promotes breast cancer proliferation via activation of the NF-κB/cyclin D1 pathway and facilitates tumor growth

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Recent research indicates that inflammatory factors play important roles in the initiation and progression of cancers, including breast cancer. Daintain/allograft inflammatory factor-1 (AIF-1) is a crucial mediator in the inflammatory response, but it has not yet been reported whether daintain/AIF-1 is involved in the development of breast cancers. In this study, immunohistochemical analysis found strong positive expression of daintain/AIF-1 in breast ductal tumor epithelia, but only weakly positive or negative expression in the adjacent histologically normal ductal epithelia. Then, the effect of daintian/AIF-1 on the proliferation of the breast cancer cell line MDA-MB-231 was explored via transduction of the daintian/AIF-1 gene into the cells, and via inhibition of the expression of daintain/AIF-1 through short interference RNA. The results demonstrated that up-regulation and down-regulation of daintain/AIF-1 expressions promoted and inhibited the proliferation of MDA-MB-231, respectively. More interestingly, daintain/AIF-1 overexpression facilitated tumor growth in female nude mice. Furthermore, we found that daintain/AIF-1 overexpression up-regulated the expression of cyclin D1 and enhanced the transcriptional activity of nuclear factor-kappa B (NF-κB), a regulator of cyclin D1 expression. In contrast, the down-regulation of daintain/AIF-1 expression decreased cyclin D1 expression and inhibited the transcriptional activity of NF-κB. These results strongly suggest that daintain/AIF-1 can promote the growth of breast tumors via activating NF-κB signaling, which consequently up-regulates the expression of cyclin D1, implying that daintain/AIF-1 may be a novel target molecule for the prognosis and therapy of breast cancer. (*Cancer Sci* 2008; 99: 952–957)

Breast cancer is the most commonly occurring cancer among women[1] and is second only to lung cancer as a cause of cancer death in women.[2] The initiation and progression of breast cancer are related to many factors. In 1863, Rudolf Virchow noted leukocytes in neoplastic tissues and made a connection between inflammation and the development of cancer.[3] In recent years, additional research has further demonstrated the association of inflammation with cancer.[4] With regard to breast cancer, Pollard *et al*. reported that suppression of macrophage infiltration by deleting the gene for colony-stimulating factor 1 inhibited the growth and metastasis of breast tumors in the mammary cancer model,[5] showing that inflammation is associated with the progression of breast cancer. Inflammatory cytokines, such as interleukin 1, interleukin 6, and tumor necrosis factor (TNF), have been shown to play important roles in the malignant progression of cancers.[6]

In the mid-1990s, we isolated and characterized a polypeptide from porcine intestines and named it 'daintain'.[7] During the same time period, Utans *et al*. identified a novel macrophage factor from rat cardiac allografts with chronic rejection and named it allograft inflammatory factor-1 (AIF-1).[8] When aligned, the amino acid sequences of daintain and AIF-1 are highly similar. Therefore, we call the polypeptide daintain/AIF-1. In humans, daintain/AIF-1 is an evolutionarily conserved, interferon-α-inducible, 143-amino acid calcium-binding protein, and it maps to the major histocompatibility complex class III region on chromosome 6p21.3, which is known for clusters of genes involved in the inflammatory response.[9] It has been reported that daintain/AIF-1 is closely associated with cardiac allograft vasculopathy, rheumatoid arthritis, inflammatory skin disorders, and systemic sclerosis.[10–13] However, it has not yet been reported whether daintain/AIF-1 is also involved in the development of cancers. In this study, we explored the association of daintain/AIF-1 with the development of breast cancer through examining the expression of daintain/AIF-1 in breast ductal tumors, and the effect of daintain/AIF-1 on the proliferation of the breast cancer cell line MDA-MB-231 *in vitro* and in nude mice.

Materials and Methods

Immunohistochemistry. Seventy-three breast biopsy samples from breast tumor patients with ductal carcinoma *in situ* (22 samples) and invasive breast ductal cancer (51 samples) were used in this study. These tissue samples were selected from the archives of the Department of Pathology, Hubei Cancer Hospital (Wuhan, China). Clinical and immunohistochemical data for these breast ductal cancer samples are presented in Table 1. The paraffin sections (5-μm thick) were used and daintain/AIF-1 monoclonal antibodies prepared by our lab[14] were applied at a 1:1000 dilution in 1% bovine serum albumin. For testifying the specific recognition between antibody and antigen, the immunoabsorption assay was performed, and antigen to antibody was in the ratio of three to one (M/M). This study was approved by the institutional ethics committee in Hubei province.

Cell culture and transfection. The human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and subcultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 50 mg/mL streptomycin at 37°C. The cDNA for daintain/AIF-1 was cloned into the pcDNA3.1(–) vector by T4 DNA ligase (Life Technologies, Gaithersburg, MD,

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Table 1. Pathological characteristics of breast ductal cancer samples ($n = 73$) in the present study

Pathological characteristics		$n^{\dagger}$	Daintain/AIF-1 (n)†	
			Positive	Negative
Estrogen receptor	Positive	32	19 (59.3)‡	13 (40.7)
	Negative	49	30 (61.2)	19 (38.8)
Human epidermal growth factor receptor 2	Positive	30	20 (66.7)	10 (33.3)
	Negative	51	29 (56.9)	22 (43.1)
Axillary nodal metastasis	Yes	38	20 (52.6)	18 (47.4)
	No	43	25 (58.1)	18 (41.9)
Histological grade	Grade I	9	6 (66.7)	3 (33.3)
	Grade II	58	34 (58.6)	24 (41.4)
	Grade III	14	10 (71.4)	4 (28.6)
Tumor size (diameter)	≤ 2 cm	36	16 (44.4)	20 (55.6)
	2.1–4 cm	45	33 (73.3)	12 (26.7)

Note: †indicates the number of samples. ‡Numbers in parentheses indicate the percentage of samples.
AIF-1, allograft inflammatory factor-1.

USA). Primer sequences for human daintain/AIF-1 were sense, 5′-*GAATTC*ACCATGAGCCAAAC-3′ and antisense, 5′-*GGATCC*-TTAGGGCAACTCAG-3′. EcoRI and BamHI sites (underlined sequences) were inserted into the primer sequences for cloning. Two pairs of short interference RNAs (siRNAs) for daintain/AIF-1 were synthesized by Sangon (Shanghai, China). The prospective 19-bp regions of human daintain/AIF-1 mRNA were targeted (5′ to 3′): construct 1, AAGACTCACCTAGAGCTAA; and construct 2, AAGAGAGGCTGGATGAGAT. They were chemically synthesized as part of a small, double-stranded 65-bp DNA insert containing the target sequence in the sense orientation, followed by a short loop region (UUCAAGAGA), the target in the antisense orientation, and six thymidines added to the 3′end. This insert was flanked by BamHI and HindIII restriction sites and cloned into the siRNA expression vector pRNAT-H1.1/Shuttle. Vector alone was used as a negative control. The reconstituted daintain/AIF-1-pcDNA3.1(–) (pcDNA-DT), empty vector pcDNA3.1(–) (pcDNA), and reconstituted daintain/AIF-1-pRNAT-H1.1/Shuttle (pRNAT-DT) and siRNA expression vector pRNAT-H1.1/Shuttle (pRNAT) were transfected, respectively, into MDA-MB-231 by lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's protocol. In order to obtain stable transfectants, 1000 μg/mL G418 (Amresco, Solon, OH, USA) was used to eliminate untransfected cells, and the transfectants were pooled and expanded in 500 μg/mL G418.

Cell proliferation assay, tumor cell inoculation, and tumor excision. For proliferation assays, equal numbers of stable transfectants were seeded into 24-well plates at a density of 2×10^4 cells per well. Media were changed on the third day, and after 1, 3, and 5 days, viable, trypan blue–excluding cells were counted using a standard hemocytometer. Female athymic nude mice (NCr-nu/nu) of 4 to 5 weeks of age were purchased from the Laboratory Animal Center of Hubei province, China. The animals were housed under specific pathogen-free conditions. MDA-MB-231 transfected with pcDNA-DT or with empty vector pcDNA alone were injected subcutaneously (lateral cervix) at 2×10^5 cells per mouse. The tumors were excised 10 weeks after cell inoculation and processed for immunohistochemistry. Daintain/AIF-1 antibody (1:1000), Ki67 antibody (1:500), and cyclin D1 antibody (1:500) were used. The antibodies for Ki67, Cyclin D1, and α-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Animal studies were approved by the Huazhong University of Science and Technology Institutional Committee for the Care and Use of Animals.

Western blot analysis. The different MDA-MB-231 that was transfected with reconstituted plasmids pcDNA-DT and pRNAT-DT, and with empty vector pcDNA and pRNAT, were synchronized through culturing the cells in FBS-free media for 48 h, then stimulated with media containing 10% FBS for the indicated times. Western blotting was used to determine the expression of cyclin D1 in the above cell lines. For detection of daintain/AIF-1 in tissues and cell lines, the extracts of breast ductal cancer tissues and adjacent histologically normal tissues, and the lysates of MDA-MB-231 transfected with reconstituted plasmids and empty vector alone were prepared, respectively, as described in the reference indicated.[8] Immunoreactive proteins were visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ, USA) according to the manufacturer's instructions.

Luciferase reporter assay. The pNF-κB-Luc vector (Stratagene, La Jolla, CA, USA) containing the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element (TATA box) plus five repeats of κB *cis*-enhancer element (TGGGGACTTTCCGC) was used. A phRL-TK vector (Promega, Madison WI, USA) containing the *Renilla* luciferase reporter gene was used for the normalization of transfection efficiency. pNF-κB-Luc and phRL-TK were cotransfected into the stable transfectants MDA-MB-231 using lipofectamine 2000 reagent. After 48 h, the cells were harvested either immediately or after stimulation with 10% FBS for 0.5 h, and the firefly luciferase activity and *Renilla* luciferase activity of the cell lysates were measured with a TD-20/20 luminometer (Turner BioSystems, Sunnyvale, CA, USA) using a dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized by *Renilla* luciferase activity to calculate the relative luciferase activity.

Statistics. The statistical significance of differential findings between experimental groups and controls was determined by the Student's t-test. Findings were regarded as significant if two-tailed P-values were less than 0.05.

Results and Discussion

Daintain/AIF-1 expressed in breast tumor tissue. Immunohistochemical tests showed that in 60% (31/51) of the invasive ductal carcinomas and in 50% (11/22) of the ductal carcinoma *in situ* examined, daintain/AIF-1 was largely located at the ductal epithelia of tumor tissues, but little or none was detected at the adjacent histologically normal ductal epithelia (Fig. 1a,b). By immunoabsorption assay, no immunostaining of daintain/AIF-1 was visualized at the location where daintain/AIF-1 was positive by immunohistochemistry (Fig. 1c), showing that the recognition between daintain/AIF-1 and its antibody is specific. In addition, Western detection also showed that daintain/AIF-1 expression in the tumor tissues was apparently more than in the adjacent histologically normal tissues (Fig. 1d). These results suggest

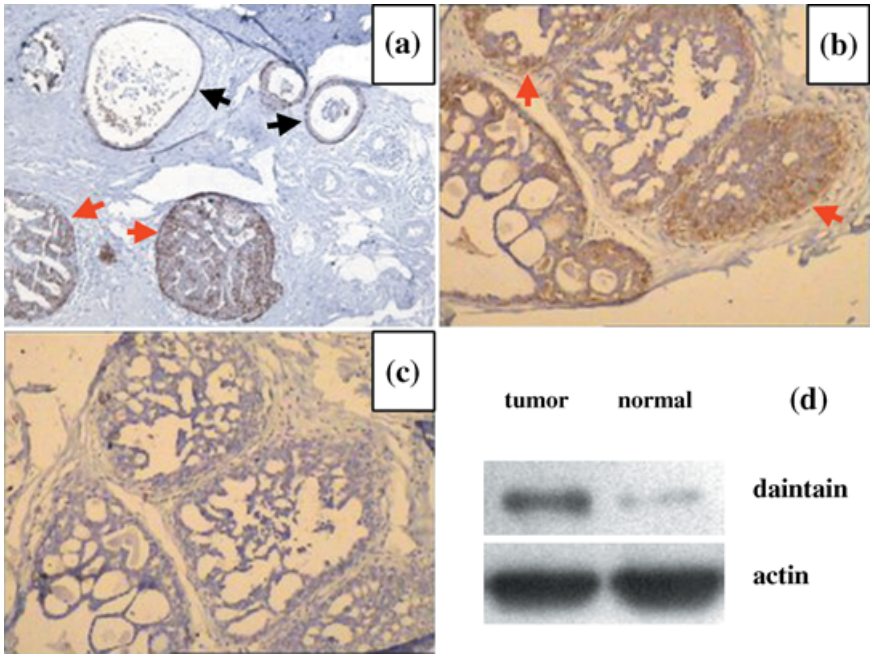

Fig. 1. Representative immunohistochemical analysis of daintain/allograft inflammatory factor-1 (AIF-1) expression in breast ductal tumors. (a) Expression of daintain/AIF-1 in invasive breast ductal tumor epithelia was observed (red arrow), and little or no expression was detected in the adjacent histologically normal ductal epithelia (black arrow). (b) Expression of daintain/AIF-1 in ductal carcinoma *in situ* (red arrow). (c) Immunoabsorption analysis of daintain/AIF-1 expression on the adjacent section of ductal carcinoma *in situ*, and no immunostaining was observed. (d) Detection of the expression of daintain/AIF-1 in breast ductal cancer tissues and adjacent histologically normal tissues. Tumor denotes breast cancer tissues, and normal denotes adjacent histologically normal tissues. Sections were counterstained with hematoxylin. Brown staining indicates antibody recognition. Magnification ×100.

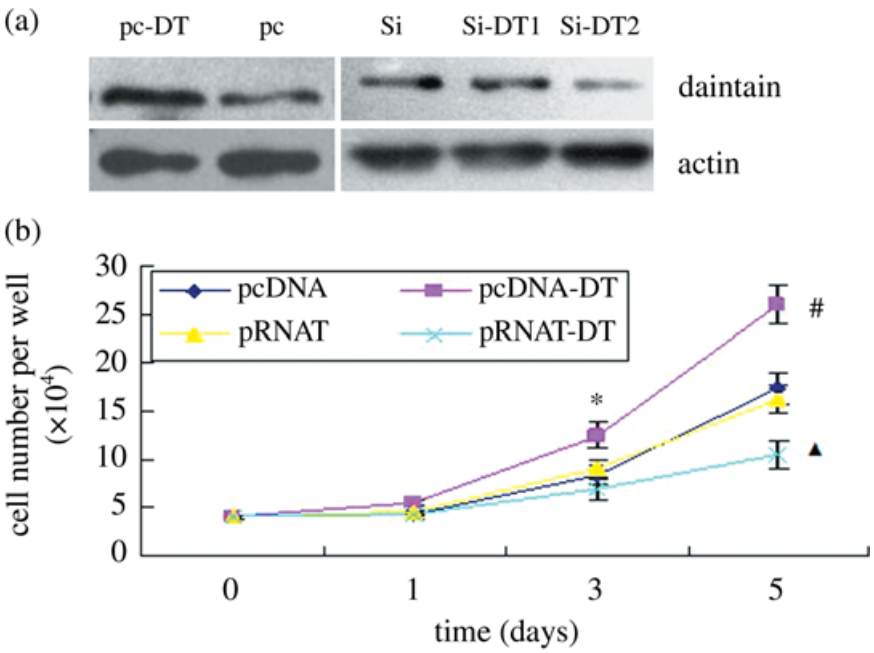

Fig. 2. Daintain/allograft inflammatory factor-1 (AIF-1) promoted proliferation of MDA-MB-231. (a) The expression of daintain/AIF-1 in MDA-MB-231 transfected with pcDNA-DT, pRNAT-DT, pcDNA, and pRNAT, respectively. pc-DT denotes MDA-MB-231 transfected with pcDNA-DT; pc denotes pcDNA; Si denotes pRNAT; Si-DT1 denotes pRNAT-DT containing construct 1; and Si-DT2 denotes pRNAT-DT containing construct 2. (b) MDA-MB-231 transfected, respectively, with pcDNA-DT, pRNAT-DT, pcDNA, and pRNAT were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, and the total cell number was counted on the first, third, and fifth days. At the third day, pcDNA-DT *versus* pcDNA was $1.2 \times 10^5 \pm 1.3$ *versus* $0.8 \times 10^5 \pm 1.0$, $^*P < 0.05$, and at the fifth day, pcDNA-DT *versus* pcDNA was $2.6 \times 10^5 \pm 2.1$ *versus* $1.7 \times 10^5 \pm 1.4$, $\#P < 0.01$. At the fifth day, pRNAT-DT *versus* pRNAT was $1.1 \times 10^5 \pm 1.5$ *versus* $1.6 \times 10^5 \pm 1.7$, $^▲P < 0.05$ ($n = 4$, error bar = SD). Data represent at least four independent experiments with similar results.

that daintain/AIF-1 might be associated with the initiation and progression of breast ductal cancers. We further investigated the relationship between daintain/AIF-1 expression and the pathological characteristics of the breast ductal cancers, and found that the percentage of daintain/AIF-1-positive tumors with a bigger diameter (2.1–4 cm) was significantly more than that with a smaller size (≤ 2 cm) (73.3% *versus* 44.4%) as shown in Table 1. Furthermore, the percentage of daintain/AIF-1-positive tumors in Grade III was also slightly higher than that in Grade II (71.4% *versus* 58.6%). The positive correlation between daintain/AIF-1 expression and tumor size and histological grade implies that daintain/AIF-1 is probably involved in the development of breast ductal cancer. In the past few years, numerous studies have shown that inflammatory factors are closely associated with the development of cancers. Whether daintain/AIF-1, an important player in inflammation, participates in the progression of breast cancer deserves to be further investigated.

Daitain/AIF-1 promoted proliferation of MDA-MB-231. In order to confirm the association of daintain/AIF-1 with breast cancer, the effect of daintain/AIF-1 on the proliferation of the human breast cancer cell line MDA-MB-231 was examined. First, we constructed two breast cancer cell lines. One stably overexpressed daintain/AIF-1 via transfection of the *daintian/AIF-1* gene into MDA-MB-231, and the other decreased the constitutive expression of daintain/AIF-1 via transfection of siRNA (Fig. 2a). It was observed that the up-regulation of daintain/AIF-1 expression promoted MDA-MB-231 proliferation, and that the down-regulation inhibited the proliferation (Fig. 2b). It has been

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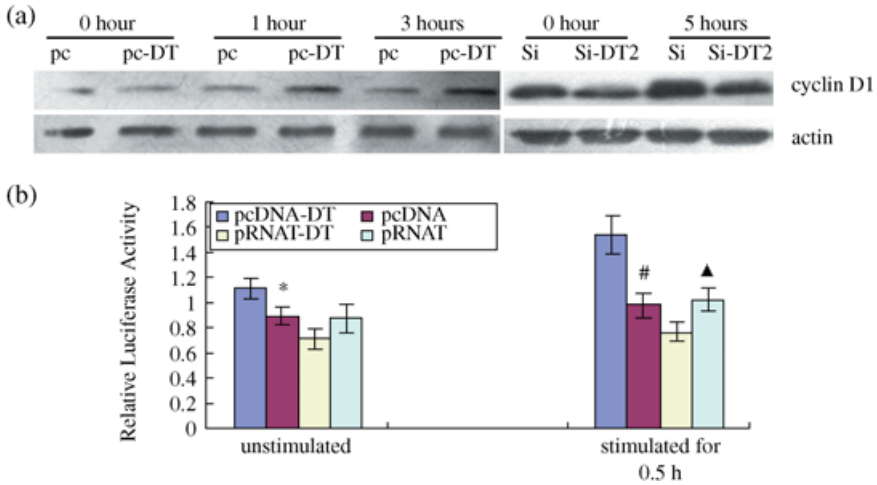

Fig. 3. Daintain/allograft inflammatory factor-1 (AIF-1) up-regulated the expression of cyclin D1 via activation of the transcriptional activity of NF-κB in MDA-MB-231. (a) The expression of cyclin D1 in MDA-MB-231 transfected with pcDNA-DT and with pcDNA alone under stimulation with 10% fetal bovine serum (FBS) for 1 and 3 h, and in MDA-MB-231 transfected with pRNAT-DT and with pRNAT alone under stimulation with 10% FBS for 5 h. (b) The relative luciferase activity in MDA-MB-231 transfected, respectively, with pcDNA-DT, pRNAT-DT, pcDNA, and pRNAT when without stimulation or under stimulation with 10% FBS for 0.5 h. pcDNA-DT *versus* pcDNA was 1.11 ± 0.05 *versus* 0.90 ± 0.03, $^{*}P < 0.05$; pcDNA-DT *versus* pcDNA was 1.54 ± 0.11 *versus* 0.98 ± 0.06, $\#P < 0.01$; pRNAT-DT *versus* pRNAT was 0.76 ± 0.02 *versus* 1.03 ± 0.04, $^{\blacktriangle}P < 0.05$ ($n = 5$, error bar = SD). Representative immunoblots were shown and repeated in triplicate on different lysate samples with similar results; the data represent three independent experiments.

reported that daintain/AIF-1 can promote the proliferation of macrophages[15] T lymphocytes,[16] and vascular smooth muscle cells (VSMCs).[17] In this study, we firstly found that daintain/AIF-1 can also promote MDA-MB-231 proliferation. The effect of daintain/AIF-1 on the proliferation of cancer cells implies that daintain/AIF-1 is probably an inflammatory factor associated with the growth of breast tumor.

Daintain/AIF-1 up-regulated cyclin D1 expression. Failure to enter the G_0 phase is a prerequisite for cell proliferation. At the early part of the G_1 phase in the cell cycle, a decision is made as to whether the cell will enter the G_0 phase or the S phase. The point of decision-making is the G_1 checkpoint, defined as the time-point after which the cell will proceed through the cell cycle independent of the presence of mitogens or antimitogenic factors.[18] Cyclin D1 is a key regulator of the G_1 checkpoint and crucial for cell proliferation.[19] To elucidate the underlying mechanism by which daintain/AIF-1 promotes the proliferation of MDA-MB-231, the effect of daintain/AIF-1 on the expression of cyclin D1 was examined. As summarized in Figure 3a, the expression of cyclin D1 was up-regulated in MDA-MB-231 transfected with pcDNA-DT under stimulation with FBS for 1 h. However, in MDA-MB-231 with pcDNA alone, cyclin D1 expression did not apparently increase under stimulation with FBS even for 3 h. In contrast, the expression of cyclin D1 was inhibited in MDA-MB-231 transfected with pRNAT-DT, and the expression level was still lower than that of the cells with pRNAT alone when under stimulation with FBS for 5 h. These results show that daintain/AIF-1 can enhance the expression of cyclin D1 in MDA-MB-231, which is in line with the effect of daintain/AIF-1 on VSMCs.[20] The effect of daintain/AIF-1 on cyclin D1 expression indicates that daintain/AIF-1 promotes the proliferation of MDA-MB-231, at least in part, through the up-regulation of cyclin D1 expression. Overexpression of cyclin D1 accelerates the cell's entry into S-phase[21] and is closely associated with the initiation and progression of cancers.[22] Therefore, the fact that daintain/AIF-1 enhances cyclin D1 expression suggests that daintain/AIF-1 participates in the development of breast cancer probably via promoting the passing of the G_1 checkpoint.

Daintain/AIF-1 potentiated the transcriptional activity of NF-κB. The expression of cyclin D1 is regulated by NF-κB through the NF-κB binding site in its promoter.[19] In order to elucidate whether the effect of daintain/AIF-1 on cyclin D1 expression is due to the activation of NF-κB, we determined the effect of the up-regulation and down-regulation of daintain/AIF-1 expression on the transcriptional activity of NF-κB using a luciferase reporter assay. As shown in Figure 3b, the results showed that the relative luciferase activity in MDA-MB-231 transfected with pcDNA-DT was higher than that in MDA-MB-231 with pcDNA alone when unstimulated with FBS (1.11 *versus* 0.90). After stimulation with FBS for 0.5 h, the relative luciferase activity in MDA-MB-231 transfected with pcDNA-DT increased 40% (1.11–1.54) compared with the activity before stimulation. However, the relative luciferase activity only increased 10% (0.90–0.98) in MDA-MB-231 transfected with pcDNA alone. On the other hand, the relative luciferase activity was lower in MDA-MB-231 transfected with pRNAT-DT in contrast to MDA-MB-231 transfected with pRNAT alone (0.71 *versus* 0.88). When both of the above cells were stimulated with FBS for 0.5 h, the relative luciferase activity had little change in MDA-MB-231 transfected with pRNAT-DT (0.71–0.76), while there was an increase from 0.88 to 1.03 in MDA-MB-231 transfected with pRNAT. The results reveal that daintain/AIF-1 can up-regulate the transcriptional activity of NF-κB, and that the effect of daintain/AIF-1 on the transcriptional activity of NF-κB is more marked when the cells are stimulated with FBS. NF-κB is a pivotal component in the inflammation-cancer linkage.[23] Inhibition of NF-κB through anti-TNF-α treatment or induction of the I κB-superrepressor in later stages of tumor development resulted in the apoptosis of transformed hepatocytes and failure to progress to hepatocellular carcinoma in *Mdr2*-knockout mice,[24] indicating that NF-κB plays an important role in tumorigenesis. The fact that daintain/AIF-1 can enhance the transcriptional activity of NF-κB implies that daintain/AIF-1 is a crucial component in the development of breast cancer.

Daintain/AIF-1 facilitated tumor growth in nude mice. We also examined the tumor-forming properties of MDA-MB-231 transfected with pcDNA-DT in nude mice. Ten weeks after subcutaneous inoculation of nude mice with MDA-MB-231 transfected with pcDNA-DT, tumors had developed in all eight mice. However, tumors only developed in four of eight mice when inoculated with MDA-MB-231 transfected with pcDNA alone. In addition, the tumors in mice inoculated with pcDNA-DT-transfected cells were apparently larger than those in mice with pcDNA alone (Fig. 4a,b). The differences in tumor formation and growth rate between the two groups indicate that daintain/AIF-1 overexpression favors the development of tumors *in vivo*, an observation that supports the role of daintain/AIF-1 in the proliferation of MDA-MB-231. Interestingly, we also found that daintain/AIF-1 overexpression enhanced the secretory activity of MDA-MB-231 *in vitro* (data not shown), suggesting that the effect of daintain/AIF-1 on breast tumor formation and growth is probably partially due to the construction of a microenvironment that is more suitable for cell

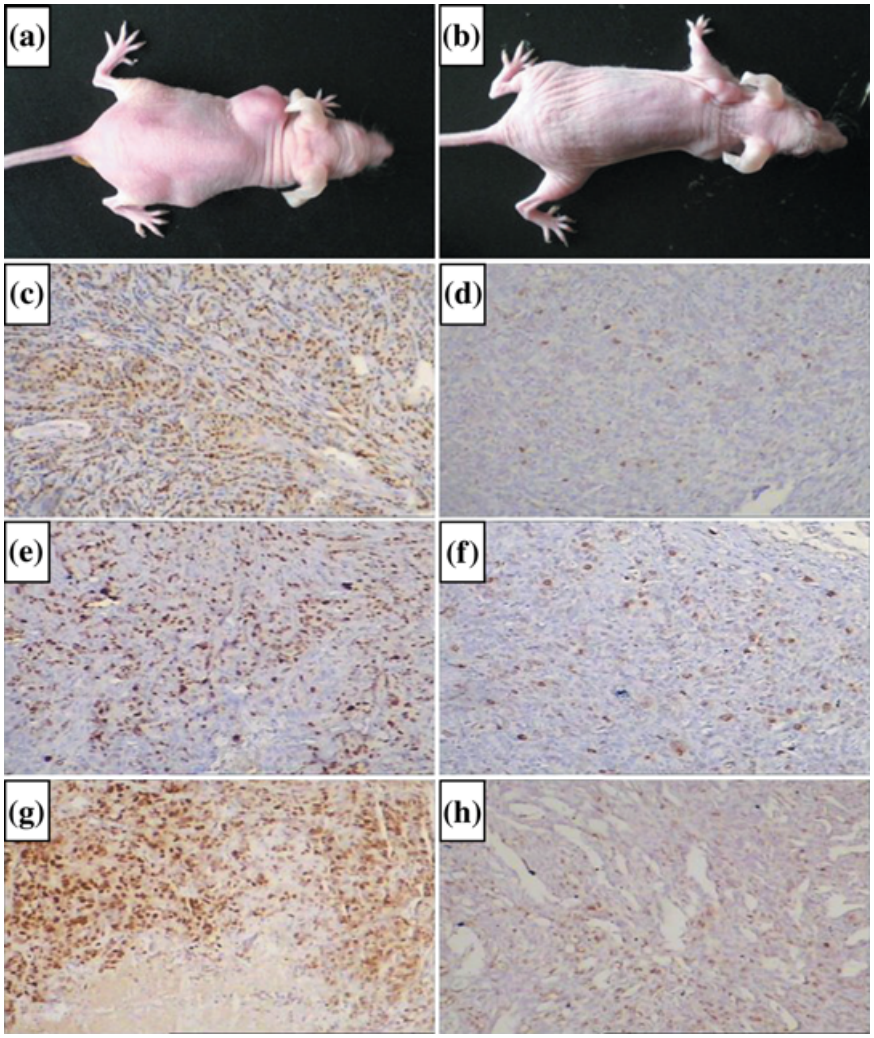

Fig. 4. (a,b) Daintain/allograft inflammatory factor-1 (AIF-1) overexpression promoted the growth of breast tumors in nude mice and (c–h) the immunohistochemical detection of daintain/AIF-1, cyclin D1, and Ki67 in tumor tissues excised from nude mice inoculated with MDA-MB-231 transfected with pcDNA-DT and with pcDNA alone. (a) Nude mice inoculated subcutaneously (lateral cervix) with MDA-MB-231 transfected with pcDNA-DT and (b) with pcDNA alone for 10 weeks. (c) Expression of daintain/AIF-1 in tumor tissues with pcDNA-DT, and (d) with pcDNA alone. (e) Cyclin D1 and (g) Ki67 expressions in tumor tissues with pcDNA-DT, and (f) cyclin D1 and (h) Ki67 expressions in tumor tissues with pcDNA alone. Brown staining indicates antibody recognition. Magnification ×100.

survival and proliferation. Further, we immunohistochemically examined the expression of both cyclin D1 and Ki67, two principal indices used clinically for the prognosis of cancer. The results showed that both cyclin D1 and Ki67 were strongly positive (Fig. 4e,g) in the tumor tissues overexpressing daintain/AIF-1 (Fig. 4c), while they were expressed at low levels (Fig. 4f,h) in tumors excised from mice inoculated with MDA-MB-231 transfected with pcDNA alone (Fig. 4d). Cyclin D1 and Ki67 are two indicators reflecting cell proliferation. The increase of the proliferation rate of cancer cells correlates strongly with poor prognosis in breast cancer.[25] The marked up-regulation of cyclin D1 and Ki67 expression in tumors overexpressing daintain/AIF-1 indicates that daintain/AIF-1 is positively correlated with malignant breast cancer.

In summary, this study describes the association of the inflammatory polypeptide daintain/AIF-1 with the development of breast cancer. First, daintain/AIF-1 was found to be specifically expressed in breast ductal tumors. Then, the up-regulation and down-regulation of daintain/AIF-1 were found, respectively, to promote and inhibit proliferation of the breast cancer cell line MDA-MB-231 via influence of the transcriptional activity of NF-κB, which in turn affected the expression of cyclin D1, a key regulator of the G_1 checkpoint. In line with the results *in vitro*, the overexpression of daintain/AIF-1 favored the growth of tumors in nude mice. These findings provide information on the association of daintain/AIF-1 with the development of breast cancer, which will be useful for the prognosis and therapy of breast cancer, and will aid the understanding of the molecular mechanism linking inflammation and breast cancer.

Acknowledgments

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