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Case Report

Evidence for an association between increased oxidative stress and derangement of FOXO1 signaling in tumorigenesis of a cellular angiofibroma with monoallelic 13q14: a case report

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Abstract: Cellular angiofibroma (CAF) is a rare soft tissue tumor characterized by random arrangement of spindle tumor cells in the stroma with short collagen bundles and thick- and hyalinized small vessels. CAFs share histological characteristics with spindle cell lipomas and mammary type myofibroblastomas. Because these tumors harbor monoallelic 13q14, common genetic and molecular mechanism for tumorigenesis is presumed. In this study, we reported a case of CAF in a 69-year-old man with monoallelic 13q14. Immunohistochemical analysis revealed that *FOXO1*, which is located in chromosome 13q14, was not expressed in the tumor. We also detected oxidative stress markers and found p38 MAPK activation, which is often induced by cellular stressors such as reactive oxygen species (ROS). Because *FOXO1* induces the expression of genes encoding enzymes that generate antioxidants, oxidative stress induced by loss of *FOXO1* expression may be common among CAFs, spindle cell lipomas, and mammary type myofibroblastomas.

Keywords: Cellular angiofibroma, monoallelic deletion, RB1, *FOXO1*, ROS, p38 MAPK

Introduction

Cellular angiofibroma (CAF) is a rare mesenchymal spindle cell tumor that arises in the vulvovaginal region of women and in the inguinoscrotal region of men [1-3]. Microscopically, spindle tumor cells exhibit random and sparse arrangement in the stroma with short collagen bundles and thick- and hyalinized small vessels [2-4]. Evidences indicate that CAFs share morphological and immunohistochemical characteristics with spindle cell lipomas, mammary type myofibroblastomas, and aggressive angiomyxomas [2, 3]. Although no specific immunohistochemical marker of CAFs was identified, monoallelic 13q14 deletion was found in the cases of CAFs, spindle cell lipomas, and mammary type myofibroblastomas [4-6], suggesting that common genetic alteration is associated with pathogenesis of these tumors [4]. *RB1* and *FOXO1,* both of which encode tumor suppressors, reside within 13q14. A large body of evidence establishes RB1 as a tumor suppressor [7, 8], and many human cancer cells harbor genetic alterations of *RB1*, such as chromosomal deletions including RB1 locus, as well as nonsense and missense mutations [9-11]. *FOXO1* is a member of the O class of forkhead transcription factors (FOX) and functions as a transcription factor associated with apoptosis, cell cycle regulation, DNA repair, and resistance to oxidative stress [12]. Because of these properties, *FOXO* proteins are classified as tumor suppressors [13, 14] and several chromosomal aberra-

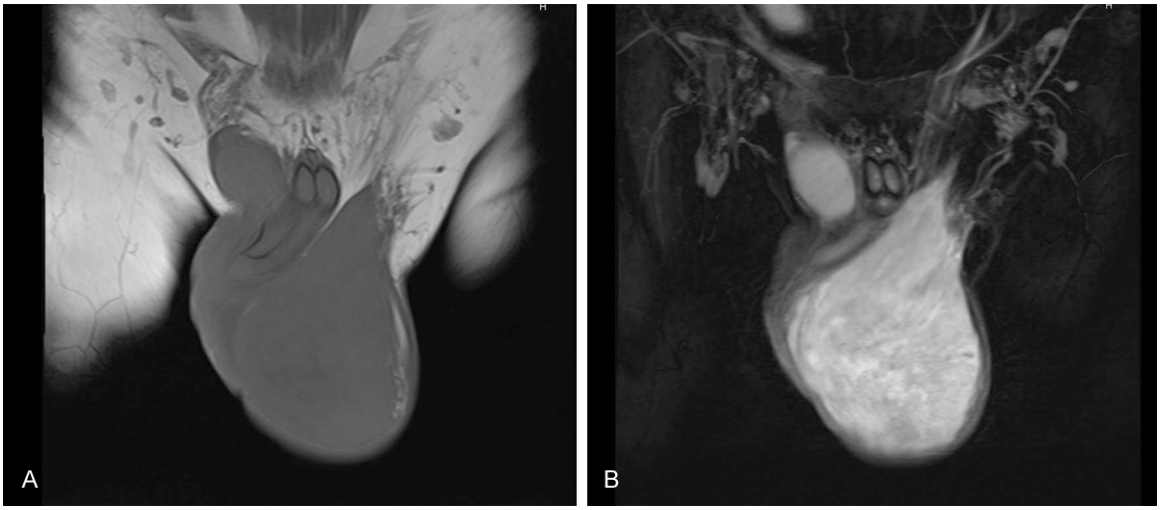

Figure 1. Magnetic resonance images of a large left scrotal tumor. A. T1-weighted images in coronal section show an isointence solid mass in the left scrotum. B. The demarcated mass with slight heterogeneous intensity signals is seen on T2-weighted images.

tion involving the *FOXO* gene family occur in alveolar rhabdomyosarcomas, acute myeloblastic leukemias and prostate carcinomas [13, 15].

In this study, we report a patient with CAF with monoallelic chromosome 13q14 and loss of *FOXO1* expression, which was accompanied by increased expression of oxidative stress markers such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (4-HNE). Activation of the p38 mitogen-activated protein kinase (p38 MAPK) pathway, which is often induced by stressors such as reactive oxygen species (ROS) [16, 17], was also demonstrated in CAF suggesting that loss of *FOXO1* function induces oxidative stress. To the best of our knowledge, this is the first report demonstrating the association between CAF and oxidative stress.

Materials and methods

Histopathological analysis

The excised tumor was fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. The sections were stained with hematoxylin and eosin (HE), azan, Alcian blue (pH 2.5), and Congo red. The paraffin sections were analyzed using immunohistochemistry. For antigen retrieval, heat induced epitope retrieval in citrate buffer (pH 6.0) was performed. Except for detecting oxidative stress markers such as 8-OHdG and 4-HNE, endogenous peroxidase was inactivated with 3% hydrogen peroxide and blocked with normal rabbit serum. After the primary antibody reaction, signal was detected by the streptavidin-biotin method. For 8-OHdG and 4-HNE detection, the sections without hydrogen peroxide treatment were blocked with normal rabbit serum. After the primary antibody reaction, goat anti mouse IgG conjugated with alkaline phosphatase was applied as secondary antibody. Fuchsin (Dako, Carpinteria, California) was used as a chromogen. Nuclear counterstaining was not performed.

Fluorescence in situ hybridization analysis

Fluorescence *in situ* hybridization (FISH) analysis was performed using a chromosome 13 (13q14)-specific probe (POSEIDON repeat-free FISH probes, KREATECH Diagnostics, Amsterdam, The Netherlands) according to the manufacturer's instructions. In brief, 4-μm thick paraffin-embedded tissue sections were deparaffinized with xylene and subsequently digested with pepsin using a commercial kit (KBI-60007 Tissue Digestion Kit I, KREATECH Diagnostics, Amsterdam, The Netherlands). Samples and probe were denatured at 80°C ± 1°C followed by hybridization at 37°C ± 1°C in a humidified chamber overnight. After post-hybridization washes, the sections were counterstained with 4',6-diamidino-2-phenylindole and examined using an Eclipse TE300 fluorescence microscope (Nikon, Tokyo, Japan).

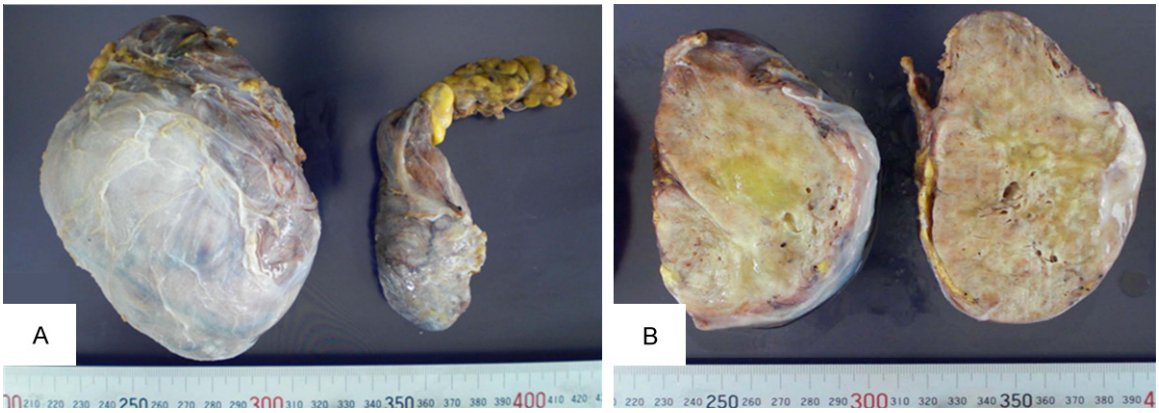

Figure 2. Macroscopic characteristics of the tumor. A. Resected tumor. The tumor (left) do not involve the testis or spermatic cord (right). B. The tumor is a well-circumscribed, solid, and white to gray with a yellow cast. Neither apparent hemorrhage nor necrosis is identified.

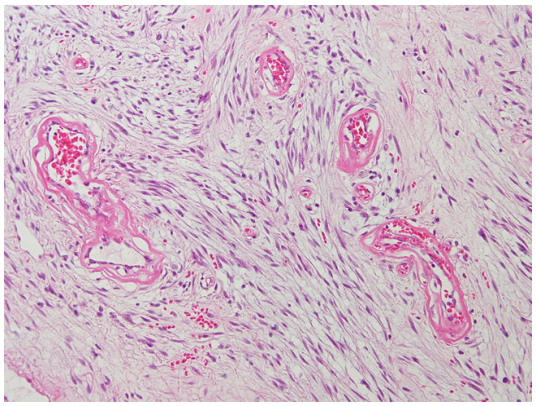

Figure 3. Histological findings of the tumor. Short spindle cells proliferate in edematous and mildly fibrous stroma with thick and hyalinized vessels.

Case report

Clinical presentation

A 69-year-old man presented with a gradually enlarging scrotal mass that had been present for approximately 20 years. Although the mass was painless, he noticed temporary rapid enlargement of his scrotal mass when he carried heavy loads of sugar cane, and the mass returned to its previous size when he did not carry heavy materials. Because rapid enlargement of scrotal mass impaired his walking, he visited the urology department in the hospital. Physical examination revealed an elastic hard mass in the subcutaneous tissue from the inguinal region to the scrotum. Magnetic resonance imaging (MRI) showed a large mass measuring 12.9 × 9.3 × 6.6 cm in the left scrotum (**Figure 1A**, **1B**). Because an epididymal tumor was suspected according to the results of MRI, orchiectomy was performed.

Pathological findings

On gross examination the tumor was a 13 × 10 × 7 cm well-circumscribed oval mass with a thin capsule. Cut section revealed a gray, tan surface with focally yellowish region, and an elastic hard consistency. Neither necrosis nor hemorrhage was present (**Figure 2A**, **2B**).

Histological examination revealed a tumor with moderate cellularity composed of ovoid or spindle tumor cells dispersed in a fibrous and myxoid stroma. Random distribution or a swirl pattern of tumor cells was noted and a fascicular arrangement of tumor cells around blood vessels was apparent in azan stained sections. The tumor cells contained oval or spindle shaped nuclei and scanty amounts of eosinophilic cytoplasm with ill-defined borders (**Figure 3**). Nuclear atypia was mild, and mitotic figures were infrequent (< 1 out of 10 high-power fields). Neither atypical mitotic figures nor nuclear pleomorphism was noted. There were numerous small to median-sized hyalinized or thick-walled blood vessels throughout the lesion. The stroma contained wispy collagen strands with edematous or myxoid changes (**Figure 3**). Mast cells were easily recognized, and small amount of fat tissue was present.

Immunohistochemically, the tumor cells were diffusely reactive for vimentin. There was focal positive-staining for CD34 (**Figure 4A**), a-smooth muscle actin, desmin, and calretinin, but negative for S-100, glial fibrillary acidic pro-

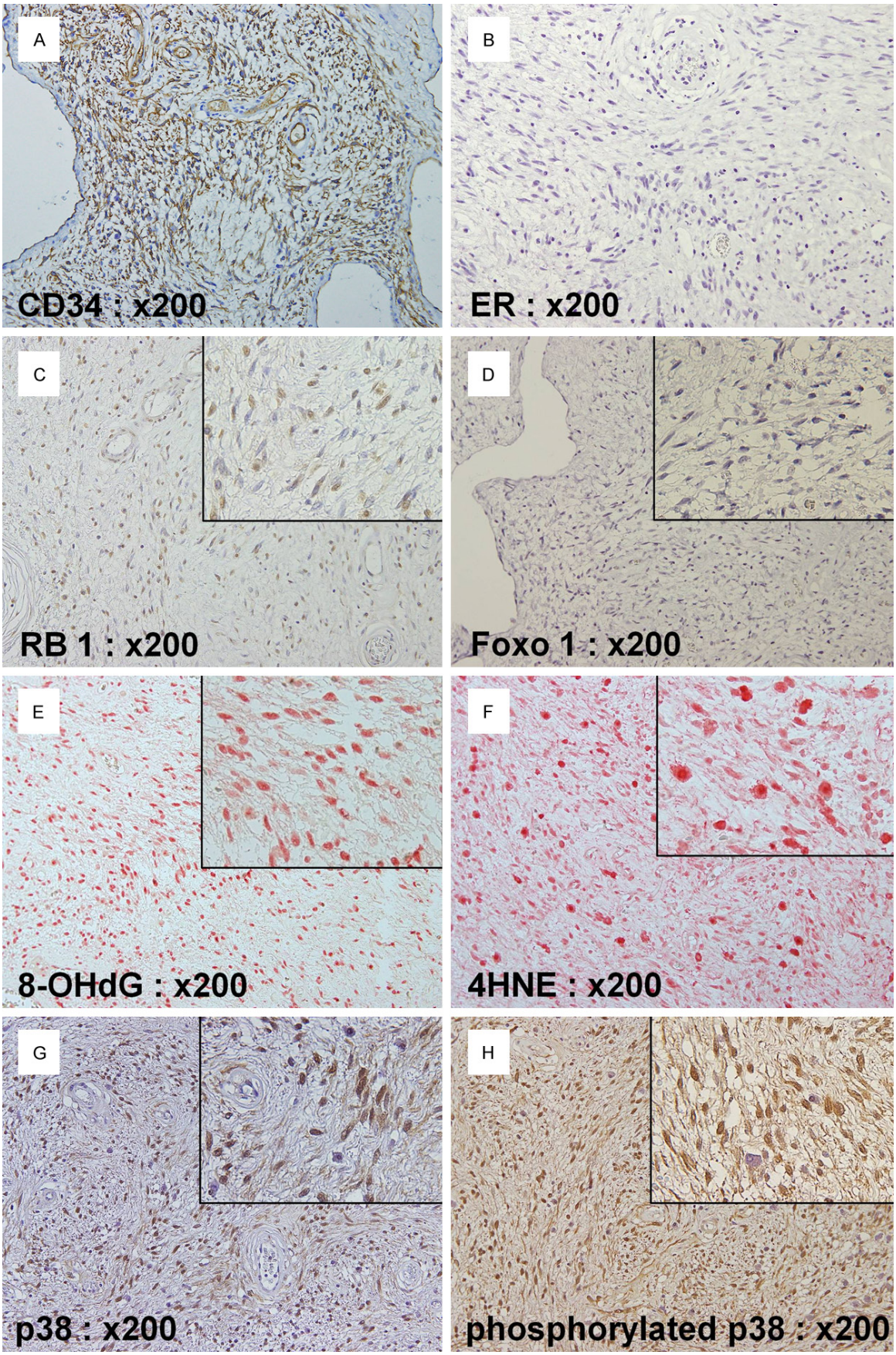
A
CD34 : x200
B
ER : x200
C
RB 1 : x200
D
Foxo 1 : x200
E
8-OHdG : x200
F
4HNE : x200
G
p38 : x200
H
phosphorylated p38 : x200

Figure 4. Immunohistochemical analysis. (A) In the lesion, focal clusters of the tumor cells expressing CD34 are noted. (B) The tumor cells stain negative for ER, and neither do PgR (data not shown). (C) Tumor cells show very low level of RB1 expression. Inset shows that the nuclei of the tumor cells are negative or weak positive for RB1, whereas infiltrating mast cells stain stronger positive signals. (D) Staining for FOXO1 is not detected in the tumor cells as evidenced in the Inset. (E) Tumor cells are positive for oxidized nucleic acids marker, 8-OHdG. Inset shows that nuclei of both tumor cells and mast cells exhibit positive signals for 8-OHdG. (F) Both tumor cells and mast cells are positive for peroxide lipids marker, 4HNE. Nuclei and cytoplasm of both tumor cells and mast cells show positive signals (Inset). (G, H) Tumor cells show p38 MAPK signal activation. Insets demonstrate that nuclei of tumor cells are positive for total p38 (G) and phosphorylated p38 (H), but mast cells and vascular endothelial cells are weak positive for both of them. Original magnifications of all the inset images are × 400.

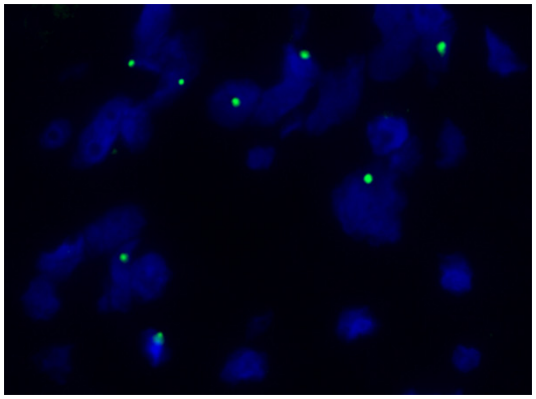

Figure 5. Fluorescent *in situ* hybridization analysis of chromosome 13q14. Monoallelic loss of 13q14 is evident in the tumor cells.

tein (GFAP), keratin, skeletal muscle actin (ACTA1), estrogen receptor (ER) (**Figure 4B**), progesterone receptor (PgR). The Ki-67 (MIB1) labeling index was less than 5%. Based on histological and immunohistochemical profiles, final diagnosis of CAF was made.

To explore the pathogenesis of CAF, transformation associated factors including oxidative stress markers, tumor suppressor genes, intracellular signal transduction factors, oncogenes, and cell cycle associated genes were evaluated by immunohistochemistry. Most tumor cells were strong positive for 8-OHdG, 4-HNE and negative or weak positive for RB1 (**Figure 4C**, **4E**, **4F**). As to expression level of RB1, infiltrating mast cells showed increased positive signals compared with that of tumor cells (**Figure 4C**). The nuclei of the tumor cells were positive for total p38 MAPK and phosphorylated p38 MAPK (**Figure 4G**, **4H**). Positive staining was not detected using antibody against FOXO1 (**Figure 4D**), KIT, p16 (CDKN2A), p21 ($p21^{WAF1}$), p27 ($p27^{KIP1}$), p53 (TP53), p65 (RELA), AKT, phosphorylated AKT, LYN, phosphoryrated LYN and β-catenin (CTNNB1).

FISH analysis using a probe specific for chromosome 13q14 detected a single signal in tumor cells (**Figure 5**) and two signals in the nuclei of normal cells, demonstrating that tumor cells were monoallelic for the 13q14 region.

Discussion

Tumorigenesis of CAF remains quite obscure. However, there are some studies reporting the association between CAF and hormone-receptors and it is postulated that CAFs that express ER and PgR arise from hormone-receptor positive mesenchymal cells [3, 18]. In the present case, the relationship between hormone-receptors and tumorigenesis is unclear, since tumor cells showed negative immunoreactivity for ER and PgR.

CAFs share some histological characteristics with spindle cell lipomas and mammary type myofibroblastomes [1-4, 6]. In addition, these tumors have common genetic alteration, i.e., monoallelic deletion of chromosome 13q14 [4-6, 19, 20]. These results suggest the presence of tumor suppressor gene(s) common to these three types of soft tissue tumor in chromosome 13q14. Therefore, we focused on RB1 and *FOXO1* as a candidate molecule involving the tumorigenesis of CAF, because both RB1 and *FOXO1* are located in 13q14 and are evidenced to function as a tumor suppressor [7, 8, 10-13, 15, 21, 22].

Chen *et al*. reported that soft tissue tumors with monoallelic 13q deletions, such as spindle cell lipomas, pleomorphic lipomas, CAFs, and majority of mammary type myofibroblastomas do not express RB1 [20]. They suggested that *RB1* might suffer a second hit or epigenetic changes which lead to the downregulation of RB1 expression and subsequent tumor development [20]. In the present case, spindle tumor cells showed negative or weak signals of RB1 whereas infiltrating mast cells exhibited increased expression. This reflects the status of chromosome 13q14, because the spindle tumor cells lost monoallele of 13q14 whereas

mast cells retain both alleles. Although studies on a mouse model with monoallelic deletion of *Rb1* demonstrate increased risk of pituitary and thyroid carcinomas [22], the mechanism of transformation of human cells with a monoallelic *RB1* deletion is unknown.

Members of the *FOXO* family of transcription factors are related to various cellular functions such as induction of apoptosis, regulation of the cell cycle, repair of DNA damage, response to oxidative stress, cell differentiation, glucose metabolism [13, 14]. Resistance to oxidative stress mediated by *FOXO* transcription factors is achieved by increasing the expression of manganese superoxide dismutase (MnSOD) and catalase [13, 14], which function as scavengers essential for oxidative detoxification. Therefore, loss of *FOXO* expression induces increased oxidant stress through ROS [23].

In the present case, the tumor cells proved to be negative for *FOXO1* expression whereas strong positive for 8-OHdG and 4-HNE, which indicates increased oxidized nucleic acids and peroxidized lipids, respectively [24, 25]. In addition, total p38 MAPK and phosphorylated form of p38 MAPK were detected in the nuclei of tumor cells, indicating activation of the p38 MAPK signaling pathway [26, 27]. Since activation of the p38 MAPK pathway is induced by cellular stressors such as ROS [16], elevated levels of oxidation of DNA and peroxidation of lipids in the present case support that increased intracellular ROS generation was caused by loss of *FOXO1* expression. Because oxidative stress in sustained inflammation induce DNA damage such as DNA strand breaks, point mutations, and aberrant DNA cross-linking, it is postulated that intracellular ROS generation causes mutations in proto-oncogenes and tumor suppressor genes [28]. In addition to mutations giving rise to genomic instability and abnormal gene expression, ROS activate signaling pathways such as nuclear factor-kappa B (NF-κB), Janus kinase 2/Signal transducer and activator of transcription 5 (JAK2/STAT5), and RAS, that are associated with malignant transformation [28-32]. Moreover, ROS can directly cause degradation of proteins and lipids, leading to inhibition of enzymatic activities required for cellular function [33, 34]. By means of above-mentioned mechanisms, ROS can lead to tumor development through cellular proliferation, tumor cell invasion, angiogenesis, and cancer stem cell survival [28]. We can cite other example indicating that monoallelic deletion at *FOXO1* region is associated with tumorigenesis. Dong *et al.* reported that monoallelic deletion at *FOXO1* region was frequently detected in 22 of the 72 (31%) of prostate cancer cases and found inhibitory effect of *FOXO1* on androgen- and androgen receptor-mediated gene regulation and cell proliferation [15]. They also indicate that *FOXO1* rarely has inactivating mutations in the prostate cancer and suggest that down-regulation of *FOXO1* is caused by monoallelic deletion [15]. Magro *et al.* showed that monoallelic deletion of *FOXO1* occurs in most mammary and vaginal myofibroblastomas [19]. Besides, inactivation of *FOXO1* by the phosphatidylinositol 3-kinase/AKT pathway in response to growth factors is well-established phenomenon [13].

In the present study, we mainly described on a probable pathogenesis of CAF from the viewpoint of monoallelic 13q14 deletion, *FOXO1* signaling, and oxidative stress. A limitation of the present study is that we dealt with only a single patient. However, our case is instructive in demonstrating a probable mechanism for the tumorigenesis of CAF. We believe that our findings will give hints on future systematic studies with sufficient number of patients concerning the tumorigenesis of CAF as well as neoplasms with monoallelic 13q14 deletion.

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Disclosure of conflict of interest

None.

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