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Gene fusion detection in formalin-fixed paraffin-embedded benign fibrous histiocytomas using fluorescence *in situ* hybridization and RNA sequencing

Charles Walther[1], Jakob Hofvander[1], Jenny Nilsson[1], Linda Magnusson[1], Henryk A Domanski[2], David Gisselsson[1,2], Johnbosco Tayebwa[1], Leona A Doyle[3], Christopher DM Fletcher[3] and Fredrik Mertens[1]

Benign fibrous histiocytomas (FH) can be subdivided into several morphological and clinical subgroups. Recently, gene fusions involving either one of two protein kinase C genes (*PRKCB* and *PRKCD*) or the *ALK* gene were described in FH. We here wanted to evaluate the frequency of *PRKCB* and *PRKCD* gene fusions in FH. Using interphase fluorescence *in situ* hybridization on sections from formalin-fixed paraffin-embedded (FFPE) tumors, 36 cases could be analyzed. *PRKCB* or *PRKCD* rearrangements were seen in five tumors: 1/7 regular, 0/3 aneurysmal, 0/6 cellular, 2/7 epithelioid, 0/1 atypical, 2/10 deep, and 0/2 metastatic lesions. We also evaluated the status of the *ALK* gene in selected cases, finding rearrangements in 3/7 epithelioid and 0/1 atypical lesions. To assess the gene fusion status of FH further, deep sequencing of RNA (RNA-Seq) was performed on FFPE tissue from eight cases with unknown gene fusion status, as well as on two FH and six soft tissue sarcomas with known gene fusions; of the latter eight positive controls, the expected fusion transcript was found in all but one, while 2/8 FH with unknown genetic status showed fusion transcripts, including a novel *KIRREL/PRKCA* chimera. Thus, also a third member of the *PRKC* family is involved in FH tumorigenesis. We conclude that gene fusions involving *PRKC* genes occur in several morphological (regular, cellular, aneurysmal, epithelioid) and clinical (cutaneous, deep) subsets of FH, but they seem to account for only a minority of the cases. In epithelioid lesions, however, rearrangements of *PRKC* or *ALK* were seen, as mutually exclusive events, in the majority (5/7) of cases. Finally, the study also shows that RNA-Seq is a promising tool for identifying gene fusions in FFPE tissues.
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Benign fibrous histiocytoma (FH) can be subdivided into several morphological and clinical subgroups. Morphological variants include cellular, aneurysmal, epithelioid, and atypical types, and clinical manifestations range from benign tumors of the skin, deep soft tissues or skeleton to, rarely, metastasizing tumors.[1–3] We recently showed that both cutaneous and deep FH may carry specific gene fusions, in which a member—either *PRKCB* and *PRKCD*—of the gene family encoding protein kinase C (PRKC) is juxtaposed with a gene encoding a membrane-associated protein.[4] The pathogenetic mechanism was assumed to involve the uncoupling of the carboxy-terminal kinase domain of the PRKC protein from its regulatory domain, and its ectopic localization through fusion with the amino-terminal part of a membrane-associated protein. It has also recently been shown that some cases of epithelioid, and possibly also atypical, FH may harbor fusions activating the ALK protein.[5,6] The connection between *ALK* rearrangements and FH was further evaluated and strengthened by Doyle *et al.*, showing that *ALK* fusions were restricted to the epithelioid subtype.[7]

To study the frequency and distribution of *PRKCB* and *PRKCD* fusions in FH, we used interphase fluorescence *in situ* hybridization (FISH) on a series of tumors that represented different morphological and clinical subsets; the results of that study prompted transcriptome sequencing (RNA-Seq) of RNA from formalin-fixed paraffin-embedded (FFPE) tissue from a second set of FH.

MATERIALS AND METHODS

Patients and Tumors

For the interphase FISH studies, 66 cases of FH were retrieved from the consultation files of one of the authors (CDM

[1]Department of Clinical Genetics, University and Regional Laboratories, Skåne University Hospital, Lund University, Lund, Sweden; [2]Department of Pathology, University and Regional Laboratories, Skåne University Hospital, Lund, Sweden and [3]Department of Pathology, Brigham and Women´s Hospital, Harvard Medical School, Boston, MA, USA
Correspondence: Dr C Walther, MD, Department of Clinical Genetics, University and Regional Laboratories, Skåne University Hospital, Lund 221 85, Sweden.
E-mail: charles.walther@med.lu.se

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Fletcher). The cases were selected to represent different clinical and morphological aspects of FH: 10 regular, 10 cellular, 10 aneurysmal, 10 atypical, 10 epithelioid, 11 deep, and 5 metastatic lesions; no prior genetic information was available on these tumors, except for two epithelioid FH that had been analyzed with regard to *ALK* gene rearrangements. The diagnostic criteria have been outlined before.[1,8–13] Two cases of regular FH with known fusions involving *PRKCB* and *PRKCD* (previously published as Cases 5 and 6 in[4]), respectively, were used as positive controls. All seven epithelioid FH that could be analyzed by FISH were also analyzed with regard to expression of the ALK protein, as described.[7]

For the RNA-Seq study, another set of six recently (2011–2013) diagnosed cutaneous regular FH was chosen. In addition, we included two cytogenetically analyzed FH from which no frozen tissue was available: one cellular/aneurysmal FH from 1990 (Case 7 in Plaszczyca *et al*.[4] and Case Lu 317 in Vanni *et al*.[14]) with an unbalanced t(3;12) and one cellular FH from 2010 with a balanced t(1;16)(p36;p11), strongly suggestive of a *PDPN/PRKCB* fusion. As positive controls, we selected two FH from 2009 with *PDPN/PRKCB* fusions (Cases 2 and 5 in Plaszczyca *et al*.[4]), four myxoid liposarcomas (MLS) from 2011 or 2014 with *FUS*/*DDIT3* fusions, one synovial sarcoma from 2013 with an *SS18*/*SSX2* fusion, and one low-grade fibromyxoid sarcoma from 2013 with an *FUS*/*CREB3L2* fusion; the fusion gene status of the positive controls had been established by G-banding, FISH, and/or RT-PCR (data not shown). Thus, the RNA-Seq study included eight FH with unknown fusion gene status and eight soft tissue tumors, including two cases of FH, with known gene fusions.

FISH

Interphase FISH for the detection of rearrangements of *PRKCB* and *PRKCD*, mapping to chromosome bands 16p12 and 3p21, respectively, was performed on 4 μm FFPE tumor sections. The bacterial artificial chromosome (BAC) probes were obtained from the BACPAC Resource Center (http://bacpac.chori.org; Supplementary Table 1). Clone preparation, hybridization, and analysis were performed as described.[15,16] On average 113 (range = 83–176) nuclei from different areas were evaluated per probe set in each case. Nuclei displaying separate green and red signals or a single 3′ signal were scored as positive. In seven epithelioid and one atypical FH, the status of the *ALK* gene was also investigated, using a commercial double color, break-apart probe (LSI ALK, Vysis, Abbott Molecular, IL, USA); in each case, 95–112 nuclei were scored. The status of the *PRKCA* gene was evaluated in Case 39, using probes described in Supplementary Table 1; 93 nuclei were scored.

RNA-Seq and RT-PCR

For RNA extraction, tumor blocks from each of the 16 cases (Cases 37–44 and PC3–10) were retrieved and tumor-representative areas were selected. Then, 10 μm sections were cut from each tumor ($n = 3$–38, depending on the size of the selected areas; the aim was to achieve a total of 450 mm^2 of tumor tissue from each case). The first two sections were discarded and the following sections were immediately put in Deparaffinization Solution (Qiagen, Valencia, CA, USA) followed by RNA extraction with Qiagen's RNeasy FFPE Kit, according to the manufacturer's recommendations. The quality and quantity of the RNA, with a particular focus on the fraction of RNA fragments >200 nt (the DV_{200}), were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). mRNA libraries were prepared from 20 to 50 ng, depending on the DV_{200} value, of the extracted RNA using the capturing chemistry of the TruSeq RNA Access Library Prep Kit (Illumina, San Diego, CA, USA), according to the manufacturer's recommendations. The quality of the mRNA library was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies) and the concentration was measured on a Qubit Fluorometer using the Qubit RNA HS Assay kit (Life Technologies, Rockville, MD, USA). With a loading concentration of 1.8 pM, paired-end 2×81 nt reads were generated in-house from the mRNA libraries using Illumina's High Output Kit (150 cycles) on a NextSeq 500 (Illumina). To identify candidate fusion transcripts from the sequence data, analyses were performed on fastq files using ChimeraScan version 0.4.5 and TopHat version 2.0.7,[17,18] as decribed.[19,20] The GRCh37/hg19 build was used as the human reference genome.

RT-PCR was performed in Case 39, using primers specific for *KIRREL* (forward, 5'-GACCGGGAGGATGACACCG-3') and *PRKCA* (reverse, 5'-GGGACTGATGACTTTGTT GCC-3'). RT-PCR and sequencing were performed as described.[16]

RESULTS

FISH

For technical reasons, interphase FISH for *PRKCB* and/or *PRKCD* rearrangements was possible to perform in only 36 of the selected FH cases (cases 1–36) and in two positive controls (PC1–2; Table 1); the remaining samples did not yield signals of sufficient quality. The distribution of positive nuclei differed between the two probe sets: the mean numbers of positive nuclei for *PRKCB* and *PRKCD* rearrangements were 8.4% and 15.5%, with standard deviations (s.d.) 7.1% and 11.3%, respectively. Cutoff values for scoring cases as positive were chosen at $>23\%$ for *PRKCB* and $>38\%$ for *PRKCD* (i.e., mean+2 s.d.). The cutoff for the commercial ALK probe was set at $>15\%$. Using these criteria, the two positive controls, i.e., tumors in which we previously had identified fusions using RT-PCR on frozen tissue,[4] were positive for the expected *PRKC* gene and negative for the other (Table 1). *PRKCB* was scored as positive in 3/34 cases and *PRKCD* was positive in 2/31 cases. The *PRKC*-positive FH cases were distributed as follows: 1/7 regular, 0/3 aneurysmal, 0/6 cellular, 2/7 epithelioid, 0/1 atypical, 2/10 deep, 0/2 metastatic (Table 1). The two epithelioid FH that had *PRKCB* rearrangements at

Table 1 Results of FISH analyses on FFPE sections from benign fibrous histiocytomas[a]

Case No.	*Subtype*	*PRKCB (%)*	*PRKCD (%)*	*ALK (%)*
PC1	*PRKCD* +	Neg (2)	**Pos (44)**	ND
PC2	*PRKCB*+	**Pos (51)**	Neg (14)	ND
1	**Regular**	Neg (9)	**Pos (47)**	ND
2	Regular	Neg (16)	Neg (12)	ND
3	Regular	Neg (8)	Neg (15)	ND
4	Regular	Neg (18)	Neg (14)	ND
5	Regular	Neg (1)	Neg (6)	ND
6	Regular	Neg (8)	Neg (10)	ND
7	Regular	Neg (6)	Neg (12)	ND
8	Aneurysmal	Neg (7)	Neg (33)	ND
9	Aneurysmal	Neg (1)	Neg (11)	ND
10	Aneurysmal	Neg (6)	Neg (14)	ND
11	Cellular	Neg (6)	Neg (17)	ND
12	Cellular	Neg (17)	Neg (15)	ND
13	Cellular	Neg (9)	ND	ND
14	Cellular	Neg (10)	ND	ND
15	Cellular	Neg (11)	Neg (7)	ND
16	Cellular	ND	Neg (9)	ND
17	**Epithelioid**	Neg (3)	Neg (10)	**Pos (58)**[b,c]
18	**Epithelioid**	ND	Neg (31)	**Pos (66)**[c]
19	**Epithelioid**	**Pos (25)**	Neg (17)	Neg (11)[b,d]
20	**Epithelioid**	Neg (11)	Neg (8)	**Pos (38)**[c]
21	Epithelioid	Neg (9)	ND	Neg (6)[c]
22	**Epithelioid**	**Pos (23)**	ND	Neg (1)[d]
23	Epithelioid	Neg (8)	ND	Neg (11)[c]
24	Atypical	Neg (7)	Neg (17)	Neg (1)
25	Deep	Neg (0)	Neg (15)	ND
26	Deep	Neg (2)	Neg (12)	ND
27	Deep	Neg (3)	Neg (17)	ND
28	Deep	Neg (3)	Neg (14)	ND
29	Deep	Neg (9)	Neg (12)	ND
30	**Deep**	Neg (7)	**Pos (56)**	ND
31	Deep	Neg (1)	Neg (1)	ND
32	Deep	Neg (3)	Neg (25)	ND
33	Deep	Neg (1)	Neg (8)	ND
34	**Deep**	**Pos (30)**	Neg (24)	ND
35	Metastatic	ND	Neg (5)	ND
36	Metastatic	Neg (4)	Neg (14)	ND

Abbreviations: ND, not done; PC, positive control. [a]Fraction of nuclei showing split signals is indicated in the parentheses. Cases with frequencies exceeding cutoff values are highlighted in bold. [b]Previously also analyzed by Doyle *et al*.,[7] yielding the same results. [c]Positive for ALK expression at immunohistochemistry. [d]Negative for ALK expression at immunohistochemistry.

FISH analysis were negative for ALK protein expression (Table 1).

The status of the *ALK* gene had previously been studied in two cases of epithelioid FH, one being negative and one positive.[7] Here, these results could be repeated, and another two cases of epithelioid FH, both negative for *PRKC* rearrangements, were scored as positive. Thus, 3/7 epithelioid and 0/1 atypical FH were ALK positive (Table 1).

The status of the *PRKCA* gene was investigated by interphase FISH on cut sections from Case 39, in which a *KIRREL/PRKCA* fusion transcript was found by both RNA-Seq and RT-PCR (described below). The FISH analysis disclosed a split signal, consistent with translocation, in 42% of the nuclei.

RNA-Seq and RT-PCR

RNA of sufficient quantity (range = 2.9–43.4 μg, median = 9.7 μg) and quality (DV_{200} value: range = 45–75%, mean = 60%) could be extracted from all 16 tumors. The number of reads obtained per case ranged from 14.5 to 64.4×10^6, median = 25.2×10^6 (Table 2). The two software used in this study identified numerous potential fusion transcripts in all cases (Supplementary Tables 2 and 3), the vast majority of which could be discarded as read-through transcripts or technical artifacts. A single, biologically relevant fusion transcript, all of which were identified by ChimeraScan, was found in nine cases; five of these were found also with TopHat (Table 2). The expected fusion transcripts were found in 5/6 sarcomas with known gene fusions (three MLS with the *FUS/DDIT3* fusion, one synovial sarcoma with the *SS18/SSX2* fusion, and one low-grade fibromyxoid sarcoma with the *FUS/CREB3L2* fusion); one MLS did not display the expected *FUS/DDIT3* fusion by any of the two software. Also the two FH with known *PDPN/PRKCB* fusions were identified at RNA-Seq of FFPE tissue. Thus, 7/8 positive controls were correctly classified. In addition, one FH with a known t(1;16) showed a *PDPN/PRKCB* fusion, in agreement with the karyotype, whereas one FH with an unbalanced t(3;12) was negative. Of the remaining six FH (Cases 37–44), five did not display any relevant fusion transcript, whereas one showed a novel *KIRREL/PRKCA* fusion (Table 2). This fusion was verified by RT-PCR, revealing an in-frame fusion of exon 13 (nt 158,093,762) of *KIRREL* with exon 9 (nt 66,732,687) of *PRKCA* (Positions according to GRCh38; Figure 1).

DISCUSSION

We recently showed that gene fusions involving the *PRKCB* and *PRKCD* genes are recurrent in FH.[4] However, that study was biased in the sense that it only included tumors that had clonal chromosomal rearrangements at G-banding analysis, potentially enriching certain genetic subgroups with a high proliferative capacity *in vitro*. Furthermore, only four cases in that study could be analyzed by molecular and/or FISH techniques. We thus wanted to explore a larger series of BFH, with tumors representing all known morphologic subtypes.

Table 2 Benign fibrous histiocytomas and soft tissue sarcomas used for RNA-Seq on FFPE tissue

Case no.	*Diagnosis*	*Year*	*Known/predicted fusion status*	*Percentage fragments >200 nt (%)*	*Read count*	*TopHat*	*Chimerascan (total No of fragments supporting, spanning fragments)*
PC3	RFH	2009	*PDPN/PRKCB*	53	20 950 432	*PDPN/PRKCB*	*PDPN/PRKCB* (51, 21)
PC4	RFH	2009	*PDPN/PRKCB*	62	16 872 619	*PDPN/PRKCB*	*PDPN/PRKCB* (44, 14)
37	CFH	2010	*PDPN/PRKCB?*	66	33 084 533	*PDPN/PRKCB*	*PDPN/PRKCB* (119, 47)
38	CAFH	1990	Unknown	53	14 533 329	Neg	Neg
39	RFH	2011	Unknown	72	23 114 062	*KIRREL/PRKCA*	*KIRREL/PRKCA* (28, 16)
40	RFH	2011	Unknown	56	17 936 136	Neg	Neg
41	RFH	2012	Unknown	58	18 311 561	Neg	Neg
42	RFH	2012	Unknown	45	33 233 957	Neg	Neg
43	RFH	2012	Unknown	72	40 665 408	Neg	Neg
44	RFH	2013	Unknown	54	60 129 479	Neg	Neg
PC5	LGFMS	2013	*FUS/CREB3L2*	68	26 917 670	Neg	*FUS/CREB3L2* (5, 0)
PC6	Syn sarc	2013	*SS18/SSX2*	55	64 428 765	*SS18/SSX2*	*SS18/SSX2* (532, 405)
PC7	MLS	2014	*FUS/DDIT3*	75	23 576 431	Neg	*FUS/DDIT3* (14, 0)
PC8	MLS	2014	*FUS/DDIT3*	54	39 879 954	Neg	*FUS/DDIT3* (16, 1)
PC9	MLS	2011	*FUS/DDIT3*	56	14 764 666	Neg	Neg
PC10	MLS	2014	*FUS/DDIT3*	55	28 966 064	Neg	*FUS/DDIT3* (12, 0)

Abbreviations: CAFH, cellular/aneurysmal fibrous histiocytoma; CFH, cellular fibrous histiocytoma; LGFMS, low-grade fibromyxoid sarcoma; MLS, myxoid liposarcoma; PC, positive control; RFH, regular fibrous histiocytoma; Syn sarc, synovial sarcoma.

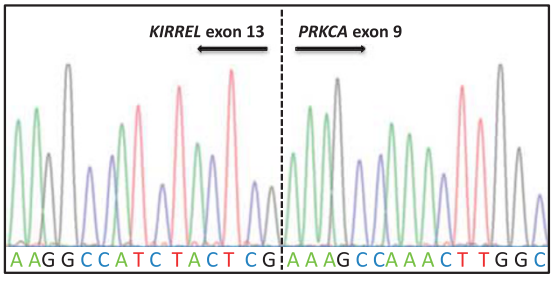

Figure 1 Chromatogram depicting the fusion between *KIRREL* and *PRKCA* in a benign fibrous histiocytoma.

For that purpose, unstained sections from 66 cases of BFH were retrieved and studied with regard to *PRKCB* and *PRKCD* rearrangements using interphase FISH. However, this analysis turned out to be unusually challenging in the sense that it was very difficult to obtain signals of sufficient quality. Because FISH analyses with commercial probes for the *ALK* gene have been successfully performed on FFPE sections from FH,[7] we assume that the technical problems were due to the probe sets we used. However, also the age of the samples could possibly have affected the results. Nevertheless, reliable results could be obtained in 36 of the cases; 5 of them were interpreted to have a *PRKCB* or a *PRKCD* rearrangement (Table 1). As this frequency (5/36, 14%) of *PRKCB*/*PRKCD*-positive cases was considerably lower than in our initial study and because the positive cases were found among several of the different morphologic/clinical subsets—regular, epithelioid, and deep lesions—we wanted to use another technique to investigate whether the discrepancy was due to poor sensitivity of FISH. With no further material being available from the intial cohort, a second set of recently diagnosed regular FH was retrieved for RNA extraction and RNA-Seq. This approach turned out to be technically highly successful, in terms of RNA quantity and quality as well as sequencing results (Table 2). With only 1/8 positive controls being negative for the expected gene fusion, it seems reasonable to assume that the results on the FH with unknown genetic status were fairly accurate; the negative case (PC9) was the positive control yielding the lowest number of reads, possibly explaining the negative results (Table 2).

The RNA-Seq analysis identified one cellular FH with the *PDPN/PRKCB* fusion that has been previously detected in FH.[4] In addition, 1/6 regular FH displayed a novel fusion between *KIRREL* and *PRKCA*, mapping to 1q23 and 17q24, respectively. Although *KIRREL* has not been implicated in gene fusions before, Bridge and co-workers recently described a recurrent *SLC44A1*/*PRKCA* fusion in papillary glioneural tumor, with the same breakpoint in *PRKCA* as in the FH of the present study.[21] Further arguments for a pathogenetic significance of the *KIRREL*/*PRKCA* fusion in the context of FH is that KIRREL, like the previously identified amino-terminal partners PDPN, CD63, and LAMTOR1, is a membrane-associated protein, retaining its membrane-binding part in the chimeric protein.[4]

We can thus conclude that *PRKC* gene rearrangements are indeed recurrent in FH and that, based on the present and our previous study, they at least occur in the regular, cellular, aneurysmal, and epithelioid subtypes, and that they may be found in both cutaneous and deep lesions; more cases of atypical and metastatic FH need to be analyzed before it can be excluded that also these entities may harbor *PRKC* fusions. The overall frequency of *PRKC*-positive FHs can only be roughly estimated. First, interphase FISH was only performed for two members of the *PRKC* gene family (*PRKCB* and *PRKCD*) but we now know from the RNA-Seq study that at least the alpha variant (*PRKCA*) can also be involved in fusions. Second, results of interphase FISH on FFPE sections are somewhat difficult to standardize, as the samples may vary significantly in technical quality. If we had lowered the cutoff values to 1 s.d. above the mean frequencies of split signals, four more cases would have been scored as positive for *PRKC* rearrangements, whereas an increase of the cutoff to 3 s.d. above mean frequencies would have turned two *PRKCD*-positive cases, including the positive control, and two *PRKCB*-positive cases into negative ones (Table 1); unfortunately, no tissue blocks for RNA extraction were available from the cases that were used for FISH. Taking the methodological shortcomings mentioned above into account, our results from FISH and RNA-Seq suggest that only some 15–25% of FH are positive for *PRKC* gene fusions. Thus, there must exist alternative pathogenetic pathways in FH. Indeed, it was recently shown by both FISH and RNA-Seq that epithelioid FH carry fusions involving the *ALK* gene—*VCL*/*ALK* and *SQSTM1*/*ALK*, respectively.[6] This finding was further expanded by Doyle and co-workers who analyzed 33 epithelioid FH by immunohistochemistry (IHC)—29/33 cases were ALK-positive, and 14/14 of these turned out to be *ALK*-rearranged also at FISH analysis; other subtypes of FH were IHC-negative for ALK.[7] Interestingly, epithelioid FH was the subtype most often (2/7) showing a *PRKC* gene rearrangement in our FISH study (Table 1), suggesting that the vast majority of these lesions harbor either an *ALK* fusion or a *PRKC* fusion. The conclusion that *ALK* and *PRKC* activation constitute alternative, and not overlapping, pathogenetic pathways in epithelioid FH was supported by the finding that the two cases with *PRKCB* rearrangement were negative for ALK expression, whereas the other five cases were positive (Table 1); there were no obvious morphological or clinical differences between *PRKC*- and ALK-positive epithelioid FH, but it should be kept in mind that very few cases were analyzed. Also two tumors diagnosed as atypical FH were recently reported to have ALK rearrangements, as shown by FISH or IHC,[5] but, in our opinion, the morphologic description and images would fit better with epithelioid FH. The single atypical FH (Case 24) that we could analyze by FISH was negative for *PRKCB*, *PRKCD*, and *ALK* rearrangements (Table 1).

The RNA-Seq part of our study adds to the still small but growing number of reports showing that the NGS technologies are applicable to RNA extracted from FFPE tissues.[22–26] Furthermore, RNA-Seq on FFPE tissue has only rarely been used to detect gene fusions.[6,23,27] The study of Sweeney and co-workers is of particular interest in the present context, as they also investigated sarcomas (three synovial sarcomas, three MLS, two Ewing sarcomas, and one clear cell sarcoma), used Illumina equipment and kits, and had results similar to ours;[23] all their cases showed the expected gene fusions, although some of the samples had to be re-analyzed at greater depth or after depletion of ribosomal RNA before obtaining positive results. Some of the methodological differences between their and our study deserve further comments. First, Sweeney and co-workers read longer sequences than we did (2×150 nt, compared with 2×81 nt in our study). In principle, longer reads should have a greater chance of covering the fusion breakpoints (spanning reads) and while all their cases had fusion spanning reads, three of our positive controls did not (Table 2). As shown in our study, however, flanking reads can also accurately identify gene fusions. Second, none of the samples analyzed by Sweeney and co-workers had been archived for more than 6 months, whereas our oldest positive sample had been archived for 5 years. Most likely, even older samples can be successfully studied with regard to gene fusion status. Hedegaard *et al.* performed a comprehensive analysis of RNA-Seq data on both FFPE and fresh frozen samples from various carcinomas that had been stored up to 244 months; the two methods showed very good correspondence in terms of up- and downregulated genes.[24] Third, Sweeney and co-workers selected reads aligning only to genes known to be involved in sarcoma-associated fusions, a so-called sarcomatome, whereas we extracted all potential fusion genes. Our strategy did not result in any potential false positives, and did not significantly increase the time needed to identify the fusion of interest. Most importantly, our unbiased approach allowed us to find a novel fusion transcript, where none of the two genes had been implicated in soft tissue tumors before. In summary, we find the results of our RNA-Seq study very promising and believe that FFPE samples can be successfully used for gene fusion detection.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Gleason BC, Fletcher CD. Deep "benign" fibrous histiocytoma: clinicopathologic analysis of 69 cases of a rare tumor indicating occasional metastatic potential. Am J Surg Pathol 2008;32:354–362.

2. Luzar B, Calonje E. Cutaneous fibrohistiocytic tumours—an update. Histopathology 2010;56:148–165.
3. Nielsen GP, Kyriakos M. Non-ossifying fibroma/benign fibrous histiocytoma of bone. In: Fletcher CD, Bridge JA, Hogendoorn PCW, Mertens F (eds). World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. IARC Press: Lyon, 2013, pp 302–304.
4. Plaszczyca A, Nilsson J, Magnusson L *et al.* Fusions involving protein kinase C and membrane-associated proteins in benign fibrous histiocytoma. Int J Biochem Cell Biol. 2014;53:475–481.
5. Szablewski V, Laurent-Roussel S, Rethers L *et al.* Atypical fibrous histiocytoma of the skin with CD30 and p80/ALK1 positivity and ALK gene rearrangement. J Cutan Pathol 2014;41:715–719.
6. Jedrych J, Nikiforova M, Kennedy TF *et al.* Epithelioid cell histiocytoma of the skin with clonal *ALK* gene rearrangement resulting in *VCL-ALK* and *SQSTM1-ALK* gene fusions. Br J Dermatol 2015;172:1427–1429.
7. Doyle LA, Marino-Enriquez A, Fletcher CD *et al.* ALK rearrangement and over-expression in epithelioid fibrous histiocytoma. Mod Pathol, in press.
8. Calonje E, Mentzel T, Fletcher CD. Cellular benign fibrous histiocytoma. Clinicopathologic analysis of a distinctive variant of cutaneous fibrous histiocytoma with frequent recurrence. Am J Surg Pathol 1994;18:668–676.
9. Singh Gomez C, Calonje E, Fletcher CD. Epithelioid benign fibrous histiocytoma of skin: clinic-pathological analysis of 20 cases of a poorly known variant. Histopathology 1994;24:123–129.
10. Calonje E, Fletcher CD. Aneurysmal benign fibrous histiocytoma: clinicopathological analysis of 40 cases of a tumour frequently misdiagnosed as a vascular neoplasm. Histopathology 1995;26:323–331.
11. Kaddu S, McMenamin M, Fletcher CD. Atypical fibrous histiocytoma of the skin. Clinicopathologic analysis of 59 cases with evidence of infrequent metastasis. Am J Surg Pathol 2002;26:35–46.
12. Doyle LA, Fletcher CD. EMA positivity in epithelioid fibrous histiocytoma: a potential diagnostic pitfall. J Cutan Pathol 2011;38:697–703.
13. Doyle LA, Fletcher CD. Metastasizing "benign" cutaneous fibrous histiocytoma. A clinicopathologic analysis of 16 cases. Am J Surg Pathol 2013;37:484–495.
14. Vanni R, Fletcher CD, Sciot R *et al.* Cytogenetic evidence of clonality in cutaneous benign fibrous histiocytomas: A report of the CHAMP study group. Histopathology 2000;37:212–217.
15. Arbajian E, Puls F, Magnusson L *et al.* Recurrent *EWSR1-CREB3L1* gene fusions in sclerosing epithelioid fibrosarcoma. Am J Surg Pathol 2014;38:801–808.
16. Walther C, Tayebwa J, Lilljebjörn H *et al.* A novel *SERPINE1-FOSB* fusion results in transcriptional up-regulation of *FOSB* in pseudomyogenic hemangioendothelioma. J Pathol 2014;232:534–540.
17. Iyer MK, Chinnaiyan AM, Maher CA. ChimeraScan: a tool for identifying chimeric transcription in sequencing data. Bioinformatics 2011;27:2903–2904.
18. Kim D, Pertea G, Trapnell C *et al.* TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 2013;14:R36.
19. Hofvander J, Tayebwa J, Nilsson J *et al.* Recurrent *PRDM10* gene fusions in undifferentiated pleomorphic sarcoma. Clin Cancer Res 2015;21:864–869.
20. Hofvander J, Tayebwa J, Nilsson J *et al.* RNA sequencing of sarcomas with simple karyotypes: identification and enrichment of fusion transcripts. Lab Invest 2015;95:603–609.
21. Bridge JA, Liu X-Q, Sumegi J *et al.* Identification of a novel, recurrent SLC44A1-PRKCA fusion in papillary glioneuronal tumor. Brain Pathol 2013;23:121–128.
22. Adiconis X, Borges-Rivera D, Satija R *et al.* Comparative analysis of RNA sequencing methods for degraded or low-input samples. Nat Methods 2013;10:623–629.
23. Sweeney RT, Zhang B, Zhu SX *et al.* Desktop transcriptome sequencing from archival tissue to identify clinically relevant translocations. Am J Surg Pathol 2013;37:796–803.
24. Hedegaard J, Thorsen K, Lund MK *et al.* Next-generation sequencing of RNA and DNA isolated from paired fresh-frozen and formalin-fixed paraffin-embedded samples of human cancer and normal tissue. PLoS ONE 2014;9:e98187.
25. Li P, Conley A, Zhang H, Kim HL. Whole-transcriptome profiling of formalin-fixed, paraffin-embedded renal cell carcinoma by RNA-seq. BMC Genomics 2014;15:1087.
26. Zhao W, He X, Hoadley KA *et al.* Comparison of RNA-Seq by poly (A) capture, ribosomal RNA depletion, and DNA microarray for expression profiling. BMC Genomics 2014;15:419.
27. Ma Y, Ambannavar R, Stephans J *et al.* Fusion transcript discovery in formalin-fixed paraffin-embedded human breast cancer tissues reveals a link to tumor progression. PLoS ONE 2014;9:e94202.