

Original Article

Expression of p16 in nodular fasciitis: an implication for self-limited and inflammatory nature of the lesion

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Abstract: Nodular fasciitis (NF) is a self-limited tumorous lesion occurring in the upper as well as lower extremities. NF is composed of a proliferation of “primary culture”-like myofibroblastic cells with nuclear atypia and large nucleoli, thus mimicking sarcoma. NF harbors a promoter-swapping fusion gene containing the entire coding region of *USP6* gene. Therefore, NF is a tumor with a fusion oncogene but self-limited. In order to explore why NF is self-limited, we examined whether myofibroblastic cells in NF express p16 protein, a gene product of *CDKN2A* gene and an inhibitor of cyclin-dependent kinase 4 (CDK4) as well as one of the hallmarks of cellular senescence. We immunohistochemically demonstrated strong and diffuse expression of p16 in myofibroblastic cells in 11 out of 15 cases of NF, and strong but partial expression in the remaining 4 of the cases. We also showed that 15 out of 15 cases of NF were immunohistochemically negative or only showed focal and faint immunopositivity for CDK4, murine double minute 2 (MDM2), and TP53 proteins. Furthermore, there were no significant changes in the copy number of *CDKN2A*, *CDK4* and *MDM2* genes, and no significant mutations in *TP53*, *RB1*, and *CDKN2A* genes in 1 case of NF selected. These data suggest a possible involvement in cell cycle arrest and presumed cellular senescence by p16 in myofibroblastic cells in NF. This may explain the self-limited as well as inflammatory nature of NF as a senescence-associated secretory phenotype.

Keywords: CDK4, MDM2, nodular fasciitis, p16, senescence, TP53

Introduction

Nodular fasciitis (NF) is a self-limited tumorous lesion occurring mainly in the upper extremities, trunk, and head and neck as well as in the lower extremities. It expands from the surface of fascia into the subcutis and rapidly enlarges up to around 2 cm within a few months [1]. Histopathologically, NF is composed of proliferation of “primary culture”-like myofibroblastic cells with nuclear atypia and large nucleoli. Due to these clinico-pathological features, NF mimics sarcoma. NF had long been thought of as an inflammatory reactive lesion. However, it was reported that NF harbors a promoter-swapping fusion gene containing the entire coding region of *ubiquitin-specific protease 6 (USP6)* gene, which facilitates a definitive diagnosis of NF [2]. A representative of the fusion gene is *MYH9-*

USP6 fusion gene, in which *MYH9* gene promoter is fused to the entire coding region of *USP6* gene, leading to its enhanced expression [2].

USP6 is a deubiquitinating enzyme. Two studies *in vitro* showed that cultured cells transfected with the *MYH9-USP6* fusion gene exhibited activation of growth factor signaling pathways such as WNT/APC/ β -catenin [3] and JAK/STAT pathways [4], and stabilization of JUN protein [5]. Therefore, *MYH9-USP6* fusion gene functions as an oncogene in NF, providing mechanistic bases of how NF grows. On the other hand, why NF is self-limited remains to be clarified. Recently, it was reported that Ewing sarcoma cells expressing *USP6* showed increased response to interferon- β and hence increased apoptosis, suggesting a growth-inhibitory effect of *USP6* expression [6].

p16 expression in nodular fasciitis

Table 1. Characteristics of selected cases of nodular fasciitis in this study

Case No.	Age (y)	Sex*	Tumor location	Tumor size at cut surface (mm)**	p16 IHC***	CDK4 IHC	MDM2 IHC	p53 IHC
1	42	F	Right finger	17 × 12	(+)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
2	61	F	Right cervical region (back)	8 × 4	(+)	(-)	(-) >> (+/-)	(-) > (+/-)
3	26	M	Right shoulder	21 × 13	(+)	(-) >> (+/-)	(-) > (+/-)	(-) > (+/-)
4	72	M	Right upper arm	4 × 3	(+) > (-)	(-) >> (+/-)	(-) > (+/-)	(-) > (+/-)
5	38	F	Left fourth finger	20 × 10	(+) > (-)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
6	27	M	Right shoulder	16 × 13	(+)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
7	42	M	Left thigh	20 × 14	(+)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
8	21	M	Right lower limb	22 × 12	(+)	(-) >> (+/-)	(-)	(-) > (+/-)
9	25	M	Right knee	27 × 17	(+)	(-) >> (+/-)	(-)	(-) > (+/-)
10	74	F	Right thigh	25 × 15	(+) > (-)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
11	22	M	Left chest	30 × 30	(+)	(-) >> (+/-)	(-) >> (+/-)	(-) > (+/-)
12	41	M	Right thigh	8 × 5	(+)	(-) >> (+/-)	(-) > (+/-)	(-) > (+)
13	49	M	Right forearm	13 × 6	(+)	(-) >> (+/-)	(-) >>> (+/-)	(-) > (+/-)
14	35	M	Right popliteal region	13 × 11	(+) > (-)	(-) >> (+/-)	(-) >>> (+/-)	(-) > (+/-)
15	37	M	Right upper arm	12 × 4	(+)	(-) >> (+/-)	(-) >>>> (+/-)	(-) > (+/-)

*F denotes "female" and M does "male". **Tumor size at cut surface was measured histologically except cases 10 and 11, the size of which was measured by imaging studies. ***IHC denotes "immunohistochemistry". (+): Positive. (+) > (-): Partially (that is, at most around 50%) positive. (-) > (+): Only focally positive. (-) > (+/-): Only focally and weakly positive. (-) >> (+/-): Only focally and faintly positive. (-) >>> (+/-): Just a few weakly positive cells. (-) >>>> (+/-): Just a few faintly positive cells. (-): Negative.

It is generally known that activation of oncogenes induces not only cell proliferation but also cellular growth arrest, which is called oncogene-induced senescence [7, 8]. It is generally accepted that one of the hallmarks of cellular senescence induced by oncogenes is the expression of p16, a product of *CDKN2A* gene, which inhibits the activity of cyclin-dependent kinase 4 (CDK4) [8].

However, p16 is also known to be expressed concomitantly with disruption of the tumor-suppressing TP53 pathway or amplification of CDK4 pathway phosphorylating RB1. For example, p16 is expressed in squamous cell carcinoma of head and neck or cervix where the TP53 pathway is disrupted by gene products of human papilloma virus [9, 10]. Other examples include gynecologic serous adenocarcinoma [11] or lung small cell carcinoma with mutations in *TP53* and/or *RB1* genes [12], and atypical lipomatous tumor (well differentiated liposarcoma) with amplification of *murine double minute 2 (MDM2)* and/or *CDK4* genes on chromosome 12 [13]. Surrogate markers for mutational or structural changes in these genes are the immunohistochemical detection of gene products of *CDK4*, *MDM2* [13], and *TP53* genes, except for *RB1* gene mutation where the expression of RB protein is lost.

In the present paper, we examined the hypothesis that a self-limited feature of NF is caused

by cellular senescence induced by the *USP6* fusion oncogene.

Materials and methods

Case selection

From the case files of our hospital, we selected 15 cases (11 males and 4 females) of NF with age from 21 to 74 (median: 38) and with size from 4 to 30 mm (median: 20 mm) in diameter. Nine cases were from the upper parts of the body, including the shoulder (n=2), neck (n=1), chest (n=1), arm (n=3), and finger (n=2), while the remaining 6 were from the lower body, including the thigh (n=3), knee (n=1), popliteal region (n=1), and lower limb (n=1). The details of the cases are summarized in **Table 1**.

Immunohistochemistry

Immunohistochemistry was performed on 3 μm-thick sections of formalin-fixed and paraffin-embedded tissues. All of the immunohistochemical procedures were performed using Leica BOND-MAX (Leica). For p16 immunostaining, antigen retrieval was carried out by placing the sections in Epitope Retrieval Solution 2 (pH 9, Leica) at 95°C for 10 minutes. E6H4 antibody (Ab) in CINtec p16 Histology (Roche) was used at 10 fold dilution. For TP53 immunohistochemistry, a mouse monoclonal Ab specific for TP53 (Leica, NCL-L-p53-DO7)

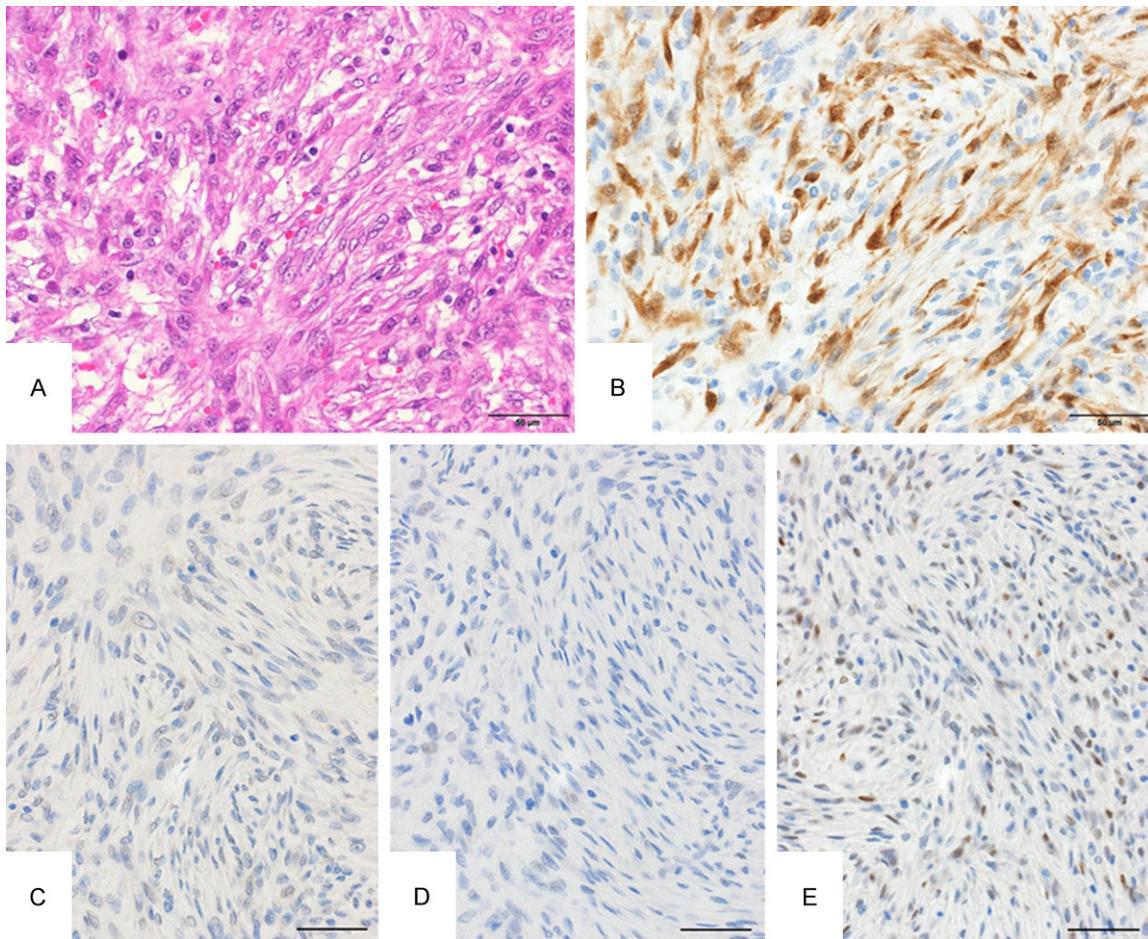


Figure 1. Representative images of hematoxylin-eosin stain and immunohistochemistry for nodular fasciitis. (A) Hematoxylin-eosin stain, (B) p16, (C) CDK4, (D) MDM2, (E) TP53. Original magnification: $\times 400$. Bar: 50 μm .

was used at 2 fold dilution and antigen retrieval was carried out by placing the sections in Epitope Retrieval Solution 2 at 95°C for 20 minutes. For MDM2 immunohistochemistry, a mouse monoclonal Ab specific for MDM2 (clone IF2, InVitrogen #182403) was used at 200 fold dilution and antigen retrieval was carried out by placing the sections in Epitope Retrieval Solution 2 at 95°C for 20 minutes. For CDK4 immunohistochemistry, a mouse monoclonal Ab specific for CDK4 (clone DCS-31, InVitrogen #AHZ0202) was used at 100 fold dilution and antigen retrieval was carried out by placing the sections in Epitope Retrieval Solution 1 (pH 6, Leica) at 95°C for 20 minutes.

Genomic analysis

Genomic changes of 1 selected case of NF were examined by next-generation sequencing

(NGS) according to OncoPrint Cancer Research Panel v2.0 (Thermo Fisher Scientific).

Results

A representative hematoxylin-eosin stained image of the NF cases is presented in **Figure 1A**. Proliferation of myofibroblastic cells with nuclear atypia and large nucleoli was observed against inflammatory background, myxoid stroma and slight hemorrhage.

We immunohistochemically demonstrated strong and diffuse expression of p16 in myofibroblastic cells in 11 out of 15 NF cases (**Figure 1B**), and strong but partial (that is, at most around 50%) expression in the remaining 4 NF cases. The results are summarized in **Table 1**.

As described above, p16 is also known to be expressed concomitantly with disruption of the

tumor-suppressing TP53 pathway or amplification of the CDK4 pathway phosphorylating RB1. In order to exclude these possibilities, we immunohistochemically examined overexpression of CDK4, MDM2, and TP53 proteins in NF. For CDK4 immunohistochemistry, 1 out of 15 NF cases was negative and the remaining 14 cases showed only focal and faint immunopositivity for CDK4 protein (**Figure 1C**). With regard to MDM2 immunohistochemistry, 2 out of 15 NF cases were immunohistochemically negative, 3 cases showed just a few number of weakly or faintly positive myofibroblastic cells, and the remaining 10 cases showed only focal and faint to weak immunopositivity for MDM2 protein (**Figure 1D**). Finally, as for TP53 immunohistochemistry, 14 out of 15 NF cases showed only focal and faint immunopositivity except one showing focal and weakly positive myofibroblastic cells (**Figure 1E**). The detail of these results is summarized in **Table 1**. These results indicate that there were no significant changes in the copy number of *CDK4* and *MDM2* genes and no significant mutations in *TP53* gene.

To confirm these immunohistochemical results, genomic changes of 1 selected NF case (Case 3 in **Table 1**) were examined by NGS according to OncoPrint Cancer Research Panel v2.0 (Thermo Fisher Scientific). There were no significant changes in the copy number of *CDKN2A*, *CDK4* and *MDM2* genes, and no significant mutations in *TP53*, *RB1*, and *CDKN2A* genes (data not shown). This result is consistent with the above immunohistochemical data, demonstrating that overexpression of CDK4, MDM2, and TP53 proteins was not observed in NF.

Discussion

We immunohistochemically demonstrated strong and diffuse expression of p16 in myofibroblastic cells in 11 out of 15 NF cases, and strong but partial expression in the remaining 4 NF cases. We also showed that 15 out of 15 NF cases were immunohistochemically negative or only showed focal and faint immunopositivity for CDK4, MDM2, and TP53 proteins. Furthermore, there were no significant changes in the copy number of *CDKN2A*, *CDK4* and *MDM2* genes, and no significant mutations in *TP53*, *RB1*, and *CDKN2A* genes in 1 case of NF selected.

Taken together, we demonstrated that p16 was expressed in NF without significant changes in the copy number of *CDK4*, and *MDM2* genes and significant mutations in *TP53* genes. Guo *et al.* also reported that no significant mutations were found in one of their NF cases by using NGS and the Ion AmpliSeq Cancer Hotspot Panel v2 targeting 50 cancer-associated genes including *CDKN2A*, *RB1*, and *TP53* [14].

Expression of p16 in NF in the present study is consistent with a possibility that cellular senescence occurs in self-limited myofibroblastic tumors as NF. Expression of p16 may be induced in NF as a consequence of cellular senescence induced by the *USP6* fusion oncogene, thus explaining a self-limited feature of NF. A similar example of p16 expression by oncogene-induced cellular senescence is seen in nevi; the occurrence of *BRAF* V600E mutation presumably transforms melanocytes into nevi where p16 expression induced by the *BRAF* oncogene causes growth arrest of nevi [7, 15]. In this sense, loss of expression of functional p16 protein encoded by *CDKN2A* gene may be critical for further transformation from nevi to malignant melanomas. Hypothetically, oncogene-induced cellular senescence similar to that seen in nevi may operate in proliferating myofibroblastic cells in NF, causing them to cease to proliferate.

However, p16 expression was reported in ischemic fasciitis, a tumorous lesion composed of myofibroblastic proliferation [16]. Myofibroblasts are reported to become prematurely senescent in myocardial fibrosis [17]. It is also reported that connective tissue growth factor secreted by fibroblasts themselves induces cellular senescence in fibroblasts [18]. Therefore, the presumed cellular senescence of myofibroblastic cells in NF may be an inherent nature of myofibroblasts themselves, and may not be induced by *USP6* fusion oncogene. Expression of p16, an inhibitor of CDK4, thus arresting the cell cycle in myofibroblasts may be a negative-feedback “brake” to prevent unlimited collagen deposition by myofibroblasts and hence fibrosis.

On the other hand, it is known that cellular senescence is also associated with secretion of various inflammatory cytokines, which is called senescence-associated secretory phe-

notype [8]. Therefore, inflammatory aspects of NF may be explained by the involvement of presumed cellular senescence of myofibroblastic cells in NF, whether it is induced by the *USP6* fusion oncogene or not.

In conclusion, we demonstrated p16 expression in NF without immunohistochemical expression of CDK4, MDM2, and TP53 proteins. These data suggest a possible involvement of cell cycle arrest and presumed cellular senescence by p16 in myofibroblastic cells in NF, which may explain the self-limited as well as inflammatory nature of NF as a senescence-associated secretory phenotype. Whether this is oncogene-induced or inherent in myofibroblasts may be an interesting problem related to not only NF *per se*, but also myofibroblastic lesions including fibrosis in general.

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Disclosure of conflict of interest

None.

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