RESEARCH ARTICLE

Fusion of the *AHRR* and *NCOA2* Genes Through a Recurrent Translocation $t(5;8)(p15;q13)$ in Soft Tissue Angiofibroma Results in Upregulation of Aryl Hydrocarbon Receptor Target Genes

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Soft tissue angiofibroma is a recently delineated tumor type of unknown cellular origin. Cytogenetic analysis of four cases showed that they shared a $t(5;8)(p15;q13)$. In three of them it was the sole change, underlining its pathogenetic significance. FISH mapping suggested the involvement of the aryl hydrocarbon receptor repressor (*AHRR*) and nuclear receptor coactivator 2 (*NCOA2*) genes in 5p15 and 8q13, respectively. RT-PCR revealed in-frame *AHRR/NCOA2* and *NCOA2/AHRR* transcripts in all four cases. Interphase FISH on paraffin-embedded tissue from 10 further cases without cytogenetic data showed that three were positive for fusion of *AHRR* and *NCOA2*. While *AHRR* has never been implicated in gene fusions before, *NCOA2* is the 3'-partner in fusions with *MYST3* and *ETV6* in leukemias and with *PAX3* and *HEY1* in sarcomas. As in the previously described fusion proteins, *NCOA2* contributes with its two activation domains to the *AHRR/NCOA2* chimera, substituting for the repressor domain of *AHRR*. Because the amino terminal part of the transcription factor *AHRR*, responsible for the recognition of xenobiotic response elements in target genes and for heterodimerization, shows extensive homology with the aryl hydrocarbon receptor (*AHR*), the fusion is predicted to upregulate the *AHR/ARNT* signaling pathway. Indeed, global gene expression analysis showed upregulation of *CYP1A1* as well as other typical target genes of this pathway, such as those encoding toll-like receptors. Apart from providing a diagnostic marker for soft tissue angiofibroma, the results also suggest that this tumor constitutes an interesting model for evaluating the cellular effects of *AHR* signaling. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

A growing number of bone and soft tissue tumors has been shown to be characterized by chromosome rearrangements, typically translocations, resulting in the creation of gene fusions (Mitelman et al., 2007; Mertens et al., 2010). As these rearrangements often are strongly associated with particular morphologic entities, they have come to serve as useful diagnostic markers (Fletcher et al., 2002). Furthermore, the fact that the translocations, at least at the cytogenetic level, often appear as the sole anomalies strongly implies that the corresponding gene fusions are essential, if not necessary, for tumorigenesis. By studying the molecular consequences of tumor-associated gene fusions in tumors, as well as in various in vitro and animal models, much has been learnt about the mechanisms behind tumor development.

A large proportion of the genes involved in fusions in bone and soft tissue tumors encode transcription factors, i.e., proteins that directly or indirectly regulate the expression of other genes. Although the cellular impact of most such fusion genes still remains poorly investigated, it seems safe to conclude that altered properties of one or both of the genes involved in the fusion result in

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TABLE 1. Clinical, Cytogenetic, and FISH Data on 14 Soft Tissue Angiofibromas

Case No. ^a	Age/sex	Site ^b	Follow-up ^c	Karyotype ^d	FISH ^e
1. (36)	65/F	L leg	34 NED	46,XX,t(5;8)(p15;q13)	+
2. (37)	66/F	U leg	16 NED	46,XX,t(5;8)(p15;q13)	+
3. (21)	65/F	Arm	18 NED	44,XX,dic(1;3)(q21;p14), del(2)(p14),t(5;8)(p15;q13), add(18)(p11),-21	ND
4. (32)	74/M	Foot	144 NED	46,XY,t(5;8)(p15;q13)	ND
5. (4)	6/M	Back	LR 9, NED 43	ND	—
6. (9)	10/F	Back	23 NED	ND	—
7. (29)	31/M	Abd wall	LTF	ND	+
8. (16)	29/F	U leg	LTF	ND	—
9. (17)	42/F	Chest wall	LTF	ND	—
10. (6)	45/F	Knee	LTF	ND	—
11. (20)	49/F	Knee	78 NED	ND	—
12. (28)	58/F	L leg	16 NED	ND	+
13. (19)	63/F	Foot	39 NED	ND	—
14. (15)	86/F	Foot	103 NED	ND	+

^aCase numbers in parentheses correspond to case numbers in Mariño-Enríquez and Fletcher (2011).

^bL leg, lower leg; U leg, upper leg; Abd wall, abdominal wall.

^cFollow-up is given in months from diagnosis. NED, no evidence of disease; LR, local recurrence; LTF, lost to follow-up.

^dND, not done.

^eInterphase FISH on cut tumor sections; + = positive for *AHRR/NCOA2* fusion signal; ND, not done; —, negative for *AHRR/NCOA2* fusion signal.

deregulated transcription of a large number of target genes. For instance, it has been shown that the Ewing sarcoma-specific chimera EWSR1/FLI1, in which the DNA-binding domain of the transcription factor FLI1 is combined with the transcriptional activation domain of EWSR1, results in up- or down-regulation of more than 1,400 genes (Smith et al., 2006).

In this study, we describe the finding of a novel fusion between two genes—*AHRR* and *NCOA2*—encoding transcription-associated factors. *AHRR* codes for the aryl hydrocarbon receptor repressor, a key regulator of the activity of the aryl hydrocarbon receptor (AHR). *NCOA2* encodes nuclear receptor coactivator 2, which is involved in the function of, e.g., nuclear hormone receptors. AHR is a crucial mediator of the cellular response to xenobiotics such as polycyclic aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), better known as dioxin (Abel and Haarmann-Stemmann, 2010; Fujii-Kuriyama and Kawajiri, 2010). Both genes have previously been implicated in tumorigenesis, *AHRR* as a putative tumor suppressor gene (Zudaire et al., 2008) and *NCOA2* as the 3'-partner in gene fusions in various leukemias and sarcomas (Carapeti et al., 1998; Liang et al., 1998; Strehl et al., 2008; Sumegi et al., 2010; Wang et al., 2012). Apart from providing new insights into the cellular function of these two genes, our results also suggest that the fusion between *AHRR* and *NCOA2* is specific for a distinct type of soft tissue tumor—soft tissue angiofibroma.

MATERIALS AND METHODS

Tumors

A total of 14 tumors from 14 patients were included in the study. Clinical information on the 14 patients is summarized in Table 1. Fresh tumor biopsies for chromosome banding analysis and RT-PCR were available from Cases 1 to 4. Cut sections from paraffin-embedded tumors were available from Cases 1, 2, and 5–14 for interphase fluorescence in situ hybridization (FISH). Cases 5–14 were retrieved from the consultation files of one of the authors (C. D. M. F.) at Brigham and Women's Hospital, Boston, MA. Metaphase spreads for FISH were available from Cases 1 and 2.

Histopathologic Features

All 14 soft tissue angiofibromas were included in a recently published clinicopathological study (Mariño-Enríquez and Fletcher, in press). In brief, the tumors were most often well-circumscribed, consisting of a patternless or vaguely lobulated spindle cell proliferation accompanied by a prominent vascular component. The cytomorphology was nondistinctive, characterized by uniformly bland fibroblastic spindle cells with unremarkable pale eosinophilic cytoplasm and short ovoid nuclei showing no atypia. The vasculature consisted of a complex network of countless small thin-walled branching blood vessels reminiscent of the "crow's feet-like" vasculature characteristic of myxoid liposarcoma. Medium

and large-sized round or branching blood vessels, with variably thick walls, were also present. The stroma of the lesion was variably collagenous or myxoid, in some cases showing a focal lymphocytic infiltrate. Of these 14 tumors, six showed at least focal positivity for epithelial membrane antigen (EMA), three showed scattered cells positive for desmin and one was focally positive for smooth muscle actin. CD34 and S100 protein were consistently negative.

Chromosome Banding Analysis

Cell culturing, harvesting and G-banding were performed as described (Mandahl, 2001) and the karyotypes were written following the recommendations of the International System for Human Cytogenetic Nomenclature (Shaffer et al., 2009).

Metaphase FISH

To map the breakpoints at 5p15 and 8q13, metaphase preparations from Cases 1 and 2 were hybridized in situ with a number of bacterial artificial chromosomes (BAC) clones obtained from the BACPAC Resource Center (<http://bacpac.chori.org>). BAC clones were selected based on their location in the UCSC Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>, hg18). Clones were propagated and the DNA was extracted using the Plasmid Miniprep Kit (BIO-RAD Laboratories, Hercules, USA) according to the manufacturer's instructions. Amplification of BAC DNA was performed using the GenomiPhi™ V2 DNA amplification kit (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. BAC DNA was labeled by nick translation with Cy3-dUTP (GE Healthcare), or Fluorescein-12-dUTP (Fermentas, Burlington, Canada). The labeled probes were precipitated, and dissolved in a standard hybridization solution. To identify unequivocally chromosomes 5 and 8, whole chromosome painting (WCP) probes (Applied Spectral Imaging, Migdal Haemek, Israel) were used. Slides and probes were denatured simultaneously by incubation at +74°C on a hot plate. After hybridization overnight at +37°C in a dark humidified chamber, the slides were washed at +74°C in 0.4×SSC with 0.05% TWEEN-20, for 2 min, dehydrated in an ethanol series, and mounted with a 2% DABCO (1,4-diazabicyclo[2.2.2]octane) solution containing 0.5 µg/ml DAPI (4'-6-Diamidino-2-phenylindole) as a counterstain.

Interphase FISH on Tumor Sections

To search for the t(5;8) and variant rearrangements involving *NCOA2*, interphase FISH was carried out on paraffin-embedded 4-µm tissue sections from Cases 5 to 14, using the BAC/PAC probes listed in Supporting Information Table 1. To detect fusion signals, three BACs covering *NCOA2* and two covering *AHRR*, respectively, were pooled to yield stronger signals. Cases 1 and 2 were used as positive controls. BAC DNA was labeled as described above. After heating to +60°C, the slides were deparaffinized in xylene for 2 × 7 min at 55°C, washed for 2 × 3 min in 100% ethanol at room temperature, and put on a +98°C hot plate. The slides were then soaked in distilled water for 2 × 3 min at room temperature and incubated in Spot-Light Heat Pretreatment Solution (Invitrogen, Carlsbad, CA) for 15 min at +95–98°C. The slides were again soaked in distilled water for 2 × 3 min at room temperature before being covered with Pepsin Solution Digest –All™3 (Invitrogen) at +37°C for 5 min. After a 2 × 3 min soak in distilled water, predenatured probes were applied to the sections, covered with a cover slip and sealed with rubber glue and heated to +74°C for 5 min and hybridized overnight in a dark humidified chamber at +37°C. Post hybridization washes were done in 2×SSC with 0.05% TWEEN-20 at +74°C for 2 min, followed by 1 min in 2×SSC with 0.05% TWEEN-20 at room temperature. Slides were dehydrated and mounted as described above.

RT-PCR Analyses

Total RNA was extracted from frozen tumor tissue using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). cDNA synthesis was conducted as described (Panagopoulos et al., 2001). PCR amplifications were performed using 1 µl cDNA as template in a final volume of 50 µl containing 1×PCR buffer, 0.25 mM of each dNTP, 1.25 mM MgCl₂, 0.5 µM of each forward and reverse primer, 1 U Platinum *Taq* DNA polymerase (Invitrogen), and run on a PCT-200 DNA Engine (MJ Research, Waltham, USA).

Primers specific for *AHRR* and *NCOA2* were designed to detect possible fusion transcripts, as well as wt *AHRR* and wt *NCOA2* transcripts (Supporting Information Table 2). Transcripts were amplified using an initial denaturation for 5 min at 95°C, followed by 35 cycles of 1 min at +95°C, 1 min at +55°C, and 1 min at +72°C and

a final extension for 10 min at +72°C. Amplified fragments were purified from agarose gels and directly sequenced using the Big Dye v1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) on an ABI-3100 Avant genetic analyzer (Applied Biosystems). The BLASTN software (<http://www.ncbi.nlm.nih.gov/blast>) was used for the analysis of *AHRR* and *NCOA2* sequence data.

Global Gene Expression Analysis

RNA from two soft tissue angiofibromas (Cases 1 and 3) was of sufficient quality for the global gene expression analysis. For comparison of expression levels, seven previously reported myxofibrosarcomas (Möller et al., 2011) were included. Extraction of total RNA from frozen tumor biopsies, RNA concentration and quality measurements and hybridization of cDNA to the Human GeneChip® Gene 1.0 ST array (Affymetrix, Santa Clara, USA) were performed as described (Möller et al., 2011). Background correction, normalization and probe summarization were done using the Robust Multichip Average (RMA) method implemented in the Expression Console software version (v) 1.0 (Affymetrix).

Western Blot

Frozen tissue from Case 1 and from a soft tissue tumor without the t(5;8) were lysed in RIPA buffer. Proteins were separated on an SDS-PAGE and blotted onto a PVDF Transfer Membrane (GE Healthcare), as described (Panagopoulos et al., 2007). For detection of the putative *NCOA2/AHRR* fusion protein and wt *AHRR*, a primary antibody (dilution 1:500) for the carboxy-terminal part (encoded by exon 12) of *AHRR* (SAB2100078; Sigma-Aldrich, Stockholm, Sweden) was used. Proteins were visualized using ECL anti-rabbit antibody (NA 9340v, dilution 1:10,000; GE Healthcare) and ECL Plus Western Blotting Detection System (RPN2132, GE Healthcare). To verify that specific bands were identified, a peptide blocking the primary *AHRR* antibody was used (AAPP21453, AVIVA, San Diego, USA). Mouse monoclonal anti- β -actin primary antibody (Sigma-Aldrich) was used as a protein loading control.

RESULTS

Cytogenetic Findings

Cases 1–4 were characterized cytogenetically after short-term culturing. A balanced

t(5;8)(p15;q13) was identified in all of them (Table 1). In Cases 1, 2, and 4, the t(5;8) was the sole anomaly (Fig. 1A). In Case 3, additional changes were found.

Metaphase and Interphase FISH

In Cases 1 and 2, breakpoints were precisely mapped by FISH with the use of a panel of BAC clones. The breakpoints at 5p15 and 8q13 were delineated by BAC clones RP11-1006P13, covering the *AHRR* locus, and RP11-479K21, harboring *NCOA2*, which both gave split signals (Figs. 1B–1E).

Interphase FISH on cut sections from 10 cases of soft tissue angiofibroma using pools of BACs covering *AHRR* and *NCOA2* yielded fusion signals in at least 10% of the nuclei in three cases (Table 1). Additional interphase FISH using a break-apart probe for the *NCOA2* gene in the seven fusion-negative cases did not reveal any split signals.

Detection and Characterization of Fusion Transcripts and of wt *AHRR* and wt *NCOA2* Transcripts

RT-PCR and subsequent sequencing in Cases 1, 2, and 3 showed that nt 1018 (the last nt of exon 9; NM_020731) of *AHRR* was joined with nt 3321 (the first nt of exon 16; NM_006540) of *NCOA2*, resulting in a chimeric in-frame *AHRR/NCOA2* transcript (Figs. 2A and 2B). In case 4, a slightly different fusion transcript was detected (Figs. 2A and 2C). Due to overlap of six nucleotides (nt 1080–1082 of *AHRR* and nt 2974–2976 of *NCOA2*) at the junction, the exact positions of the breakpoints could not be determined. However, intronic breaks are more likely, suggesting that nt 1080 (last nt of exon 10) of *AHRR* was joined to nt 2975 (first nt of exon 14) of *NCOA2* (Fig. 2C).

Also the reciprocal *NCOA2/AHRR* fusion transcripts were detected in all four cases (Figs. 2D–2F). In all cases, two *NCOA2/AHRR* fusion transcripts were detected. Sequencing and blast analysis of the bands in Cases 1–3 revealed that the difference between the two transcripts was the presence or absence of exon 11 of *AHRR*; the transcript without *AHRR* exon 11 had a premature stop codon (not shown). The breakpoints in the *NCOA2/AHRR* fusion transcripts were the same as for the *AHRR/NCOA2* transcripts, and all were in-frame (Figs. 2E and 2F).

In all *AHRR/NCOA2* and reciprocal *NCOA2/AHRR* fusion transcripts, the entire exon 8 of *AHRR* was absent.

Figure 1. A: Representative karyogram from a soft tissue angiofibroma (Case 1), showing a balanced translocation $t(5;8)(p15;q13)$ as the sole anomaly. Arrows indicate breakpoints. B–E: Metaphase FISH analysis revealing split signals with BAC probes RP11-1006P3, cover-

ing the *AHRR* locus (green signals), and RP11-479K21, covering the *NCOA2* locus (red signal). Chromosome 8 is shown in blue. B, D: Case 1. C and E) Case 2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RT-PCR for the expression of wt *AHRR* and *NCOA2* showed that both genes were expressed (not shown) and that exon 8 was absent also from wt *AHRR*; thus, it was the active form *AHRR* that was expressed (Karchner et al., 2009).

Global Gene Expression Analysis

Two soft tissue angiofibromas, Cases 1 and 3, were also analyzed using global gene expression profiling. Bearing in mind the small number of

Figure 2. RT-PCR analysis of the *AHRR/NCOA2* and *NCOA2/AHRR* fusion transcripts. A: Detection of the *AHRR/NCOA2* transcripts with forward primer *AHRR218F* and reverse primer *NCOA23807R*. Lane 1: Case 3, Lane 2: Case 4, Lane 3: blank, Lane 4: negative control, M: 1kb ladder, M1:100bp ladder. Fusion transcripts were detected in all four cases (Cases 1 and 2 not shown). B, C: Partial chromatograms depicting fusion junctions (arrows) in cases 1 and 4. D: Detection of

reciprocal *NCOA2/AHRR* transcripts with forward primer *NCOA23063F* and reverse primer *AHRR1324R*. M: 1kb ladder, Lanes 1 and 2: Case 4, Lanes 3 and 4: Case 2. Fusion transcripts were detected in all four cases (Cases 1 and 3 not shown). E, F: Partial chromatograms depicting fusion junctions (arrows) in Cases 3 and 4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cases studied, no attempt to analyze gene expression differences at a global level was made. Instead, the data were used to study the expression levels of specific genes, implicated as direct targets of AHR signaling in two recent review articles (Abel and Haarmann-Stemmann, 2010; Fujii-Kuriyama and Kawajiri, 2010). The Affymetrix expression values are provided in Supporting Information Table 3. In brief, the expression levels of *AHR*, *AHRR*, *ARNT*, and *NCOA2* were

similar in the soft tissue angiofibromas and the seven myxofibrosarcomas used as controls. Several well-known target genes of AHR signaling were, however, expressed at higher levels in soft tissue angiofibromas. In particular, *CYP1A1* expression was clearly increased, and also, e.g., *CYP1B1*, *IL18*, *NQO1* and eight of ten genes encoding toll-like receptors showed higher expression values in both soft tissue angiofibromas compared to all seven control tumors. No

increase was seen for other potential AHR targets, such as *FOXP3* and *PON1*. (Supporting Information Table 3).

Western Blot

The expected size of wt AHRR is 90 kDa (Sigma-Aldrich), and the predicted size of the putative NCOA2/AHRR chimera 157kDa (http://web.expasy.org/compute_pi/). Western blot analysis identified an ~90 kDa band in Case 1 as well as in the control tumor; this band disappeared when run with blocking peptide. A band slightly larger than 150 kDa was identified in Case 1 but not in the control. However, when run with the blocking peptide, this band did not disappear, suggesting that this signal was unspecific. No other band corresponding to the putative fusion chimera was identified (Supporting Information Fig. 1)

DISCUSSION

In this study, we used FISH and various molecular genetic approaches to characterize the consequences of a balanced chromosome translocation t(5;8)(p15;q13) that was found to be recurrent in a recently described soft tissue tumor—soft tissue angiofibroma (Mariño-Enríquez and Fletcher, in press). The starting material was four cytogenetically characterized tumors, all of which displayed the seemingly balanced t(5;8). The breakpoints were mapped by metaphase FISH, followed by RT-PCR which disclosed a fusion between the *AHRR* and *NCOA2* genes; in-frame *AHRR/NCOA2* as well as *NCOA2/AHRR* fusion transcripts were found in all four cases (Figs. 2 and 3C and 3D). As all these cases had been selected on the basis of their cytogenetic profiles, a series of paraffin-embedded soft tissue angiofibromas was studied by interphase FISH to assess the frequency of the fusion in unselected tumors. Sections were available from 10 tumors, representing different tumor locations and age groups; three were found to be positive for fusion signals. Bearing in mind that interphase FISH on cut sections is less sensitive than FISH on cultured cells, it seems safe to conclude that the fusion between *AHRR* and *NCOA2* is present in a substantial proportion of soft tissue angiofibromas. However, it is also clear that there is a large subset that probably is fusion-negative, indicating genetic heterogeneity. As we did not detect any cases with split signals for *NCOA2*, the *AHRR/NCOA2*-negative cases are unlikely to harbor

variant *NCOA2* fusion events. The number of genetically analyzed soft tissue angiofibromas is still low, making it difficult to discuss potential clinico-pathologic correlations. It could be noted, however, that the *AHRR/NCOA2*-positive cases were somewhat older than the negative ones—median age 65 years (range 29–86) *vs* 42 years (6–62). In particular, the two pediatric tumors were fusion-negative.

Both genes involved in the fusion have been implicated in tumorigenesis before. *AHRR*, mapping to 5p15, encodes a member of the growing family of basic helix-loop-helix/Per-ARNT-sim (bHLH/PAS) transcription factors (Fig. 3A). The gene consists of 12 exons, with the start codon in exon 2. Intron 1 contains a functional regulatory sequence called xenobiotic response element (XRE). The bHLH domain of the AHRR protein is located in the aminoterminal part (encoded by exon 3), followed by the PAS domain (exons 5 and 6). The carboxyterminal part has a repression domain (Baba et al., 2001; Watanabe et al., 2001). Together with the aryl hydrocarbon receptor (AHR), AHRR forms a negative regulatory loop in the cellular response to dioxin or other aryl hydrocarbons (Watanabe et al., 2001; Karchner et al., 2009; Puga et al., 2009; Fujii-Kuriyama and Kawajiri, 2010). When activated by exogenous ligands or other stimuli, AHR dimerizes with ARNT (AHR nuclear translocator), translocates to the nucleus, and forms a complex with histone acetyltransferases and chromatin remodelling factors, which in turn binds to XRE in target genes (Abel and Haarmann-Stemann, 2010; Fujii-Kuriyama and Kawajiri, 2010). This complex then recruits a number of coactivators, including NCOA2, resulting in transcriptional up-regulation of target genes. Also the *AHRR* promoter has a functional XRE and is thus activated by AHR-ARNT. The AHRR protein then competes with AHR for dimerization with ARNT. This AHRR-ARNT heterodimer in turn binds to the XRE of target genes, including *AHRR* itself, down-regulating them by recruiting co-repressors. Thus, AHR, AHRR, ARNT, and multiple other proteins are involved in a complex regulatory network, and there are probably other mechanisms by which AHRR regulates AHR apart from competing for dimerization with ARNT and/or by binding to XRE (Evans et al., 2008).

Zudaire et al. (2008) recently provided evidence for *AHRR* having a tumor suppressor function. They showed that it was transcriptionally down-regulated, typically through hypermethylation of

Figure 3. Schematic representation of (A) wt AHRR, (B) wt NCOA2, and (C, D) predicted chimeric AHRR/NCOA2 and NCOA2/AHRR proteins. Arrowheads indicate the breakage/fusion points. (C upper) Predicted AHRR/NCOA2 chimera in Cases 1–3. (C lower) Predicted AHRR/NCOA2 chimera in Case 4. (D upper) Predicted NCOA2/AHRR chimera in Cases 1–3. (D lower) Predicted

NCOA2/AHRR chimera in Case 4. E: Breakpoints in NCOA2 in reported neoplasia-associated NCOA2 chimeras. bHLH, basic helix-loop-helix domain; PAS, Per-Arnt-Sim domain; NID, nuclear interaction domain; AD, activation domain; CID, CREB binding protein interacting domain.

the promoter region, in a variety of carcinomas, including malignancies of the colon, breast, lung, stomach, cervix, and ovary. Knockdown of *AHRR* gene expression in a human lung cancer cell line enhanced cell growth *in vitro* as well as *in vivo* in a mouse model (Zudaire et al., 2008). Furthermore, overexpression of *AHRR* in a breast cancer cell line resulted in growth inhibition (Kanno et al., 2006). Possibly, the oncogenic effect of down-regulation of *AHRR* is due to reduced control of *AHR* expression; transgenic mice constitutively expressing *Ahr* develop stomach and liver carcinomas upon exposure to dioxin (Andersson et al., 2002; Moennikes et al., 2004).

NCOA2 (also known as *TIF2* and *SRC2*), mapping to 8q13, encodes one of three members of the p160 steroid receptor coactivator (SRC) gene family (Fig. 3B). The proteins (NCOA1-3) encoded by the SRC gene family are all about 160 kDa in size and contain three structural domains (Xu et al., 2009). The N-terminal bHLH/PAS domain is the most conserved region, and can bind to DNA as well as heterodimerize with a variety of proteins with similar regions to enhance transcription of nuclear hormone receptor target genes. The central region of the SRC proteins contains three LXXLL motifs, which interact with the nuclear hormone receptors. The

C-terminus has two transcriptional activation domains (AD1 and AD2). AD1, also known as CREB binding protein (CREBBP) interacting domain (CID), binds other coactivators, such as CREBBP and EP300 (E1A binding protein p300), which leads to chromatin remodeling. AD2 is responsible for interaction with histone methyltransferases, coactivator-associated arginine methyltransferase1 and PRMT1 (Xu et al., 2009).

All SRC proteins have been implicated in neoplasia (Xu et al., 2009). *NCOA2* has been identified as the 3'-partner in a number of neoplasia-associated fusion genes: *MYST3/NCOA2* through inv(8)(p11q13) in acute myeloid and biphenotypic leukemias, *ETV6/NCOA2* through t(8;12)(q13;p13) in acute biphenotypic leukemias, *PAX3/NCOA2* through t(2;8)(q36;q13) in alveolar and embryonal rhabdomyosarcomas, and *HEY1/NCOA2* in mesenchymal chondrosarcoma (Carapeti et al., 1998; Liang et al., 1998; Strehl et al., 2008; Sumegi et al., 2010; Wang et al., 2012). In none of these neoplasms was the reciprocal transcript (i.e., with *NCOA2* as the 5'-partner) detected, and in all of them the two activating domains of *NCOA2* were retained in the fusion protein. Using various constructs, it has been shown that deletion of either domain, in particular the CID, from the *MYST3/NCOA2* and *PAX3/NCOA2* chimeras significantly reduces or abolishes the oncogenic effect (Deguchi et al., 2003; Sumegi et al., 2010). Based on their extensive analyses, Deguchi et al. (2003) suggested that the essential outcome of the *MYST3/NCOA2* fusion was a *MYST3* gain of function through the recruitment of CBP by the CID of *NCOA2*.

The four cases of soft tissue angiofibroma with t(5;8) that could be investigated by RT-PCR in the present study resulted in in-frame fusions of *AHRR* with *NCOA2*: exon 9 with exon 16 in Cases 1–3 and exon 10 with exon 14 in Case 4; in all four cases the transcript data were compatible with intronic breakpoints and would (excluding the spliced out exon 8 of *AHRR*) result in proteins consisting of 718 and 855 aa, respectively (Fig. 3C). The breakpoints in *NCOA2* were more distal in Cases 1–3 than in the previously described fusion genes, which included the 3'-parts from exon 12 in *PAX3/NCOA2*, exon 13 in *MYST3/NCOA2* and *HEY1/NCOA2*, and exon 15 in *ETV6/NCOA2* (Fig. 3E). Despite the slightly more distal breakpoint in Cases 1–3, all four predicted *AHRR/NCOA2* chimeras would contain both transcriptional activation domains (encoded by exons 16–17 and 19–20, respectively) of

NCOA2. The lack of exon 15 provides further support for the notion that it is the two activation domains, in particular the AD1/CID domain, of *NCOA2* that are important for neoplastic transformation (Deguchi et al., 2003; Sumegi et al., 2010). As in the previously described *NCOA2*-fusions, the *AHRR/NCOA2* chimera would lack the central LXXLL motifs responsible for interaction with nuclear hormone receptors. The consistent lack of exon 8 in all detected fusion transcripts as well as in wt *AHRR* is compatible with the finding that the functionally active isoform of *AHRR* lacks the 18 aa encoded by exon 8 (Karchner et al., 2009).

Bearing in mind the extensive homology of the amino-terminal parts of AHR and *AHRR*, corresponding to the first ~275 aa and including the bHLH and PAS-A domains (Hahn et al., 2009), a reasonable assumption is that the *AHRR/NCOA2* chimeras should be able to dimerize with the same proteins, notably ARNT, and target the same genes as wt AHR and wt *AHRR*. These target genes should show enrichment of XRE sequences, and because of the replacement of the Carboxy-terminal repression domain of *AHRR* with the two activation domains of *NCOA2*, the effect on the transcriptional activity of the target genes would be opposite to that achieved by wt *AHRR*. Thus, in essence the outcome would be a “gain of function” mutation of AHR. To support this conclusion, we looked at the expression levels of 39 genes that are known to be either involved in AHR signalling and/or direct targets of AHR (Abel and Haarmann-Stemmann, 2010; Fujii-Kuriyama and Kawajiri, 2010; Supporting Information Table 3). The prototypical target of TCDD-activated AHR is cytochrome P 1A1 (*CYP1A1*) (Abel and Haarmann-Stemmann, 2010; Fujii-Kuriyama and Kawajiri, 2010). Indeed, the expression of *CYP1A1* was much higher in the two soft tissue angiofibromas than in the seven myxofibrosarcomas used as controls. Also *CYP1B1*, as well as most genes encoding toll-like receptors, showed higher expression. On the other hand, some other known targets, like *CYP1A2*, were not convincingly overexpressed, possibly reflecting that some AHR target genes are only activated in certain cell types. Furthermore, although the increased expression of *CYP1A1* strongly suggests that the *AHRR/NCOA2* chimera targets and activates some of the genes activated by AHR, the addition of activation domains from *NCOA2* is a likely source of differences in transcriptional responses.

Considering the complexity of the signaling networks in which AHR, AHRR and NCOA2 are active, the relative importance of the domains contributed by the two partners of the chimera will have to be evaluated in experimental models.

Although the number of cases described is still limited, it is of interest to note that there is a female predominance (12 men, 25 women) among patients (Mariño-Enríquez and Fletcher, *in press*), an otherwise unusual phenomenon among soft tissue tumors. The skewed sex distribution suggests that hormonal influences may be pathogenetically important. Indeed, *in vitro* studies have shown that the AHR/ARNT dimer directly interacts with estrogen receptor alpha (ER- α) and beta (ER- β), thereby influencing estrogen-dependent gene expression (Kanno et al., 2006; Abel and Haarmann-Stemmann, 2010). Another characteristic of soft tissue angiofibroma is the prominent presence of thin-walled, branching blood vessels. AHR has a well-established role in angiogenesis, exemplified by e.g., reduced ability of Ahr-deficient endothelial cells to branch and to form tubular structures, and with increased vascularisation upon activation of Ahr (Roman et al., 2009). Possibly, the effects on angiogenesis are mediated through interaction with vascular endothelial growth factor and stromal components, such as transforming growth factor beta (TGFB). The exact mechanisms by which AHR and TGFB interact are, however, not known.

Attempts to sort out the pathogenetic roles of AHRR and NCOA2 in the fusion protein and the impact of AHRR/NCOA2 on AHR signalling are further complicated by the fact that wt NCOA2 enhances the AHR/ARNT-mediated transcriptional activity (Beischlag et al., 2002). Furthermore, in contrast to all previously reported gene fusions involving NCOA2, also the reciprocal transcript—NCOA2/AHRR—was expressed in all four soft tissue angiofibromas with t(5;8). To find out whether also the NCOA2/AHRR transcript was translated, Western blot analysis was performed using an antibody against the carboxy-terminal part of AHRR. Although this analysis was hampered by the fact that we could only predict the size of this fusion chimera, we could not find any unequivocal support for the expression of the reciprocal chimera (Supporting Information Fig. 1). Despite this finding, and the fact that NCOA2 has always been the 3'-partner in previously reported gene fusions, we believe further studies are

needed before a pathogenetic role also of the NCOA2/AHRR transcript can be dismissed.

In summary, we here show that a substantial subset of a recently delineated tumor type known as soft tissue angiofibroma is characterized by a t(5;8)(p15;q13), resulting in the expression of in-frame AHRR/NCOA2 and NCOA2/AHRR fusion transcripts. As these tumors seem to follow a benign clinical course, but easily could be mistaken morphologically for malignant tumors, such as myxofibrosarcoma, genetic analysis at the chromosome or transcript level could serve as a valuable diagnostic tool. The t(5;8) was the sole cytogenetic anomaly in three of four investigated cases, strongly suggesting that it is important for tumorigenesis. On the basis of previously reported fusion genes involving the p160 SRC gene family, we assume that it is the AHRR/NCOA2 transcript that is pathogenetically significant, an assumption supported by the finding of strong expression of CYP1A1 and higher expression of other known AHR target genes, such as those encoding toll-like receptors. However, the relative impact of the reciprocal NCOA2/AHRR transcript, as well as of the individual domains contributed by each partner in the chimeric proteins, will have to be studied in experimental models. It also remains to be determined by which genetic pathways AHRR/NCOA2-negative soft tissue angiofibromas develop.

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