Metabolic Signature of Atypical Fibroxanthoma and Pleomorphic Dermal Sarcoma: Expression of Hypoxia-inducible Factor-1α and Several of Its Downstream Targets

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ABSTRACT Metabolic reprogramming mediated by hypoxia-inducible factors play a crucial role in many human cancers. HIF-1α is activated under hypoxic conditions and is considered a key regulator of oxygen homoeostasis during tumor proliferation under hypoxia. Aim of this research was to analyze the immunohistochemical expression of HIF-1α, VEGF-A, Glut-1, MCT4, and CAIX in atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS). 21 paraffin-embedded AFX and 22 PDS were analysed by immunohistochemistry, namely HIF-1α, VEGF-A (referred to as VEGF throughout the manuscript), Glut-1, MCT4, and CAIX. To quantify the protein expression, we considered the percentage of positive tumor cells (0: 0%, 1: up to 1%, 2: 2-10%, 3: 11-50%, 4: >50%) in relation to the staining intensity (0: negative, 1: low, 2: medium, 3: strong). HIF-1α expression (mean ± SD) in AFX (9.33±2.92) was significantly stronger than that in PDS (5.90±4.38; *P*= 0.007), whereas the expression of VEGF, Glut-1, MCT4, and CAIX did not show differences between AFX and PDS. When comparing all tumors without subgroup stratification, the expression of HIF-1α (*P*= 0.044) and MCT4 (*P*= 0.036) was significantly stronger in ulcerated tumors than in tumors without ulceration. Our findings provide the first evidence that HIF-1α-induced metabolic reprogramming may contribute to the pathogenesis of AFX and PDS. HIF-1α expression seems to be higher in AFX than in PDS, and ulcerated tumors show higher expression levels of HIF-1α and MCT4 irrespective of the diagnosis.

KEY WORDS: atypical fibroxanthoma, pleomorphic dermal sarcoma, HIF-1alpha, immunohistochemistry, metabolic signature

INTRODUCTION

Atypical fibroxanthoma (AFX) and pleomorphic dermal sarcoma (PDS) are rapidly growing cutaneous tumors typically affecting the UV-light exposed skin in elderly patients (1-3). Both tumors share many histological features (1). However, whereas AFX is per definition restricted to the dermis, PDS reveals invasion of deeper structures and often exhibits tumor necrosis as well as lymphovascular and perineural

invasion (1,4-6). AFX and PDS are commonly positive for CD10, CD68, and CD99 (1,4,7-11) and negative for cytokeratins (12,13), S100, CD34, and desmin (14). Nevertheless, there is very limited evidence on the pathogenetic aspects that are responsible for the higher biological aggressiveness of PDS, which is reflected in the fact that AFX generally does not recur after total excision, whereas PDS recurs locally in up to 50% and even metastasizes in up to 20% of cases (13,15).

Metabolic reprogramming and altered gene expression resulting from decreased oxygen availability enable tumors to adapt to their hypoxic environment (16). A key element in this context is hypoxia-inducible factor-1α (HIF-1α), an oxygen-sensitive transcription factor crucial for cancer cell progression under hypoxic conditions (16,17). Following HIF-1α activation, a number of proteins such as glucose transporter-1 (Glut-1), monocarboxylate transporter 4 (MCT4), carbonic anhydrase IX (CAIX), and vascular endothelial growth factor (VEGF) are upregulated and initiate carcinogenic processes such as angiogenesis, metabolic switching (glycolysis instead of oxidative phosphorylation), and cell proliferation (16,18,19).

HIF-1α overexpression has been reported in a variety of solid tumors and their metastases (19,20) and was associated with tumor invasiveness (21-23). Upregulation of Glut-1 contributes to an enhanced glucose metabolism in extensively proliferating cancer cells (16,24). MCT4 is upregulated in many tumors and mediates cellular lactate and proton efflux that characterizes the hyperglycolytic and acid-resistant metabolic phenotype of cancer cells (25-27). The pH regulator CAIX, another key element of metabolic reprogramming in tumor cells, is associated with metastatic spread and poor prognosis in various human tumors (25,28,29). VEGF represents a central downstream target of HIF-1α that acts as key driver of physiologic and pathologic (lymph-) angiogenesis (19,30). VEGF overexpression was reported in various human cutaneous tumors, indicating a critical role of VEGF in angiogenesis and tumor development in skin cancer (31).

Herein we analysed the metabolic signatures of AFX and PDS in order to shed more light on their different biological behavior. To the best of our knowledge, this is the first immunohistochemical study analyzing the expression of HIF-1α and several of its downstream targets in AFX and PDS.

PATIENTS AND METHODS

Specimens

Formalin-fixated, paraffin-embedded AFX (n=21) and PDS (n=22) were collected from the dermatopathology archives of the Departments for Dermatology at the University Hospitals in Cologne and Heidelberg and analysed by immunohistochemistry. Clinical data were obtained from patient charts. The study was approved by the local ethical research committees and was in accordance with the Helsinki Declaration of 1975 (revised in 2000). Clinical and histological data are summarized in Table 1.

Immunohistochemistry

Table 2 shows the details regarding each antibody, clone, source, dilution, and pretreatment. The expression of all studied proteins was evaluated using a quantification score (QS) calculated by multiplying the relative proportion of positive tumor cells (levels of positivity: 0: 0%, 1: up to 1%, 2: 2-10%, 3: 11-50%, and 4: >50%) with the value of the staining intensity (level of intensity: 0: negative, 1: low, 2: medium, and 3: strong). Immunohistochemical scoring was performed double-blinded by two independent dermatopathologists (FT and WH) without knowledge of the section number or any clinical data. All cases in which different scores were calculated were clarified in a follow-up joint review of the slides and discussed between the authors.

Statistical methods

Statistical analysis was performed using the SPSS statistical package (v24.0, SPSS Inc., Chicago, Illinois, USA). Group differences in the expression of the different proteins were tested using the Mann-Whitney U test with exact *P*-values. In order to illustrate the distribution of the different proteins expressed in AFX and PDS, the data are presented as box plots with medians, interquartile ranges, and ranges. Spearman's rank correlation coefficients were calculated to explore the correlation between the different protein expression scores and the clinical (age, sex) and histological (ulceration) data. A linear regression was calculated in order to evaluate the simultaneous influence of several predictors on HIF-1α expression. *P*-values <0.05 were considered statistically significant.

Table 1. Clinical and histological data

Clinical and histological data			
Feature		**AFX**	**PDS**
Age	Mean ± SD	77.7±7.8 years	82.8±6.8 years
Sex	female/male	2/19	1/21
Ulceration	Yes versus no	15:6	11:11

SD: standard deviation; AFX: atypical fibroxanthomas; PDS: pleomorphic dermal sarcomas

RESULTS

Clinical data

The mean (± standard deviation (SD)) age of patients with AFX (female/male: 2/19) was 77.71 (±7.79) years (range: 61-85 years) and the mean (± SD) age of patients with PDS (female/male: 1/22) was 82.83 (±6.84) years (range: 68-91 years). Patient demographics are summarized **in** detail in Table 1.

HIF-1α expression

HIF-1α expression (mean ± SD) in AFX (9.33±2.92) was significantly stronger than that in PDS (5.90±4.38; *P*=0.007). The expression of HIF-1α tended to be higher at the invading edges of tumor margins in AFX and PDS, whereas the central parts of the tumors tended to show weaker HIF-1α expression. Figure 1a depicts an AFX and Figure 1b shows a PDS with HIF-1α expression.

VEGF expression

VEGF expression (mean ± SD) in AFX (4.61±3.26) was not significantly different from that in PDS (4.31 ± 3.92; *P*=0.571). Figure 1c depicts an AFX with moderate and Figure 1d shows a PDS with strong VEGF expression.

Glut-1 expression

Interestingly, Glut-1 expression was found in none of the AFX samples and in only 2 of 22 PDS samples (9%). Glut-1 expression (mean ± SD) in AFX (0.00±0.00) was not significantly different from that in PDS (0.27±0.93; *P*=0.49).

MCT4 expression

MCT4 was expressed in all AFX samples and in 21 of 22 PDS samples (95%). MCT4 expression (mean ± SD) in AFX (7.90±3.47) did not differ significantly from that in PDS (8.31±3.84; *P*=0.573). Figure 1e and Figure 1f show an AFX and a PDS sample with strong MCT4 expression.

CAIX expression

CAIX was expressed in 10 of 21 AFX samples (48%) and in 9 of 22 PDS samples (41%). CAIX expression (mean ± SD) in AFX (2.66±3.24) did not differ significantly from that in PDS (2.04±3.25; *P*=0.479). Figure 1g and Figure 1h depict an AFX and a PDS with focal CAIX expression.

Clinical and histological data

When using the Mann-Whitney U test, the age (mean ± SD) showed a statistically significant difference between AFX (77.71±7.79) and PDS (82.83±6.84), (*P*=0.046). When using the Fishers exact test, the presence of tumor ulceration was not significantly correlated with the diagnosis of AFX or PDS (*P*=0.215).

Statistical correlation analyses

Protein expression levels

Spearman's rank correlation upon comparing AFX cases revealed that MCT4 expression was positively correlated with CAIX expression (correlation coefficient: 0.632, *P*=0.002). Spearman's rank correlation in comparing PDS cases revealed that HIF-1α expression was positively correlated with MCT4 expression (correlation coefficient: 0.591, *P*=0.004) and CAIX expression (correlation coefficient: 0.453, *P*=0.034). Furthermore, MCT4 expression was positively correlated with CAIX expression (correlation coefficient: 0.486, *P*=0.022).

Protein expression levels and clinical and histological data

When employing the Mann-Whitney U test, the expression of HIF-1α and MCT4 was significantly stronger in ulcerated tumors than in tumors without ulceration (*P*=0.044 and 0.036 respectively), independently of the diagnosis. CAIX expression was significantly stronger in male patients than in female patients (*P*=0.021). As only 3 of the 43 patients were women, the significance of these results must be interpreted cautiously. The expression levels of none of the other proteins were found to be significantly associated with patient age or sex.

Table 2. Primary antibodies used for immunostaining

Antibody	Clone	Source	Company	Dilution	Antigen Retrieval
VEGF	EP1176Y	rabbit	Zytomed systems, Berlin,Germany	1:200	pH 9.0
HIF-1alpha	polyclonal	rabbit	Bio-Techne, Minneapolis, USA	1:50	pH 9.0
Glut-1	SPM498	mouse	Zytomed systems, Berlin,Germany	1:400	pH 6.1
MCT4	D-1	mouse	Santa Cruz Biotechnology,Heidelberg, Germany	1:100	pH 9.0
CAIX	polyclonal	rabbit	Abcam, Cambridge, USA	1:200	pH 6.1

Linear regression

A linear regression model (predictors: diagnosis, age, and ulceration) resulted in R^2=0.222. While diagnosis, age, and ulceration were significantly associated with HIF-1α expression in bivariate analyses, in this multivariate analysis only diagnosis showed a significant influence on HIF-1α expression (*P*=0.023).

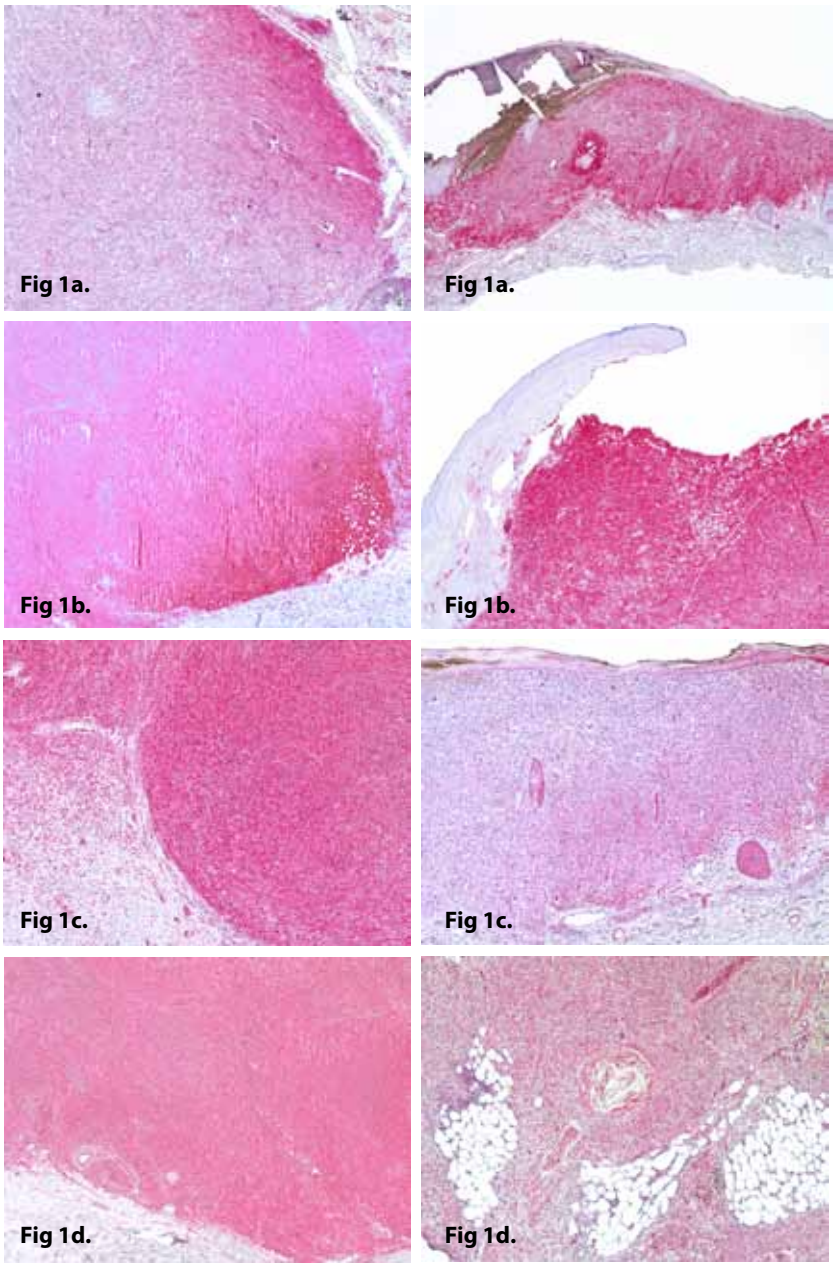

Figure 1. Expression of HIF-1α and its downstream factors in atypical fibroxanthoma and pleomorphic dermal sarcoma.a) Sample showing an AFX with moderate HIF-1α expression. (HIF-1α staining, original magnification x 50).

b) Section showing a PDS with moderate HIF-1α expression (HIF-1α staining, original magnification ×25). Note that the peripheral parts of the tumor reveal a stronger HIF-1α expression than the central parts.

c) Sample showing an AFX with moderate VEGF expression (VEGF staining, original magnification ×100).

d) Section showing a PDS with strong VEGF expression (VEGF staining, original magnification ×25).

e) Sample revealing strong MCT4 expression in an AFX (MCT4 staining, original magnification ×50).

f) Section revealing a PDS with strong MCT4 expression (MCT4 staining, original magnification ×25).

g) Sample revealing focally a moderate CAIX expression in an AFX (CAIX staining, original magnification ×25).

h) Section showing moderate CAIX expression in a PDS (CAIX staining, original magnification ×25).

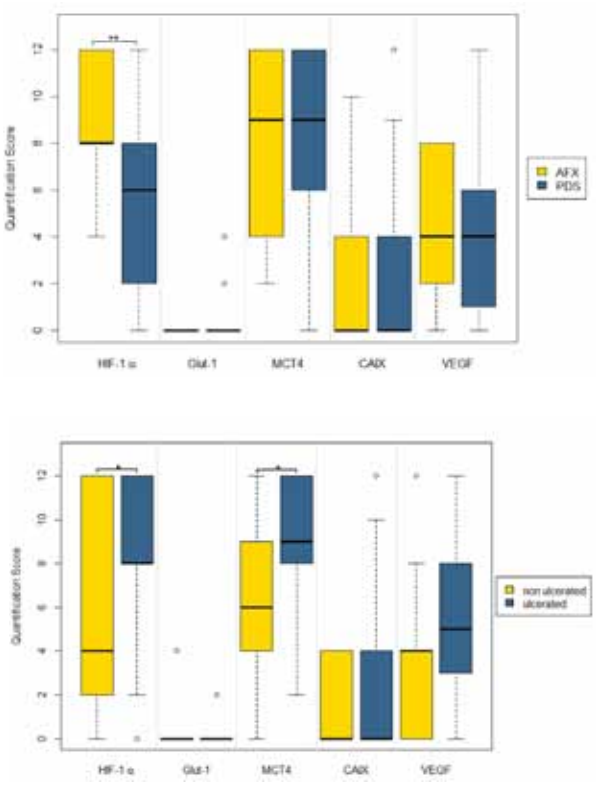

Figure 2a. Expression scores of HIF-1α, VEGF, Glut-1, MCT4 and CAIX in atypical fibroxanthomas and pleomorphic dermal sarcomas. **P*<0.05, ***P*<0.01

Figure 2b. Expression scores of HIF-1α, VEGF, Glut-1, MCT4 and CAIX in ulcerated and non-ulcerated tumors.

**P*<0.05, ***P*<0.01

DISCUSSION

Oncogene pathway analyses in AFX and PDS revealed similar oncogene expression profiles such as PT53, CCND1, and CDK4 overexpression (15). Given the various histological and genetic similarities, it is now widely accepted that AFX represents the non-infiltrating precursor lesion of PDS. In the present study, we analysed the expression of HIF-1α and several of its central downstream targets, namely Glut-1, MCT4, CAIX, and VEGF, in order to shed more light on metabolic reprogramming and angiogenesis in AFX and PDS. HIF-1α is activated under hypoxic conditions and considered a key regulator of oxygen homoeostasis during tumor proliferation under hypoxia (16,17). We found HIF-1α expression in all of the investigated AFX samples and in 82% of the PDS samples, strongly suggesting that HIF-1α may contribute to the pathogenesis of AFX and PDS. Our results are in accordance with other studies analyzing HIF-1α expression in skin tumors such as melanoma, basal cell carcinoma, or cutaneous squamous cell carcinoma (16,23). Zhong *et al*. found the highest density of HIF-1α-positive cells in numerous human cancers at the invading edges of tumor margins and adjacent to necrotic or strongly vascularized regions (20). In many samples, we also found HIF-1α expression predominantly in the invading edges of tumor margins. Interestingly, HIF-1α showed higher expression levels in ulcerated tumors. This might reflect a correlation of rapid tumor growth and decreased oxygen availability leading to ulceration. Additionally, we found stronger HIF-1α in AFX compared with PDS. At first glance these results might seem paradoxical, as AFX is considered a precursor of PDS. Nevertheless, there are several hypothetical explanations for these results. First, AFX might grow faster and therefore might present a higher level of tumor hypoxia than PDS, although the overall biological behavior of PDS is more aggressive. Second, the expression levels of a protein as measured by immunohistochemistry do not necessarily adequately reflect the protein activity. Third, we analysed a rather small number of tumors, so that the statistical results must be interpreted with caution and have to be confirmed in larger cohorts.

HIF-1α inhibition reflects a promising therapeutic option for cancer treatment (19). There are some comprehensive overviews of HIF-1α-related therapeutic options on record (19,22). In short, they describe different ways of of HIF-1α activity inhibition such as reducing mRNA, protein, DNA-binding capacity, or transcriptional activity (19). Small molecule inhibitors such like the mTOR inhibitor rapamycin seem to be most effective in blocking HIF-1α activity (19). Vincristine and 2-methoxyestradiol, inhibitors of farnesyl transferase, VEGFR and Raf inhibitors, and the topoisomerase I inhibitor topotecan are other substances capable of diminishing HIF-1α levels (19,22). However, further studies focusing on therapeutic strategies targeting HIF-1α and/or its downstream targets in locally advanced or metastasized cases of AFX and PDS are needed.

In order to satisfy an increased need for glucose, proliferating tumor cells can increase their expression of glucose transporters such as Glut-1. Nevertheless, studies analyzing Glut-1 expression in skin cancer are rare. Glut-1 was not expressed in any of the AFX samples and in only 2 of the PDS samples. These data are in accordance with the findings of Orrock *et al*., as they also found AFX to be consistently Glut-1 negative (32). These data provide evidence that upregulation of Glut-1 does not contribute to metabolic reprogramming in AFX and PDS. Nevertheless, further studies focusing on other glucose transporters (such as Glut-3) in these entities are warranted.

In the present study, MCT4 was expressed in both AFX (100% of the samples) and PDS (95% of the

samples). A central function of monocarboxylate transporters, including MCT4, is the prevention of a toxic build-up of intracellular lactate by mediating the efflux of lactate together with protons (33). Our findings point towards an upregulation of MCT4 during metabolic reprogramming in AFX and PDS, especially in ulcerated tumors.

CAIX, a HIF-1α inducible pH regulator, was expressed in 48% of the AFX and in 41% of the PDS samples. Enhanced glucose metabolism by cancer cells lowers the intracellular pH (acidosis) by increasing levels of lactate (29). However, acidosis disrupts cancer cell proliferation, migration, invasion, and metastasis (29). PH homeostasis under hypoxic conditions is sustained by CAIX, an enzyme whose membrane-bound overexpression has been described in various human cancers including carcinoma of the breast, kidney, lung, colon, cervix, and malignant melanoma (28,29). Furthermore, CAIX overexpression is associated with poorer outcomes (28,29). We found a positive correlation between HIF-1α and MCT4 as well as CAIX expression. These findings make it quite plausible that the upregulation of HIF-1α consecutively leads to an enhanced expression of its downstream targets MCT4 and CAIX.

VEGF, a central downstream protein of HIF-1α, is responsible for physiologic and pathologic (lymph-) angiogenesis and reflects a potential therapeutic target (30). VEGF acts on endothelial cells predominantly in a paracrine fashion (31). Interestingly, VEGF may additionally alter the survival and proliferation of tumor cells via an autocrine loop, thus influencing skin carcinogenesis (31). We found tumoral VEGF expression in 81% of the investigated AFX samples and in 82% of PDS samples. This upregulation of VEGF in AFX and PDS may be interpreted as an attempt of the tumors to adapt to their hypoxic micromilieu by stimulating angiogenesis.

Taken together, HIF-1α and its downstream targets MCT4, CAIX, and VEGF were upregulated in AFX and PDS and were frequently found in peripheral areas of the analysed tumors. Ulcerated tumors in particular presented higher expression levels of HIF-1α and its downstream target MCT4. These findings may reflect the phenomenon that rapidly proliferating tumor cells at the tumor periphery are not (yet) accompanied by a well-established tumor vasculature, leading to hypoxia, and may also reflect the association of rapid tumor growth, hypoxia, and ulceration.

Despite some limitations (i.e., retrospective analysis, no clinical follow-up data, relatively small sample number), our study provides the first evidence that the metabolic signatures of AFX and PDS may show some differences and that the expression of HIF-1α, MCT4, CAIX, and VEGF may play a role in the pathogenesis of these two tumors.

CONSLUSION

Our observations improve the knowledge on the metabolic signature of AFX and PDS. This might assist in developing new treatment modalities targeting HIF-1α and/or its downstream factors. Nevertheless, prospective multicenter studies investigating a higher number of tumors and the inclusion of follow-up data are needed to confirm the present findings.

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