DACH1 Expression in Osteosarcoma and Its Relationship with Proliferation and Angiogenesis

Peng Ren · Ming-zhi Gong · Zhi-yong Wang ·
Peng Zhang · Peng Chen · Wan-li Ma · Cheng-jun Zhou

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Abstract The aim of this study was to investigate the expression of DACH1 in osteosarcoma as well as its relationship with cell proliferation and angiogenesis in the tumor. DACH1 expression was detected by immunohistochemical staining in the serial sections of the osteosarcoma. The microvessel density (MVD) was counted by CD34 immunohistochemical staining, and immunohistochemical staining of PCNA staining showed the cell proliferation. The impacts of DACH1 expression on tumor proliferation and angiogenesis were evaluated by statistics. The DACH1 had different expression patterns in different osteosarcoma. Conventional osteosarcoma showed stronger DACH1 staining (conventional vs. parosteal: $P=0.037$; conventional vs. periosteal: $P=0.028$) and more PCNA-positive tumor cells than parosteal and periosteal osteosarcoma (conventional vs. parosteal: $P=0.041$; conventional vs. periosteal: $P=0.045$), the difference was significant. In addition, conventional osteosarcoma showed more cytoplasmic staining of DACH1 than parosteal and periosteal

(conventional vs. parosteal: $P=0.023$; conventional vs. periosteal: $P=0.030$). Parosteal and periosteal osteosarcoma showed no significant difference in DACH1 expression and cell proliferation index. On the other hand, DACH1 different expression patterns showed significantly different impacts on angiogenesis. In spite of the different subtypes of osteosarcoma, the MVD showed a significant difference in cytoplasmic and nuclear expression patterns of DACH1 (nuclear expression vs. cytoplasmic expression: 5.72 ± 1.19 vs. 9.65 ± 1.24 , $P=0.042$). Moreover, in the conventional osteosarcoma, the MVD also showed a significant difference in DACH1 cytoplasmic and nuclear staining (nuclear expression vs. cytoplasmic expression: 5.58 ± 0.71 vs. 13.65 ± 1.30 , $P=0.019$). However, the DACH1 expression intensity showed no significant different impacts on MVD of all kinds of osteosarcoma. DACH1 had different expression patterns and intensity. Cytoplasmic and nuclear expression of DACH1 might play different roles in cell proliferation and angiogenesis of osteosarcoma. Cytoplasmic DACH1 might promote cell proliferation and be associated with angiogenesis.

Peng Ren and Ming-zhi Gong contributed equally to this work.

C.-j. Zhou (✉)
Department of Pathology, The Second Hospital,
Shandong University,
247#, BeiYuan Street,
Jinan, Shandong 250033, People's Republic China
e-mail: liuqian1976@hotmail.com

P. Ren · M.-z. Gong · Z.-y. Wang · P. Zhang · P. Chen · W.-l. Ma
Department of Osteology, The Second Hospital,
Shandong University,
247#, BeiYuan Street,
Jinan, Shandong 250033, People's Republic China

P. Ren · M.-z. Gong · Z.-y. Wang · P. Zhang · P. Chen · W.-l. Ma ·
C.-j. Zhou
Department of Pathology,
Shandong University School of Medicine,
44#, Wenhua Xi Road,
Jinan, Shandong 250012, People's Republic China

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Introduction

The *Drosophila dachshund* (*dac*) gene is the founding member of the DACH subfamily of nuclear proteins, which play an essential role in promoting differentiation of the *Drosophila* eye and limb [1]. *Dachshund* is expressed prior to photoreceptor differentiation and is required for retinal morphogenesis. *Dachshund* itself is sufficient for inducing retinal fates since targeted misexpression results in ectopic eye formation from non-neuronal tissues [2, 3]. The development of the compound eye in *Drosophila* is regulated by several genes,

such as twin of eyeless, eyeless, sine oculis, eyes absent, and dachshund [4].

The dachshund (*dac*) gene was regarded as a dominant suppressor of the ellipse mutation, a hypermorphic allele of the epidermal growth factor receptor (EGFR); the *dac* gene plays a key role in metazoan development. The *dac* is a key component of the retinal determination gene network (RDGN), which plays an important role in *Drosophila* gonadal development and governs cell fate [5–8].

DACH1 suppresses the FGF-2-stimulated RANKL expression in marrow stromal/preosteoblast cells, and FGF-2 promotes HSF-2 nuclear transport and colocalization with DACH1 in marrow stromal cells. Coexpression of NCoR with DACH1 significantly decreased and siRNA suppression of NCoR in DACH1 cotransfected cells increased RANKL promoter activity. Furthermore, DACH1 coexpression with NCoR significantly decreased the RANKL mRNA expression in marrow stromal cells. DACH1 conducts an important role in negative regulation of RANKL gene expression in marrow stromal/preosteoblast cells in the bone microenvironment [9].

Recent studies have demonstrated an important role for human dachshund in tumorigenesis, in particular, breast, prostate and ovarian cancer. The molecular mechanisms by which DACH1 regulates differentiation and tumorigenesis are discussed herein.

It has been previously shown that DACH1 represses the cyclin D1 expression in breast cancer cells, increases nuclear DACH1 expression correlated inversely with cellular mitosis, and predicts improved breast cancer patient survival [10]. DACH1 and estrogen receptor- α (ERA) expressions were inversely correlated in human breast cancer. Nuclear DACH1 expression inhibited estradiol (E2)-induced DNA synthesis and cellular proliferation. DACH1 bound ERA in immunoprecipitation-Western blotting, associated with ERA in chromatin immunoprecipitation, and repressed ERA transcriptional activity, requiring a conserved DS domain. Proteomic analysis identified proline, glutamic acid, and leucine rich protein 1 (PELP1) as a DACH1-binding protein. The DACH1 COOH terminus was required for binding to PELP1. DACH1 repressed induction of ERA signaling. E2 recruited ERA and disengaged corepressors from DACH1 at an endogenous ER response element, allowing PELP1 to serve as an ERA coactivator. DACH1 expression, which is lost in poor prognosis human breast cancer, functions as an endogenous inhibitor of the ERA function [11].

In ovarian cancers, the genes that inhibited TGF- β signaling (DACH1, EVI1, and BMP7) were up-regulated. The expression of a dominant-negative DACH1 into an ovarian cancer cell line that expresses high levels of endogenous DACH1 and is non-responsive to TGF- β signaling partially restored TGF- β signaling [12].

Decreased DACH1 expression was significantly correlated with FIGO surgical stages, peritoneal cytology, lymph node positivity, and histological type, but not histological grade, depth of myometrial, and patient age in endometrial cancer. DACH1 positivity is associated with an increased 5-year survival in all patients in endometrial cancers, which suggested that the loss of DACH1 expression might be involved in endometrial cancer progression [13].

DACH1 transduction of mammary epithelial cells regulated secretion of cytokines and chemokines, implicating DACH1 in heterotypic signaling. DACH1 inhibited cellular migration and invasion of oncogene (Ras, Myc, ErbB2, c-Raf) transformed human breast epithelial cells. Experiments had demonstrated that IL-8 is a critical target of DACH1 mediating breast cancer migration and metastasis in vivo. DACH1 bound the endogenous IL-8 promoter in ChIP assays and repressed the IL-8 promoter through the AP-1 and NF- κ B binding sites [14].

DACH1 inhibited prostate cancer cellular DNA synthesis, growth in colony forming assays, and blocked contact-independent growth in soft agar assays. DACH1 inhibited the androgen receptor (AR) activity and inhibited the ligand-dependent activity of AR mutations identified in patients with androgen insensitive prostate cancer [15].

DACH1 expression was also identified and decreased in MPNSTs [16]. Conventional osteosarcoma is universally fatal and pulmonary metastases are the most frequent sites. Parosteal osteosarcoma is a low grade osteosarcoma which arises on the surface of bone. Prognosis is excellent with 91 % overall survival at 5 years. Periosteal osteosarcoma is an intermediate grade chondroblastic osteosarcoma arising on the surface of bone. Periosteal osteosarcoma is associated with a better prognosis than conventional osteosarcoma. In the present study, the parosteal and periosteal osteosarcoma have better prognosis than the conventional osteosarcoma, but the reasons remain unknown, except for the histological differentiation [17]. So we want to know the expression and impacts of DACH1 on different osteosarcoma. However, DACH1 has not been studied in osteosarcoma and the roles it conducts in tumorigenesis remain unknown. Here we examined the expression of DACH1 as well as MVD via immunohistochemical staining, thereby investigating their impacts on cell proliferation and angiogenesis in osteosarcoma.

Materials and Methods

Antibodies

The rabbit polyclonal anti-DACH1 antibody (Abcam ab31588, dilution 1:100), rabbit antihuman polyclonal antibody CD31 (ab28364, dilution 1:50), and mouse monoclonal

Table 1 DACH1 expression in osteosarcoma and its relationship with cell proliferation

Variable	n	DACH1			PCNA			DACH1	
		H	I	L	H	I	L	N	C
Conventional	66	^a 59	5	2	^b 61	5	0	55	^c 11
Parosteal	47	12	32	3	18	25	4	45	2
Periosteal	53	10	37	6	21	26	6	49	4

H; high expression; I intermediate expression; L low expression

^a Compared with parosteal and periosteal osteosarcoma, conventional osteosarcoma showed higher DACH1 expression ($P<0.05$).

^b Compared with parosteal and periosteal osteosarcoma, conventional osteosarcoma showed more PCNA-positive cells ($P<0.05$).

^c Compared with parosteal and periosteal osteosarcoma, conventional osteosarcoma showed more cytoplasmic expression of DACH1 ($P<0.05$).

anti-PCNA antibody (ab29, dilution 1:500) were all purchased from Abcam plc. (Cambridge, UK).

Patients

The study included 166 patients, including 66 patients with conventional osteosarcoma, 47 patients with parosteal osteosarcoma, and 53 patients with periosteal osteosarcoma who all underwent primary surgical resection between 2002 and 2009 at Qilu Hospital of Shandong University (Table 1). All the diagnoses were made following the pathology and genetics tumors of soft tissue and bone [17] by three pathologists.

Immunohistochemistry Staining

Immunohistochemistry was performed on 4- μ m-thick routinely processed paraffin sections in series. DACH1 was detected with a rabbit polyclonal anti-DACH1 antibody (Abcam ab31588, dilution 1:100); PCNA was examined with a mouse monoclonal anti-PCNA antibody (ab29, dilution 1:500). CD31

was observed with a rabbit antihuman polyclonal antibody CD31 (ab28364, dilution 1:50). Sections were dewaxed, and endogenous peroxidase was blocked by immersing the slides in a 3 % solution of hydrogen peroxide in methanol for 10 min. This was followed by a step of antigen retrieval. The slides were immersed in 0.01 mol/L citrate buffer solution (pH 6.0) and placed in a microwave oven for 25 min. Following a wash in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4), the sections were covered with normal serum in a humidity chamber for 30 min at room temperature. Excess serum was rinsed off with 0.01 mol/L PBS, and the sections were incubated with the primary antibody in a humidity chamber for 45 min at room temperature. Then, sections were rinsed with PBS before being incubated with the biotinylated second antibody in a humidity chamber for 40 min at 37 °C. After rinsing with PBS, the streptavidin–peroxidase complex reagent was added. Slides were incubated for 45 min at room temperature, then washed in 0.01 mol/L PBS, and covered with 3,3-diaminobenzidine tetrahydrochloride solution for 15 min under a microscope. Sections were then immersed in running tap water, counterstained with hematoxylin for 1 min, followed by tap water bath, immersion in a series of alcohol baths of increasing concentrations, and xylene, then covered with coverslips. Negative controls were performed, in which the primary antibody was omitted. The evaluation of DACH1 and PCNA staining was performed independently by three pathologists. Slides with equivocal evaluation were reevaluated, and a consensus was reached. For each sample, at least 3000 carcinoma cells were evaluated for the immunohistochemical staining. We examined the sections at 200 $\times$ magnification, and the carcinoma cells with cytoplasmic or nuclear staining were determined. DACH1 and PCNA expressions were classified as “low” (<33 % positive carcinoma cells) expression, “intermediate” expression (≥ 33 % and <66 % positive carcinoma cells), and “high” (≥ 66 % positive carcinoma cells) expression [18] (Table 1). Negative controls were performed, in which the primary antibody was omitted, and the positive controls were also performed with glioma.

Table 2 DACH1 expression in osteosarcoma and its relationship with angiogenesis

Variable	MVD				
	DACH1		DACH1		
	N	C	H	I	L
Conventional	5.58 $\pm$ 0.71	13.65 $\pm$ 1.30 ^a	9.92 $\pm$ 1.73	8.89 $\pm$ 1.36	7.50 $\pm$ 1.22
Parosteal	5.54 $\pm$ 0.88	6.75 $\pm$ 1.26	8.27 $\pm$ 1.40	9.02 $\pm$ 1.29	8.60 $\pm$ 1.56
Periosteal	5.36 $\pm$ 1.01	5.93 $\pm$ 1.19	7.96 $\pm$ 1.36	8.02 $\pm$ 1.17	8.12 $\pm$ 1.55
Total	5.72 $\pm$ 1.19	9.65 $\pm$ 1.24 [#]	8.95 $\pm$ 1.31	8.91 $\pm$ 1.12	8.50 $\pm$ 1.20

^a Co-mpared with nuclear expression, the cytoplasmic expression of DACH1 showed more MVD in conventional osteosarcoma ($P<0.05$).

[#] Despite the histologic types, the cytoplasmic expression of DACH1 showed more MVD in osteosarcoma ($P<0.05$).

Evaluation of MVD in DACH1 and PCNA Staining Areas

Microvessel density (MVD) was assessed in the DACH1-positive and PCNA-positive tumor areas. Vessel counts were performed under light microscope based on staining of CD31. Five areas of maximal MVD were identified by screening (magnification 40×). The number of vessels was counted within a counting grid at 400× magnification (40× objective and 10× ocular). For the blood vessel counts, any stained endothelial cell or cell cluster separated from another microvessel structure was considered a countable microvessel. But a lumen was not necessary for a structure to be counted as a microvessel. The number of vessels was expressed as the mean value of counted microvessels in five evaluated grids in areas of maximum vessel density. Data were shown as the mean±SD (Table 2).

Statistical Analysis

Statistical analysis was performed using the SPSS 11.0 software package for Windows. Measures of central tendency and dispersion were determined. The Mann–Whitney test was used to analyze the data. Differences were considered significant at $P<0.05$ level.

Results

DACH1 Expression in Osteosarcoma and its Relationship with Cell Proliferation

DACH1 was expressed in osteosarcoma including high-grade malignance and low-grade malignance. As a whole, DACH1 was expressed in osteosarcoma with nuclear staining pattern, but there was also some difference. In 66 cases of conventional osteosarcoma, 59 cases showed DACH1 high expression, five cases showed intermediate expression, and two cases showed low expression. Simultaneously, 61 out of 66 conventional osteosarcoma showed high cell proliferation index labeled by PCNA. Of 66 cases of conventional osteosarcoma, 55 cases showed DACH1 nuclei staining and 11 cases showed cytoplasmic staining. By contrast, the parosteal and periosteal osteosarcoma showed weak DACH1 expression and less nuclear staining, also less cell proliferation index stained by PCNA. In 47 cases of parosteal osteosarcoma, 12 cases showed high DACH1 expression, 32 cases intermediate expression and three cases low expression. At the same time, 18 cases showed high expression of PCNA, and 25 cases showed intermediate PCNA expression, four cases showed low PCNA expression. In 53 cases of periosteal osteosarcoma,

Fig. 1 a–d: DACH1 is expressed in osteosarcoma: DACH1 is expressed with cytoplasmic staining (a) and with nuclear staining in conventional osteosarcoma (b), and DACH1 is expressed with nuclear staining in parosteal (c) and periosteal (d) osteosarcoma. **e–h:** PCNA-positive cells are showed in conventional (e: DACH1 expression with

cytoplasmic staining, **f:** DACH1 expression with nuclear staining), parosteal (g) and periosteal (h) osteosarcoma. **i–l:** MVD is showed in conventional (i: DACH1 expression with cytoplasmic staining, **j:** DACH1 expression with nuclear staining), parosteal (k) and periosteal (l) osteosarcoma. **a–j** 400×, **k–l** 200×

10 cases showed high DACH1 expression, and 37 cases intermediate DACH1 expression, six cases low DACH1 expression. Meanwhile, 21 cases showed high PCNA expression, and 26 cases showed intermediate PCNA expression, six cases showed low PCNA expression. The parosteal and periosteal osteosarcoma all showed less DACH1 expression with cytoplasmic staining. The DACH1 had different expression patterns in different osteosarcoma. Conventional osteosarcoma showed stronger DACH1 staining (conventional vs. parosteal: $P=0.037$; conventional vs. periosteal: $P=0.028$) and more PCNA-positive tumor cells than parosteal and periosteal osteosarcoma (conventional vs. parosteal: $P=0.041$; conventional vs. periosteal: $P=0.045$), the difference was significant. In addition, conventional osteosarcoma showed more cytoplasmic staining of DACH1 than parosteal and periosteal (conventional vs. parosteal: $P=0.023$; conventional vs. periosteal: $P=0.030$). Parosteal and periosteal osteosarcoma showed no significant difference in DACH1 expression and cell proliferation index (Fig. 1 a–h) (Table 1).

DACH1 Expression and its Relationship with Angiogenesis in Osteosarcoma

DACH1 different expression patterns showed significantly different impacts on angiogenesis. In spite of the different subtypes of osteosarcoma, the MVD showed a significant difference in cytoplasmic and nuclear expression patterns of DACH1 (nuclear expression vs. cytoplasmic expression: 5.72 ± 1.19 vs. 9.65 ± 1.24 , $P=0.042$). Moreover, in the conventional osteosarcoma, the MVD also showed a significant difference in DACH1 cytoplasmic and nuclear staining (nuclear expression vs. cytoplasmic expression: 5.58 ± 0.71 vs. 13.65 ± 1.30 , $P=0.019$). However, the DACH1 expression intensity showed no significant different impacts on MVD of all kinds of osteosarcoma (Fig. 1 i–l) (Table 2).

Discussion

DACH1 (Dachshund homolog 1) is a key component of the RDGN and regulates gene expression either indirectly as a cointegrator or through direct DNA binding. The current studies have demonstrated the mechanisms governing DACH1-mediated transcriptional repression via DNA sequence specific binding. DACH1 repressed gene transcription driven by the DACH1-responsive element [19].

The mammalian homolog of the *Drosophila* dachshund gene (DACH1) has been reported as a tumor suppressor in human breast and prostate cancers. It downregulates the EGFR and cyclin D1 [20].

The cell fate determination factor Dachshund was cloned as a dominant inhibitor of the hyperactive EFGR ellipse. The expression of Dachshund is deletion in human breast

cancer associated with poor prognosis. Breast tumor initiating cells (TIC) may be involved in cancer progression and therapy resistance. Endogenous DACH1 is reduced in breast cancer cell lines with high expression of TIC markers and in patient samples of the basal breast cancer phenotype [21].

Forced expression of DACH1 decreases cell proliferation of a series of glioma cell lines. Gene expression analysis and chromatin immunoprecipitation assay have revealed that fibroblast growth factor 2 (FGF2/bFGF) is transcriptionally repressed by DACH1 [22].

RT-PCR analysis showed that DACH1 was down regulated by treatment with BMP-2 in prostate cancer [23].

Our study showed that the DACH1 had different expression patterns in different osteosarcoma. Conventional osteosarcoma showed stronger DACH1 staining and more PCNA-positive tumor cells than parosteal and periosteal osteosarcoma; the difference was significant. In addition, conventional osteosarcoma showed more cytoplasmic staining of DACH1 than parosteal and periosteal. Parosteal and periosteal osteosarcoma showed no significant difference in DACH1 expression and cell proliferation index. On the other hand, DACH1 different expression patterns showed significantly different impacts on angiogenesis. In spite of the different subtypes of osteosarcoma, the MVD showed a significant difference in cytoplasmic and nuclear expression patterns of DACH1 [24]. Moreover, in the conventional osteosarcoma, the MVD also showed a significant difference in DACH1 cytoplasmic and nuclear staining. However, the DACH1 expression intensity showed no significant different impacts on MVD of all kinds of osteosarcoma. So we thought that DACH1 had different expression patterns in conventional, parosteal, and periosteal osteosarcoma. The cytoplasmic and nuclear expression of DACH1 might conduct different roles in cell proliferation and angiogenesis of osteosarcoma. Cytoplasmic DACH1 might be a mutant expression and promote cell proliferation and be associated with angiogenesis. In our next study, the cytoplasmic and nuclear expression of DACH1 should be deeply investigated to reveal the mechanism of its different impacts on cell proliferation and angiogenesis.

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