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### Citation for published version:

Griewank, KG, Wiesner, T, Murali, R, Pischler, C, Müller, H, Koelsche, C, Möller, I, Franklin, C, Cosgarea, I, Sucker, A, Schadendorf, D, Schaller, J, Horn, S, Brenn, T & Mentzel, T 2017, 'Atypical fibroxanthoma and pleomorphic dermal sarcoma harbor frequent NOTCH1/2 and FAT1 mutations and similar DNA copy number alteration profiles', *Modern Pathology*. <https://doi.org/10.1038/modpathol.2017.146>

### Digital Object Identifier (DOI):

[10.1038/modpathol.2017.146](https://doi.org/10.1038/modpathol.2017.146)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Modern Pathology

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**Atypical fibroxanthoma and pleomorphic dermal sarcoma harbor frequent *NOTCH1/2* and *FAT1* mutations and similar DNA copy number alteration profiles**

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**Running Title:** Genetic alterations in atypical fibroxanthoma and pleomorphic dermal sarcoma

**Key Words:** Atypical fibroxanthoma, Pleomorphic dermal sarcoma, Undifferentiated pleomorphic sarcoma, Malignant fibrous histiocytoma, Dermatology, Cancer genetics

## Abstract

Atypical fibroxanthomas and pleomorphic dermal sarcomas are tumors arising in sun-damaged skin of elderly patients. They have differing prognoses and are currently distinguished using histological criteria, such as invasion of deeper tissue structures, necrosis and lymphovascular or perineural invasion. To investigate the as-yet poorly understood genetics of these tumors, 41 atypical fibroxanthomas and 40 pleomorphic dermal sarcomas were subjected to targeted next-generation sequencing approaches as well as DNA copy number analysis by comparative genomic hybridization. In an analysis of the entire coding region of 341 oncogenes and tumor suppressor genes in 13 atypical fibroxanthomas using an established hybridization-based next-generation sequencing approach, we found that these tumors harbor a large number of mutations. Gene alterations were identified in more than half of the analyzed samples in *FAT1*, *NOTCH1/2*, *CDKN2A*, *TP53* and the *TERT* promoter. The presence of these alterations was verified in 26 atypical fibroxanthoma

and 35 pleomorphic dermal sarcoma samples by targeted amplicon-based next-generation sequencing. Similar mutation profiles in *FAT1*, *NOTCH1/2*, *CDKN2A*, *TP53* and the *TERT* promoter were identified in both atypical fibroxanthoma and pleomorphic dermal sarcoma. Activating *RAS* mutations (G12, G13) identified in 3 pleomorphic dermal sarcoma were not found in atypical fibroxanthoma. Comprehensive DNA copy number analysis demonstrated a wide array of different copy number gains and losses, with similar profiles in atypical fibroxanthoma and pleomorphic dermal sarcoma. In summary, atypical fibroxanthoma and pleomorphic dermal sarcoma are highly mutated tumors with recurrent mutations in *FAT1*, *NOTCH1/2*, *CDKN2A*, *TP53* and the *TERT* promoter and a range of DNA copy

number alterations. These findings suggest that atypical fibroxanthomas and pleomorphic dermal sarcomas are genetically related, potentially representing two ends of a common tumor spectrum and distinguishing these entities is at present still best performed using histological criteria.

## Introduction

Atypical fibroxanthoma and pleomorphic dermal sarcoma are rare mesenchymal tumors typically arising in sun-damaged skin of elderly patients. The pathogenesis of these tumors is not well understood.

Atypical fibroxanthoma are tumors which occur in sun-damaged skin, primarily in the head and neck region of elderly patients <sup>1-4</sup>. More frequent in males, these tumors are often well circumscribed, rapidly-growing, and demonstrate an exophytic growth pattern. Known risk factors include UV-exposure, irradiation, xeroderma pigmentosum, and organ transplantation <sup>5</sup>. Histologically, the tumors are composed of atypical spindled and pleomorphic tumor cells including tumor giant cells. Primarily located within the dermis, tumors may have limited extension into the subcutis. High mitotic activity is customary, with presentation of frequent atypical mitotic figures <sup>2</sup>. Atypical fibroxanthoma do not invade the deep soft tissue, and despite increased proliferative activity, histological features such as necrosis, lymphovascular and/or perineural invasion are not observed. A diagnosis of atypical fibroxanthoma requires exclusion of other neoplasms, in particular, melanoma, squamous cell carcinoma, and leiomyosarcoma. Atypical fibroxanthoma generally has a good prognosis, and usually complete excision and regular follow-up are recommended <sup>1</sup>.

Pleomorphic dermal sarcomas demonstrate similar morphology to atypical fibroxanthoma, but present more aggressive histological features such as extensive involvement of the subcutis and/or deeper structures, areas of tumor necrosis, and lymphovascular or perineural invasion <sup>6</sup>. Pleomorphic dermal sarcoma display more aggressive clinical behavior than atypical fibroxanthoma and are categorized as tumors with low-grade malignant potential based on their potential for local recurrence and metastasis <sup>7</sup>. The tumors now defined as pleomorphic dermal

sarcoma have also been referred to as cutaneous undifferentiated pleomorphic sarcomas or as superficial malignant fibrous histiocytomas in the past<sup>8-10</sup>. There remains a lack of effective therapies for metastasized pleomorphic dermal sarcoma.

Distinguishing atypical fibroxanthoma from pleomorphic dermal sarcoma is critical due to their differences in clinical behavior. Discriminating these entities based on cell morphology alone is not possible as their cytologic features are similar. Histological criteria applied to distinguish pleomorphic dermal sarcoma from atypical fibroxanthoma are: larger tumor size; extensive infiltration of subcutis, invasion of fascia or muscle; necrosis; and vascular or perineural invasion<sup>2, 6</sup>. Attempts to differentiate between atypical fibroxanthoma and pleomorphic dermal sarcoma based on biopsy specimens should be avoided as these may not demonstrate the deepest extent of tumor involvement or miss histological criteria such as necrosis or vascular/perineural invasion present in other regions of the tumor. Attempts to identify immunohistochemical markers facilitating the distinction of atypical fibroxanthoma from pleomorphic dermal sarcoma (e.g. CD99 and LN-2<sup>11, 12</sup>) have not proven useful in routine practice<sup>1-3, 13</sup>.

Little is known of the genetic events leading to the development of atypical fibroxanthoma and pleomorphic dermal sarcoma. Previous small studies identified UV-signature mutations in *TP53* in atypical fibroxanthoma (7/10<sup>14</sup> and 4/6<sup>15</sup> cases) and pleomorphic dermal sarcoma (1/4 cases, diagnosed as 'malignant fibrous histiocytoma'<sup>15</sup>), as well as one *HRAS* and one *KRAS* mutation in 8 pleomorphic dermal sarcoma (diagnosed as 'malignant fibrous histiocytoma') analyzed<sup>16</sup>. In another study, pleomorphic dermal sarcoma (diagnosed as 'undifferentiated pleomorphic sarcoma') were found to harbor more frequent DNA copy number alterations than atypical fibroxanthoma<sup>17</sup>. We have reported high frequencies of

*TERT* promoter mutations in atypical fibroxanthoma and pleomorphic dermal sarcoma<sup>18</sup>. A recent study of 5 atypical fibroxanthoma and 5 pleomorphic dermal sarcoma demonstrated frequent *TP53* mutations as well as individual *CDKN2A*, *HRAS*, *KNSTRN* and *PIK3CA* gene mutations<sup>19</sup>.

The goal of our study was to try and identify recurrent gene mutations in atypical fibroxanthoma and pleomorphic dermal sarcoma applying newer sequencing technologies and genome-wide DNA copy number analysis. In addition to generally gaining a better understanding of the pathogenesis of these tumor entities, we attempted to determine if genetic alterations may be an additional diagnostic aid in distinguishing these tumors.

## **Materials and Methods**

### **Sample selection**

Atypical fibroxanthoma and pleomorphic dermal sarcoma tumor samples were obtained from the tissue archives of Dermatopathology Duisburg (24 atypical fibroxanthoma) and Dermatopathology Friedrichshafen (17 atypical fibroxanthoma, 20 pleomorphic dermal sarcoma), Germany, as well as from the Department of Pathology, Western General Hospital, The University of Edinburgh, Edinburgh, UK (20 pleomorphic dermal sarcoma, which were previously described by Miller et al.<sup>7</sup>). The study was done in accordance with the guidelines set forth by the ethics committee of the University of Duisburg-Essen.

### **Histopathology and Immunohistochemistry**

Histologic sections of all tumors were reviewed, and the diagnoses confirmed by at least two dermatopathologists (T.B., T.M., K.G.G., J.S.). Available clinical and

pathologic data was analyzed, including age, sex, site, size, depth, polyploid architecture, tumor mitotic rate, ulceration, necrosis, subcutis invasion, smooth muscle or fascia invasion, infiltrative or pushing border, lymphovascular invasion, and perineural invasion. Immunohistochemical markers applied to all tumors, including: pan-cytokeratin (MNF116; 1/500, AE1/AE3; 1/50), CD31 (JC70A; 1/100) or CD34 (QBEnd-10; 1/50), S100 (polyclonal; 1/2000), desmin (D33; 1/100), smooth muscle actin (ASMA 1A4; 1/500), and Melan-A (A103; 1/1000). The described antibodies were obtained from DAKO Hamburg/Germany (Duisburg and Friedrichshafen cases). Stains in the cases from Edinburgh were performed as described previously<sup>7</sup>.

### **DNA isolation**

10µm-thick sections were cut from formalin-fixed, paraffin-embedded tumor tissues. Sections were deparaffinized and tumor-bearing areas manually macrodissected according to routine procedures. Isolation of genomic DNA was performed applying the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

### **DNA copy number analysis**

Array-based comparative genomic hybridization was used to perform analysis of DNA copy number aberrations. The methods for hybridization and analysis, including GISTIC 2.0 statistical analysis, have been described previously<sup>20-23</sup>. In individual cases whole genome amplification was performed using Sigma's GenomePlex® Single Cell Whole Genome Amplification Kit as described previously

## **Hybridization-capture based Next-Generation Sequencing for known oncogene mutations**

Custom DNA probes were designed for targeted sequencing of all exons and selected introns of 341 oncogenes and tumor suppressor genes (MSK-IMPACT assay)<sup>25</sup>. Briefly, genomic DNA from tumor samples was used to prepare barcoded libraries using the KAPA HTP protocol (Kapa Biosystems, Wilmington, MA) and the Biomek FX system (Beckman Coulter, Brea, CA). Libraries were pooled, captured and subsequently sequenced on an Illumina HiSeq 2500 system as paired-end reads. Sequenced reads were trimmed to remove vestigial adaptor sequences using TrimGalore<sup>26</sup>, and were aligned to the hg19 human reference genome using BWA<sup>27</sup>. PCR duplicates were removed from the alignment output, and the aligned reads were subjected to local indel realignment and base quality recalibration using GATK<sup>28</sup>. Somatic variant calling was performed using MuTect<sup>29</sup> for single nucleotide variants (SNVs) and SomaticIndelDetector<sup>28</sup> for indels. Significant copy number gains and losses were detected by requiring a greater-than two-fold change in normalized coverage between tumor and a comparator reference fresh frozen paraffin embedded normal. Somatic structural variants were detected using DELLY<sup>30</sup>, requiring both paired-end and split-read support.

## **Amplicon based Targeted Next Generation Sequencing (amplicon next-generation sequencing)**

A custom amplicon-based sequencing panel covering 11 genes (*HRAS*, *KRAS*, *NRAS*, *CDKN2A*, *FAT1*, *KNSTRN*, *TP53*, *NOTCH1*, *NOTCH2*, *PIK3CA* and *TSC2*) was designed (Table 1). This panel included the genes most frequently found

mutated in atypical fibroxanthoma in our prior screen (impact next-generation sequencing), as well as the *RAS* genes which were previously reported to be mutated in pleomorphic dermal sarcomas <sup>16</sup>. Library preparation was performed applying the GeneRead Library Prep Kit from QIAGEN<sup>®</sup> according to the manufacturer's instructions. NEB Next Ultra DNA Library Prep Mastermix Set and NEBNext Multiplex Oligos for Illumina from New England Biolabs were used for adapter ligation and barcoding individual samples. Sequencing was performed on an Illumina MiSeq next generation sequencer, sequenced up to 24 samples in parallel. Sequencing the sample cohort, an average coverage of 2803 reads was achieved with 82% of the target area having a minimum coverage of 30 reads.

### **Sequence analysis**

CLC Cancer Research Workbench from QIAGEN<sup>®</sup> was applied as previously reported <sup>31</sup> for sequence analysis. Briefly, the CLC workflow included adapter trimming and read pair merging before mapping to the human reference genome (hg19). Subsequently insertions and deletions as well as single nucleotide variant detection, local realignment and primer trimming followed. Various databases were cross-referenced (COSMIC, ClinVar, dbSNP, 1000 Genomes Project, HAPMAP and PhastCons-Conservation\_scores\_hg19) regarding information on mutation type, single nucleotide polymorphisms and conservation scores. Csv files were further analyzed manually screening for protein coding mutations predicted to result in non-synonymous amino acid changes. Mutations were considered if the overall coverage of the mutation site was  $\geq 30$  reads,  $\geq 10$  reads reported the mutated variant and the frequency of mutated reads was  $\geq 10\%$ .

## **Direct (Sanger) sequencing**

PCR amplification of the *TERT* promoter region was done as previously described<sup>18</sup>. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and used as templates for sequencing. The sequencing chromatogram files were examined using Chromas software (version 2.01, University of Sussex, Brighton, United Kingdom) or Sequencher software (version 5.1, Gene Codes Corporation, Ann Arbor, MI, USA).

## **Associations of gene mutation status with clinical and pathologic parameters**

We investigated associations of mutation status with available clinical and pathological parameters using non-parametric tests, chi-squared tests and Fisher exact tests as appropriate. All statistical analyses were performed using PASW Statistics 18 software (International Business Machines Corp., Armonk NY, USA). A p-value of  $p \leq 0.05$  was considered statistically significant.

## **Results**

### **Study cohort**

Eighty-one (41 atypical fibroxanthoma and 40 pleomorphic dermal sarcoma) tumors were analyzed, of which 77 were primary tumor samples and 4 were recurrent pleomorphic dermal sarcoma. 67 patients were male, 14 were female. The median age at diagnosis was 82 (range 47-102) years overall; 79 (range 65-93) years for atypical fibroxanthoma and 84 (range 47-102) years for pleomorphic dermal sarcoma. An overview of the different genetic analysis applied to each sample is given in Supplemental Table 1.

## **Clinical and pathologic features of atypical fibroxanthoma and pleomorphic dermal sarcoma**

Clinical and pathologic features are listed in Table 1. Tumors were negative for most listed immunohistochemical markers. Focal or patchy smooth muscle actin expression was seen in 14 (34%) atypical fibroxanthoma and 11 (28%) pleomorphic dermal sarcoma. As previously reported<sup>7</sup>, 10 (25%) pleomorphic dermal sarcoma cases showed focal expression of CD31 and one pleomorphic dermal sarcoma exhibited limited aberrant expression of Melan-A (S100 was negative).

## **Genomic findings**

### **Hybridization-capture based next-generation sequencing for known oncogene mutations**

A considerable number of mutations were identified in the 13 atypical fibroxanthoma tumor samples analyzed with an overall mean sequencing depth of 492 fold. The average number of mutations identified in each sample was 55, the range was between 34 and 94 mutations. Genes mutated in more than 3 tumors are demonstrated in Figure 1 (all mutations identified are listed in Supplemental Table 2). Particularly frequent were *TP53* mutations, identified in all samples, with 10/13 (77%) samples harboring inactivating mutations. *TERT* promoter mutations were identified in 12/13 (92%) samples. In *FAT1*, 8 of 13 (62%) samples harbored mutations, 7 (87.5%) were inactivating. Other apparent alterations affected *CDKN2A* with 6 of 13 (46%) mutations, 5 (83%) inactivating. *NOTCH1* was mutated in 11 of 13 (85%) samples, also with 5 (45%) inactivating mutations (5). Although the panel

covers most known activating mutations in human cancer <sup>25</sup>, no recurrent known functionally activating protein-coding mutations were identified in these tumors.

### **Targeted amplicon-based next-generation sequencing (amplicon next-generation sequencing)**

To further assess and validate the distribution of many of the identified recurrent mutations a custom panel was designed and applied covering the most frequently mutated genes (Table 1). In addition to genes identified in the described screen for oncogene mutations, the *RAS* genes, previously reported to be mutated in pleomorphic dermal sarcoma <sup>16</sup>, and *KNSTRN*, a gene reported mutated in squamous cell carcinoma <sup>32</sup>, were included in the panel. Overall 26 atypical fibroxanthoma and 35 pleomorphic dermal sarcoma were sequenced. The mutation profiles of both tumor entities were similar (Figure 2, Supplemental Table 3). High numbers of *TP53* mutations in 22/26 (85%) atypical fibroxanthoma and 31/35 (89%) pleomorphic dermal sarcoma, *NOTCH1* mutations in 14/26 (54%) atypical fibroxanthoma and 22/35 (63%) pleomorphic dermal sarcoma, *NOTCH2* mutations in 11/26 (42%) atypical fibroxanthoma and 17/35 (49%) pleomorphic dermal sarcoma as well as *FAT1* mutations in 15/26 (58%) atypical fibroxanthoma and 24/35 (69%) pleomorphic dermal sarcoma were identified. Less frequently detected were *TSC2*, *PIK3CA* and *CDKN2A* mutations (Figure 2).

### **UV-signature mutation analysis**

To assess the role of UV-exposure in the pathogenesis of atypical fibroxanthoma and pleomorphic dermal sarcoma, an analysis of mutations identified in the *TP53* gene was performed, assessing the amount of potentially UV-induced

mutations. An approach which has been previously utilized for cutaneous tumors<sup>14, 33-35</sup>. In 61 tumors, 93 mutations in *TP53* were identified, 37 in 25 atypical fibroxanthoma and 56 in 36 pleomorphic dermal sarcoma. The frequency of mutations with a UV-signature was high in both tumors, with C>T (G>A) and CC>TT (GG>AA) mutations being detected in 43% (16/37) and 14% (5/37) of mutations in atypical fibroxanthoma and 57% (32/56) and 7% (4/56) of mutations in pleomorphic dermal sarcoma, respectively.

### ***TERT* Promoter**

The *TERT* promoter was analyzed by Sanger sequencing as previously described<sup>18</sup>. The majority of tumors 19/20 (95%) atypical fibroxanthoma and 18/24 (75%) pleomorphic dermal sarcoma where sequencing was successful, demonstrated at least one known activating hot-spot mutation in the *TERT* promoter.

### **Copy number alterations**

Copy number information was performed by comparative genomic hybridization in 42 samples, 20 atypical fibroxanthoma and 22 pleomorphic dermal sarcoma samples. The overall results are demonstrated in Figure 3. Tumors showed a considerable number of alterations. Evident differences in terms of alteration profiles between atypical fibroxanthoma and pleomorphic dermal sarcoma were not apparent. Common were losses of 8p, 9p and 9q. Larger deletions involving most of Chr. 13, 16 and 18 were also apparent. Gains were less frequent however identified in Chr. 1q, 8q, 17q, 19p. The profile of alterations was very similar in atypical fibroxanthoma and pleomorphic dermal sarcoma (Figure 3).

## **Associations of clinicopathologic and mutation status in atypical fibroxanthoma and pleomorphic dermal sarcoma**

The complete analysis is presented in Table 2. Histological criteria used to distinguish atypical fibroxanthoma from pleomorphic dermal sarcoma were found to be clearly associated with tumor type, including: necrosis ( $p < 0.001$ ), invasion into subcutis ( $p < 0.001$ ), invasion into fascia/skeletal muscle ( $p < 0.001$ ), border ( $p < 0.001$ ), lymphovascular invasion ( $p = 0.001$ ) and perineural invasion ( $p < 0.001$ ). Differences in terms of gene mutation status between atypical fibroxanthoma and pleomorphic dermal sarcoma demonstrated in Table 3 were not found to be statistically significant. An analysis of gene mutation status with individual histopathological criteria (Supplemental Table 4) demonstrated no correlations with mutation status with the exception of age with *NOTCH2* ( $p = 0.05$ ), tumor depth with *TP53* ( $p = 0.05$ ), and necrosis with *TERT* promoter ( $p = 0.002$ ) mutation status (shown in detail in Supplemental Table 4).

## **Discussion**

The genetic underpinnings of atypical fibroxanthoma and pleomorphic dermal sarcoma, as well as the relationship between these two tumor entities has been a question of debate for many years. We believe the findings of our study contribute to a better understanding of these entities, demonstrating that these tumors harbor a large number of common genetic alterations. In addition to known *TERT* promoter and *TP53* mutations, alterations in *CDKN2A*, *FAT1*, *NOTCH1* and *NOTCH2* were identified particularly frequently.

The comprehensive screen for known cancer mutations in 341 known cancer genes demonstrated a very high frequency of mutations overall. A selection of these

genes is shown in Figure 1 (only genes mutated in at least 3 tumors are included). Recurrent gene mutations known to activate signaling pathways such as the MAPK pathway were not identified. Neither were recurrent hot-spot mutations in genes other than the *TERT* promoter<sup>18</sup>. What was striking is that in the large array of frequently mutated genes, some genes were mutated in a high percentage of samples, including frequent loss-of-function mutations.

*TERT* promoter mutations, originally identified in cutaneous melanoma<sup>36, 37</sup> were subsequently identified in a wide array of cancers<sup>38</sup> and previously shown by our group to occur in the majority (>70%) of atypical fibroxanthoma and pleomorphic dermal sarcoma samples<sup>18</sup>. This finding was validated in the present study (Figure 1 + 2).

*TP53* has been known to be altered in atypical fibroxanthoma and pleomorphic dermal sarcoma for more than two decades<sup>14, 15</sup>. Our study validates this finding, demonstrating *TP53* mutations in the majority of all tumor samples (atypical fibroxanthoma MSK-IMPACT next-generation sequencing = 100%, atypical fibroxanthoma amplicon next-generation sequencing = 89%, pleomorphic dermal sarcoma amplicon next-generation sequencing = 86%) with a considerable number of samples harboring inactivation mutations (atypical fibroxanthoma MSK-IMPACT next-generation sequencing = 77%, atypical fibroxanthoma amplicon next-generation sequencing = 36%, pleomorphic dermal sarcoma amplicon next-generation sequencing = 31%). Additionally, losses of the *TP53* gene locus on 17p did occur. These findings validate *TP53* as a highly altered and important gene in atypical fibroxanthoma and pleomorphic dermal sarcoma.

*FAT1* mutations were previously identified in various cancers including glioblastoma, colorectal and head and neck cancer. Both losses and mutations were

reported<sup>39</sup>. In our data, both frequent losses of Chr. 4q (Figure 3) as well as a very high frequency of gene mutations were detected. In all screens performed, the mutation frequency was ~60% of tumors (atypical fibroxanthoma MSK-IMPACT next-generation sequencing 62%, atypical fibroxanthoma amplicon next-generation sequencing = 58%, pleomorphic dermal sarcoma amplicon next-generation sequencing = 67%) the percentage of inactivating *FAT1* mutations >40% (atypical fibroxanthoma impact next-generation sequencing 64%, atypical fibroxanthoma amplicon next-generation sequencing = 42%, pleomorphic dermal sarcoma amplicon next-generation sequencing = 44%). *FAT1* inactivation has been associated with increased  $\beta$ -catenin and Wnt signaling<sup>39</sup>, suggesting Wnt signaling may play an important role in atypical fibroxanthoma and pleomorphic dermal sarcoma pathogenesis.

*NOTCH* signaling, playing a critical role in tissue development, has long been known to play a relevant role in cancer pathogenesis<sup>40-42</sup>. Originally associated with activating genetic alterations in hematological malignancies<sup>43</sup>, recurrent loss-of-function alterations have also been identified in a wide range of malignancies, including head and neck and cutaneous squamous cell carcinoma<sup>40, 44-46</sup>. Very similar to cutaneous squamous cell carcinoma, we identified the highest frequency of mutations in *NOTCH1* (atypical fibroxanthoma MSK-IMPACT next-generation sequencing = 85%, atypical fibroxanthoma amplicon next-generation sequencing = 54%, pleomorphic dermal sarcoma amplicon NGS = 66%) followed by *NOTCH2* (atypical fibroxanthoma MSK-IMPACT next-generation sequencing = 62%, atypical fibroxanthoma amplicon next-generation sequencing = 42%, pleomorphic dermal sarcoma amplicon next-generation sequencing = 49%). Both genes also demonstrated a considerable amount of inactivating mutations (Figure 1 + 2).

Chromosomal losses of the gene loci (*NOTCH1* 9q, *NOTCH2* 1p) were rare and probably are not of considerable relevance.

*CDKN2A* which codes for p14 and p16 is one of the most frequently lost tumor suppressors in human neoplasias<sup>47</sup> and is clearly also of relevance in atypical fibroxanthoma and pleomorphic dermal sarcoma. Not only was it found mutated in around half of the samples in the atypical fibroxanthoma impact next-generation sequencing screen (6 of 13 = 46%) but copy number analysis (comparative genomic hybridization) also demonstrated the locus on Chr. 9p to be lost in around half of atypical fibroxanthoma and pleomorphic dermal sarcoma tumor samples (Figure 3). The low mutation rate detected in the amplicon next-generation sequencing screen, is most likely due to poor coverage of the gene in our sequencing panel (Table 1) and losses of the gene locus being more frequent than focal gene mutations.

The analysis of C>T (G>A) and CC>TT (GG > AA) mutations associated with UV-exposure in the *TP53* gene showed these were frequent both in atypical fibroxanthoma and pleomorphic dermal sarcoma, representing more than half of the identified alterations in both tumor entities (57% in atypical fibroxanthoma and 64% in pleomorphic dermal sarcoma). Interestingly, C>T (G>A) alterations were detected more frequently in pleomorphic dermal sarcoma than atypical fibroxanthoma (57% and 43% of mutations, respectively), however CC>TT (GG>AA) were more frequent in atypical fibroxanthoma than pleomorphic dermal sarcoma (14% and 7% of mutations, respectively). Whereas C>T alterations are frequently UV-induced, there association with UV-exposure is not as strong as is the case for CC>TT alterations, which are considered to be virtually pathognomonic for UV-exposure<sup>33, 48</sup>. The overall high frequency of these mutations (both C>T and CC>TT) in both atypical

fibroxanthoma and pleomorphic dermal sarcoma clearly supports UV-exposure being a relevant pathogenic event in both of these tumor entities.

No associations between mutation status and diagnosis of atypical fibroxanthoma and pleomorphic dermal sarcoma were observed. The rare associations identified in our study with individual clinicopathological parameters: age with *NOTCH2* mutation status (p=0.05); tumor depth and *TP53* (p=0.05) mutation status; and necrosis and *TERT* promoter mutation status (p=0.002) (Supplemental Table 4) will need to be validated in larger independent cohorts.

Alterations in many genes including those recurrently detected in atypical fibroxanthoma and pleomorphic dermal sarcoma (*TP53*, *CDKN2A*, *FAT1*, *NOTCH1/2* and the *TERT* promoter) have been reported by various groups in cutaneous squamous cell carcinoma<sup>32, 49, 50</sup>. Given that these tumors (cutaneous squamous cell carcinomas, atypical fibroxanthoma and pleomorphic dermal sarcoma) all arise in areas of high sun exposure, it is likely that the similarities in mutation profiles are in part due to a common UV-induced pathogenesis of these entities. The signaling pathways essential for tumor development in these tumors may also be similar. While this is an intriguing hypothesis, additional genetic and functional studies will be required to fully reveal similarities and differences in the pathogenesis of these tumors.

One of the goals of our study was to try and identify genetic differences distinguishing atypical fibroxanthoma and pleomorphic dermal sarcoma. Our study finds little evidence for genetic differences between these entities. The copy number profiles determined here in a relatively large cohort of tumors (atypical fibroxanthoma = 20, pleomorphic dermal sarcoma = 22) are very similar, allowing no apparent separation of these entities. Genetically both tumor groups harbored similar

frequencies of *FAT1*, *TP53*, *CDKN2A*, *TERT* promoter and *NOTCH* alterations. What is interesting, is that activating *RAS* mutations, previously identified as being present in pleomorphic dermal sarcoma (diagnosed then as MFH) but not atypical fibroxanthoma were also found to be distributed in a similar fashion in our cohort. However, these activating mutations (2 *HRAS* G12S, G13V and 1 *KRAS* G12D) were rare, present only in 3 of 35 (9%) of pleomorphic dermal sarcoma. As such it seems unlikely determining *RAS* mutation status will be of use in clinical distinction between atypical fibroxanthoma and pleomorphic dermal sarcoma.

Considering the high mutation frequencies and copy number alterations present in both atypical fibroxanthoma and pleomorphic dermal sarcoma, our study suggests it will remain difficult to fully understand the key genetic mechanisms involved in the development of these tumors. Whole-exome or whole-genome sequencing may offer more insights, however distinguishing relevant driver mutations from the accompanying very high number of passenger mutations could prove a considerable challenge. To which extent alterations can be identified allowing a clear distinction of atypical fibroxanthoma and pleomorphic dermal sarcoma will remain to be seen.

As most mutations identified in our screen are assumed to be loss-of-function mutations, it may prove difficult identifying effective treatment strategies targeting cell intrinsic signaling pathways in atypical fibroxanthoma or pleomorphic dermal sarcoma. On the other hand, given that high mutational load is associated with better responses to immunotherapy with anti-CTLA4 and anti-PD1 immune checkpoint blockade therapies in many different cancer entities<sup>51-54</sup>, our findings may suggest immunotherapy as a promising therapeutic approach for recurrent or metastatic pleomorphic dermal sarcoma.

Weaknesses of our study are that only a selection of genes were analyzed. Whole exome or whole genome approaches should prove valuable in the future, however considering the high mutation frequency we observed, large numbers of tumors will need to be assessed to identify relevant recurrent events. Obtaining the necessary number of tissue samples (preferably fresh-frozen with paired germline DNA) and performing a meaningful bioinformatics analysis may prove challenging. The lack of available paired normal tissue in our study is another considerable caveat. Although most germline variants will have been successfully excluded by cross-referencing SNP databases, we cannot exclude the possibility that occasional germ-line variants were interpreted as somatic mutations. As a result, the frequency of somatic mutations we report is likely to be somewhat higher than is actually the case. However, the comparable results obtained in our study through two separate NGS approaches, capture (MSK-IMPACT NGS) and amplicon (amplicon NGS) and analyzed by two different bioinformatics approaches, supports the validity and accuracy of the somatic mutation profiles we report. However, larger future studies with paired germline DNA will be valuable to validate our findings and further elucidate the genetic landscape of these tumor entities.

Despite certain shortcomings, our study is the most comprehensive to date identifying both a number of previously unrecognized recurrent gene mutations (i.e. *NOTCH1*, *NOTCH2* and *FAT1*) and a fairly comprehensive picture of copy number alterations in these tumors. Our findings do not fully resolve the long-standing debate as to the relationship between atypical fibroxanthoma and pleomorphic dermal sarcoma. However, we do believe the similar gene mutations and copy number profiles identified argue strongly the tumors are related, potentially representing entities along a common tumor spectrum. Whereas future studies may manage to

identify robust genetic or other biomarkers allowing a clear diagnostic distinction of these two tumor entities, our current findings suggest the established histological criteria for distinguishing these two entities (e.g. larger tumor size with extensive involvement of the subcutis, musculature or fascia, perineural or perivascular invasion, or tissue necrosis) will remain of paramount importance in determining the prognosis and correct clinical management of affected patients.

In summary, our data demonstrate that atypical fibroxanthoma and pleomorphic dermal sarcoma harbor a large number of genetic alterations, including frequent *TP53*, *CDKN2A*, *TERT* promoter, *NOTCH1* and *NOTCH2* and *FAT1* gene alterations and a wide range of copy number alterations. Rare activating *RAS* mutations were only present in pleomorphic dermal sarcoma (9% of tumors). Whereas we believe these findings offer valuable insights into the pathogenesis of these tumors, it remains to be shown if they will be of diagnostic or therapeutic value in a clinical setting.

## **Financial disclosure**

The presented study was supported by a grant from the Mercur-Stiftung. This research was funded in part through the NIH/NCI Cancer Center Support Grant P30 CA008748. The funders were not involved in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplemental material is available on Modern Pathology's web site.

## **Competing interests**

None to declare

## **Acknowledgements**

We would like to thank Nadine Stadler and Iris Moll for excellent technical assistance.

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## **FIGURE LEGENDS**

### **Figure 1. Distribution of mutations in atypical fibroxanthomas**

Demonstrated is data from 13 atypical fibroxanthomas sequenced with a hybridization based screen covering 341 oncogenes and tumor suppressors. All exons of the genes were analyzed. The genes presented are those found to have been mutated in at least 3 samples. The mutation type is according to the legend. No mutation identified (wild-type) is signified by no fill, missense mutations of unclear consequence by grey filled boxes, loss of function (non-sense or frameshift) mutations are signified by red filled boxes, and activating mutations in the *TERT* promoter by blue filled boxes.

### **Figure 2. Distribution of mutations in atypical fibroxanthomas and pleomorphic dermal sarcomas**

Presented are the results of the amplicon based sequencing approach. All genes screened for in the amplicon panel are presented. Mutation type identified is presented in the figure legend.

### **Figure 3. Copy number profiles in atypical fibroxanthomas and pleomorphic dermal sarcomas**

Shown here are the comparative genomic hybridization results of 20 atypical fibroxanthoma shown on top, 22 pleomorphic dermal sarcomas shown in the middle panel and all 42 samples together, shown on the bottom panel. All groups were analyzed identically with Agilent software. Alterations are displayed as penetrance plots, gains in red, losses in green.