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Recurrent *CIC* Gene Abnormalities in Angiosarcomas: A Molecular Study of 120 Cases with Concurrent Investigation of *PLCG1*, *KDR*, *MYC*, and *FLT4* Gene Alterations

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Abstract

Angiosarcoma (AS) is a rare sarcoma subtype showing considerable clinicopathologic and genetic heterogeneity. Most radiation-induced AS show *MYC* gene amplifications, with a subset of cases harboring *KDR*, *PTPRB* and *PLCG1* mutations. Despite recent advances, the genetic abnormalities of most primary AS remain undefined. Whole transcriptome sequencing was initiated in 2 index cases of primary soft tissue AS with epithelioid morphology occurring in young adults for novel gene discovery. The candidate abnormalities were validated and then screened by targeted sequencing and FISH in a large cohort of 120 well-characterized AS. Findings were subsequently correlated with the status of *KDR*, *PLCG1*, *MYC* and *FLT4* gene abnormalities. The clinicopathologic relevance and prognostic significance of these genetic changes were analyzed by statistical methods. Concurrent *CIC* mutations and *CIC* rearrangements were identified in both index cases, with a *CIC-LEUTX* fusion detected in one case. Upon screening, an additional visceral AS in a young adult had a complex *CIC* rearrangement, while 6 others harbored only *CIC* mutations. All 3 *CIC*-rearranged AS lacked vasoformation and had a solid growth of round, epithelioid to rhabdoid cells, showing immunoreactivity for CD31 and ERG and sharing a transcriptional signature with other round cell sarcomas, including *CIC*-rearranged tumors. Overall, *CIC* abnormalities occurred in 9% (9/98) of cases, affecting younger patients with primary AS, with an inferior disease-free survival. In contrast, *PLCG1* and *KDR* mutations occurred in both primary and secondary AS, accounting for 9.5% and 7%, respectively, with a predilection for breast and bone/viscera location, regardless of *MYC* status. *MYC* amplification

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was present in most secondary AS related to breast cancer (91%) compared to other causes (25%) or primary AS (7%). *FLT4*-amplified AS lacked *PLCG1/KDR* mutations, occurring predominantly in *MYC*-amplified population, and showed poor prognosis.

Keywords

angiosarcoma; CIC; KDR; PLCG1; MYC; FLT4

INTRODUCTION

Angiosarcomas (AS) are mesenchymal tumors recapitulating endothelial cell differentiation and displaying variable degree of vascular formation.¹ Although AS account for only 2% of soft tissue sarcomas, they have a highly aggressive behavior and dismal overall survival.² The age range at diagnosis varies significantly, with a peak incidence in the 7th decade. AS are extremely rare in children and in young adults, outside of the breast location. Furthermore, AS occur in a wide range of anatomic sites, including, skin (mainly head and neck and post-radiation), breast, soft tissue, bone, and viscera. Although most AS present *de novo*, a significant number of patients develop AS secondary to radiotherapy, and less commonly to chronic lymphedema or toxic carcinogens. Moreover, there is a broad spectrum of morphologic appearances, spanning from well-formed vasoformative spaces with minimal cytologic atypia to solid sheets of epithelioid or spindled cells lacking vasoformation.¹

Cytogenetically, most AS are characterized by complex karyotypes, without recurrent chromosomal changes being described.^{3,4} By gene expression profiling, AS show distinctive up-regulation of genes related to angiogenesis and endothelial cell receptors compared to other sarcoma types.^{5,6} Recurrent somatic mutations involving angiogenic signaling pathways, such as *KDR*, *PTPRB*, and *PLCG1*, have been identified in about 40% of AS, while only rare mutations were reported in *RAS*, *PIK3CA*, *TP53*, *FLT4*, and *TIE1*.⁶⁻⁹ *PTPRB* mutations with or without co-existing *PLCG1* mutations have been reported exclusively in secondary or *MYC*-amplified AS.⁸ In contrast, *KDR* mutations have been reported in both radiation-induced AS after breast cancer and primary AS (breast and heart).^{6,9} High level of *MYC* amplification is the hallmark of most post-radiation and chronic lymphedema-associated AS, with only a small subset of primary AS sharing this abnormality.¹⁰⁻¹² Analysis of *MYC* amplification by fluorescence in situ hybridization (FISH) or *MYC* expression by immunohistochemistry has been confirmed by a number of studies as a reliable ancillary technique to distinguish radiation-related AS from other radiation-induced conditions, particularly atypical vascular lesions (AVL).¹¹⁻¹⁴

Despite this recent progress accelerated by the next generation sequencing methodology, the underlying pathogenesis of most AS remains undefined, particularly of primary AS and AS occurring in younger patients. In this study we sought to investigate further novel genetic abnormalities by whole transcriptome sequencing of 2 index cases of primary soft tissue AS displaying an epithelioid phenotype. The candidate abnormalities were then screened in a large and diverse cohort of AS spanning different clinicopathologic features and compared

to other known genetic events, such as *PLCG1/KDR* mutations and *MYC/FLT4* amplification.

MATERIALS AND METHODS

Patient Collection

The Department of Pathology files at Memorial Sloan Kettering Cancer Center, New York were searched for the diagnosis of AS. A total of 120 cases had adequate material available for further molecular analysis, with 62 cases being included in our prior studies.^{6,7,11,15} The diagnosis was confirmed by the presence of vasoformative features microscopically and/or immunoreactivity for endothelial cell markers, i.e. CD31 and ERG. The relevant clinical information and follow-up was obtained from the electronic medical records. This study was approved by the Institutional Review Board 02-060.

RNA Sequencing and Data Mining using FusionSeq and Mutation Detection Algorithms

In 5 primary AS, including the 2 index cases (AS1, AS2) of soft tissue epithelioid AS, lacking vasoformative features and the remaining 3 cases with conventional histology (2 scalp, AS25, AS32, and 1 breast, AS110) for comparison, RNA was extracted from frozen tissue and subjected to transcriptome sequencing. Total RNA was prepared in accordance with the standard Illumina mRNA sample preparation instructions. Paired-end RNA-sequencing at read lengths of 50 or 51 bp was performed on the HiSeq 2000 platform. All reads were independently aligned with the STAR alignment software against the human genome sequence (hg19) and a splice junction library, simultaneously.¹⁶ The mapped reads were analyzed with FusionSeq method to identify potential fusion transcripts.¹⁷ Potential mutated genes were searched by MuTect (var 1.15)¹⁸ and VarScan (var 2.3.8)¹⁹ variant callers with the annotation added by Variant Effect Predictor tool provided by Ensembl. The candidate fusion genes and mutations were validated subsequently.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

An aliquot of the RNA extracted by Trizol Reagent (Invitrogen, Carlsbad, CA) was used to confirm the potential fusion transcripts. After cDNA synthesis by SuperScript® III First-Strand Synthesis Kit (Invitrogen), PCR was performed using the Clontech Advantage 2 PCR Enzyme System kit (Clontech, Mountain View, CA) with the following primers: *CIC* exon 19 forward primer, 5'-CTGGTCATGCAGCTCTTTCAGG-3', and *LEUTX* exon 3 reverse primer, 5'-GTGTCATCTTCCCCTGGCAAGTC-3'. Amplified products were sent for sequencing confirmation.

Targeted Sequencing for Hot Spots Mutations

Genomic DNA was extracted from frozen or formalin-fixed paraffin-embedded tissue by the phenol/chloroform protocol or the QIAamp DNA FFPE Tissue Kit (Qiagen), respectively. Based on the RNA sequencing results and the reviewed literature, the hotspot targeted regions included *CIC* exons 5, 15, 18–20²⁰, *PLCG1* exons 11 and 18^{8,21}, and *KDR* exons 15, 16, and 24^{6,9}. The products were amplified with Clontech Advantage 2 PCR Enzyme System (see Supplemental Table S1 for PCR primers). All mutations were verified bi-directionally by Sanger sequencing.

Fluorescence in situ Hybridization (FISH)

FISH on interphase nuclei of tissue sections was performed by applying custom probes from bacterial artificial chromosomes (BACs) flanking or targeting *CIC*, *MYC*, *FLT4* genes (Supplemental Table S2). BAC clones were chosen according to UCSC genome browser and obtained from BACPAC sources of Children's Hospital of Oakland Research Institute (CHORI) (Oakland, CA). DNA from individual BACs was isolated and labeled with different fluorochromes in a nick translation reaction. The slides were pretreated, denatured, and hybridized by probes. After overnight incubation, the slides were serially washed and mounted with DAPI in an antifade solution.²² Two hundred successive nuclei were examined using a Zeiss fluorescence microscope (Zeiss Axioplan, Oberkochen, Germany), controlled by Isis 5 software (Metasystems, Newton, MA). A positive score for gene rearrangement was interpreted when at least 20% of the nuclei showed a break-apart signal. Nuclei with incomplete set of signals were omitted. An amplification was defined as the presence of >10 signals (ratio to control centromeric probe >10) or tight clustered signals characteristic of homogeneous staining regions (hsr), as previously described.^{10,15}

Western Blotting for CIC protein expression

Frozen tissue from the 2 index AS cases and 10 control samples, including 5 *CIC* wild-type AS, 2 *CIC-DUX4* positive small blue round cell tumors (SBRCTs), 3 gastrointestinal stromal tumors (GISTs), were homogenized with Tissue Protein Extraction Reagent (Thermo Scientific, Waltham, MA) supplemented with protease inhibitors. Electrophoresis and immunoblotting were performed with the polyclonal anti-CIC antibody (ABN446, 1:1,000, Millipore, Billerica, MA). A β -actin expression was used as reference with the monoclonal anti-actin antibody (8H10D10, 1:10,000, Cell Signaling, Danvers, MA). Following hybridization with the secondary antibodies, the blots were incubated with Immun-Star horseradish peroxidase luminal/enhancer (Bio-Rad, Hercules, CA) and exposed onto Kodak Biomax MR Film (Eastman Kodak, Rochester, NY).

Statistical Analysis

Statistical analysis was conducted using SPSS software (version 20; IBM, New York, NY). The associations between the clinicopathological factors and the genetic status were evaluated by Pearson's χ^2 test, Fisher exact test, or Mann-Whitney U test according to the variable attribute. The disease free survival (DFS) was measured from the date of surgery to the date of recurrence; and overall survival (OS) from the date of surgery to the last follow-up or death. Kaplan-Meier estimate was performed to calculate the DFS and OS, and the statistical significance of different variables was examined by the log-rank test. The significant parameters in Kaplan-Meier analysis were subjected to multivariate Cox regression model to examine the statistic significance. Two-sided p values were calculated and, p < 0.05 was considered to be significant for all statistical analyses.

RESULTS

The entire cohort consisted of 34 (28%) men and 86 (72%) women, with a mean age of 58 years at diagnosis (median 62 years; range 13–91). There were 73 (61%) primary and 47 (39%) secondary AS. The anatomic locations for primary AS included breast (22, 30%),

head and neck (14, 19%), extremity (12, 16%), viscera (11, 15%), trunk (9, 12%), and body cavity (5, 7%). Aside from the mammary and visceral cases, the remaining primary AS were classified into cutaneous (14, 19%), soft tissue (18, 25%), and intra-osseous (8, 11%) subtypes. Secondary AS occurred following radiation for breast cancer (33) or other tumors (9), or in the setting of long-standing lymphedema (5). The average interval between radiotherapy and AS diagnosis was 11.3 years (median 8.5 years, range 4–70 years). Secondary AS occurred in the breast/chest wall (35, 76%; 33 s/p radiation for breast cancer, 1 s/p radiation for hemangiomatosis, 1 with chronic lymphedema due to thoracic surgery), extremity (6, 13%), head and neck (2), internal organs (2), and trunk (1).

***CIC* gene rearrangements identified in a subset of epithelioid AS in young adults, lacking vasoformative features**

The fusion candidate identified by FusionSeq analysis in AS1 was a novel intra-chromosomal *CIC-LEUTX* fusion; the 2 genes being 2.5 Mb apart at 19q13.2 locus with similar directions of transcription (Fig. 1A). The chimeric transcript was confirmed by RT-PCR and composed of *CIC* exon 20 in-frame fused to *LEUTX* exon 3 (Fig. 1B). Due to the close vicinity of *CIC* and *LEUTX* genes, a 3-color FISH assay was employed with an additional *PEPD* gene probe, 6.2 Mb away from *LEUTX*. The result confirmed the *CIC* rearrangements, with a complex pattern (Fig. 1C).

In AS2, the Fusionseq algorithm did not reveal a robust fusion candidate, although a few chimeric *CIC-DUX* reads were identified. The FISH break-apart assay confirmed the presence of *CIC* rearrangements, with small and constant gaps, typical for an intra-chromosomal translocation (Fig. 1D) and similar to the complex pattern seen in AS1. The FISH fusion assay using *CIC* and *DUX4* probes did not confirm a fused signal between these 2 genes (data not shown).

Forty-eight additional AS were screened by FISH for *CIC* gene abnormalities, with emphasis on young age, soft tissue location and epithelioid morphology. One additional *CIC*-rearrangement was found in a renal AS (AS3) from a 21 year-old female. In fact, 2 of the 3 *CIC*-rearranged AS occurred in patients younger than the age of 30 (Table 1). Morphologically, all 3 shared an epithelioid phenotype, composed of nests and solid sheets without identifiable vascular structures (Fig. 2A–D). The tumor cells ranged from a primitive small round to a polygonal appearance, with pale eosinophilic to amphophilic cytoplasm and vesicular nuclei with conspicuous nucleoli (Fig. 2A–C). AS3 showed a distinctive rhabdoid phenotype, with eccentric nuclei and paranuclear densely eosinophilic cytoplasm, arranged in a pseudo-alveolar pattern (Fig. 2D). Despite brisk mitotic activity (>10MF/10 HPFs) and geographic areas of necrosis, none showed significant nuclear pleomorphism. Immunohistochemically, all 3 cases demonstrated diffuse and strong CD31 and ERG expression and focal cytokeratin reactivity (Fig. 2E–F). The AS3 in addition showed CD34 positivity, while AS2 displayed focal membranous CD99 staining. S100 protein and desmin were negative in all cases. As AS3 showed loss of INI1 expression, raising the possibility of a proximal-type epithelioid sarcoma, further FISH analysis performed showed no *SMARCB1* gene abnormalities (data not shown).²³

***CIC* missense mutations occur preferentially in primary AS of younger patients**

The MuTect and VarScan algorithms applied on the RNAseq data identified co-existent missense *CIC* mutations in both index cases, but not in the remaining 3 AS with classic vasoformative morphology. AS1 harbored a *CIC* exon 15 mutation and AS2 a *CIC* exon 18 mutation, both being validated by Sanger sequencing in the tumor DNA, but not present in the germline DNA extracted from normal tissue (Fig. 1E). As additional hot spot mutations were reported in oligodendroglioma, including *CIC* exons 5, 19, and 20²⁴, we screened an additional 95 AS for these 5 exons by direct sequencing. The results showed an additional 6 cases carrying 7 different missense mutations (Table 1). The mutations were distributed in *CIC* exons' 15 and 18–20, but, in contrast to oligodendroglioma, no *CIC* mutations were identified in exon 5, the site of the highly conserved HMG (high-mobility group) box domain. AS6 had two synchronous *CIC* exon 19 mutations and AS7 harbored a homozygous exon 19 mutation (Fig. 1F, G). No *CIC* mutation was present in AS3.

In total we identified 9 (9%) AS harboring various *CIC* alterations, including gene rearrangements and/or missense mutations; their clinicopathologic features being summarized in Table 1. There were 4 males and 5 females, with a mean age of 41 years (versus 59 years in *CIC*-wild type cases, $p = 0.02$). Soft tissue AS was the most common type affected by this abnormality, followed by visceral, cutaneous, and breast locations. All except one were primary AS; only one case being possibly related to radiation (AS5, spleen), having a remote history of chemoradiation for ovarian germ cell tumor 35 years prior. Nevertheless, none of the AS with *CIC*-alterations demonstrated *MYC* amplification. All except one lacked other genetic abnormalities; only one primary breast AS (AS4) showed coexisting *CIC* and *PLCG1* mutations.

Aside from the index cases, there were 2 additional *CIC*-mutant AS displaying an epithelioid phenotype, with limited vasoformation: a deep-seated soft tissue AS from the neck in a 21 year-old man, harboring 2 distinct *CIC* mutations (AS6) and a cutaneous scalp AS bearing a homozygous *CIC* mutation (AS7) (Fig. 3A, B). The remaining 4 cases with single heterozygous *CIC* mutations exhibited a more classic morphology, with well to moderately formed vascular channels and pleomorphic cytology (Fig. 3C, D). The breast AS with concurrent *PLCG1* and *CIC* mutations demonstrated well-differentiated anastomosing vascular channels (Fig. 3D). Thus, 3 of 6 (50%) AS cases presenting outside the breast location in adults younger than 30 years showed the presence of *CIC* gene abnormalities.

***CIC*-rearranged AS show up-regulation of PEA3 family of transcription factors and overexpression of *CIC* protein**

By unsupervised hierarchical clustering of RNA sequencing data of >100 cases of various sarcoma types, the 2 index AS cases with *CIC*-gene rearrangements showed an overlapping gene signature with a group of SBRCTs, including *CIC-DUX4* positive cases (data not shown). Remarkably, there was a significant overexpression of PEA3 family of *ETS* genes, such as *ETV1*, *ETV4*, and *ETV5*, as well as *WT1* and *VGF*, commonly seen in the *CIC-DUX4* signature of SBRCT (Fig. 4A and Supplemental Table S3).^{25–27} Additionally, only a modest upregulation of angiogenesis and endothelial related genes, such as *TIE1*, *FLT4*, and *PECAM1* (CD31), were noted.

The Western blot revealed high CIC protein expression in the 2 index AS cases, at similar levels as the 2 *CIC-DUX4*-positive SBRCTs, whereas the control group of conventional AS and GISTs showed low, if any, protein levels (Fig. 4B).

***PLCG1/KDR* mutations are mutually exclusive but occur in similar AS subsets**

A total of 11 (9.5%) AS harbored *PLCG1* mutations of the 116 informative cases (Supplemental Table S4). All except one occurred in females, with a mean age of 53 years (range 23–82). The *PLCG1*-mutated AS were evenly distributed among primary (6 cases) and secondary AS (5 cases), the latter being all related to radiation for breast cancer. Among the primary AS there were 4 mammary and 2 visceral, one each in the heart and spleen. Most *PLCG1* mutations (9, 82%) occurred in exon 18 (p.R707Q, 8; p.R707L), affecting the auto-inhibitory Src homology 2 (cSH2) domain, with only 2 in exon 11 (p.S345F), within the PLCx catalytic domain. Both *PLCG1* p.S345F mutations occurred in primary AS, one each in breast and spleen. These mutations were mutually exclusive from *KDR* mutations, but all 5 *PLCG1*-mutated secondary AS showed co-existent *MYC* amplifications. Histologically, the *PLCG1*-mutated AS exhibited a wide morphologic spectrum, ranging from well formed vascular spaces to solid sheets of spindled to epithelioid endothelial cells, with low to high grade nuclear features.

KDR missense mutations were found in 8 (7%) of 113 informative cases (Supplemental Table S4), seen exclusively in female patients (mean age, 56 years; range 23–77). Five *KDR* mutations occurred in primary breast AS and 2 in secondary AS after radiotherapy for breast cancer, co-exhibiting *MYC* amplification. Only one *KDR*-mutant AS occurred outside the breast/chest wall area, in the lumbar spine. The most common hot spot mutation was *KDR* exon 16 (codon 771; transmembrane domain) seen in 5 cases. Additional less common spots included *KDR* exon 15 mutation (codon 717 and 731, extracellular domain Ig-like C2 type 7) in 2 cases and *KDR* exon 24 (codon 1065, kinase domain) in 1 case. In keeping with our previous study, *KDR*-mutated AS demonstrated a wide spectrum of low to high grade phenotype, with variable vasoformation and solid growth.⁶

As both *KDR* and *PLCG1* genes are involved in the VEGFR2 (vascular endothelial growth factor receptor 2) signaling pathway, it is not surprising that activating *KDR/PLCG1* mutations are present in similar AS subsets, such as primary or secondary breast AS (15/52, 29%) and bone/viscera (3/19, 16%) locations ($p = 0.012$).

***MYC* and *FLT4* amplifications occur preferentially in AS related to breast cancer radiotherapy**

The status of *MYC* gene copy number abnormalities was analyzed by FISH in 112 informative cases. *MYC* gene amplification was identified in 39 cases. It was detected in the overwhelming majority of AS secondary to radiation for breast cancer (28/31, 90%) and AS related to chronic lymphedema (4/4, 100%). The incidence was significantly lower in AS secondary to radiation for other tumor types (2/8, 25%), such as lymphoma, hemangiomatosis, salivary gland carcinoma, germ cell tumor, and prostatic cancer. The lowest incidence was seen in primary AS (5/69, 7%), occurring only in breast, bone, soft tissue, and skin. *MYC* amplification was significantly associated with female gender ($p =$

0.002) and older age ($p < 0.001$), most likely related to the high frequency of AS related to radiation for breast cancer and the lag time of post-radiation sarcoma. *FLT4* copy number status was also assessed in 110 cases, showing gene amplifications in 6 AS (5.5%), all except one being co-amplified with *MYC* (5/39 versus 1/71, $p = 0.022$). All except one *FLT4*-amplified cases occurred in secondary AS either related to radiation therapy for breast cancer or lymphedema. Only one case occurred in a primary scalp cutaneous AS. None of them had *PLCG1/KDR* mutations. Histologically, *FLT4*-amplified AS exhibited mostly a solid, high-grade histology with variable epithelioid and spindled cell phenotype.

A summary of all molecular findings are depicted in Supplemental Fig. S1, providing an overview of the genetic landscape of AS subsets.

Survival Analysis

Among 108 cases with follow-up information, the median follow-up duration was 27.3 months (range, 0.3–234). Tumor recurrence occurred in 67 cases (70%), at a median disease-free duration of 16 months. Fifty patients (46%) died of disease and the median overall survival duration was 55 months (95% CI, 37.1–72.3), with a 5-year survival rate of 44.5%. Among the different anatomic locations, the intra-osseous/visceral AS showed the shortest OS ($p < 0.001$, a median 22 mo, for intra-osseous AS; 10 mo for visceral AS)(Supplemental Table S5). Secondary AS and the presence of *CIC* alterations conferred an inferior DFS and remained statistically significant by multivariate Cox regression model ($p = 0.007$, HR 2.691, 95% CI 1.314–5.512; $p = 0.006$, HR 3.462, 95% CI 1.419–8.443, respectively). The presence of *MYC* amplification had only a trend towards worse DFS ($p = 0.083$) and OS ($p = 0.062$), but the presence of *FLT4* amplification was associated with a short OS ($p = 0.037$).

DISCUSSION

Despite significant progress being made on the discovery of molecular abnormalities driving AS pathogenesis, the pace of further understanding is hindered by the broad clinical and pathologic heterogeneity of this disease. Even at the molecular level, AS emerges as a multifaceted disease, with mostly non-overlapping genetic signatures across clinical subsets; the majority of the alterations described to date, such as *MYC/FLT4* amplifications and *PLCG1/PTPRB* mutations, affecting secondary AS. Although at the gene expression level, AS subtypes share a distinct transcriptional profile with up-regulation of angiogenic factors and endothelial receptors, compared to other tumor types^{1,2,5,6}, this feature is most likely reflective of its line of differentiation, rather than overlapping oncogenic driver events. A particular limited knowledge surrounds primary AS, such as cutaneous or soft tissue locations, where no recurrent mutations have been identified to date. Furthermore, no insight has been gained in AS occurring in younger patients, which are often highly aggressive and exhibit a high grade epithelioid or spindled phenotype with scarce vasoformative features.²⁸ Thus, our study focused on these orphan subsets, taking advantage of two index cases of primary soft tissue AS occurring in young patients with available material for paired-end RNA sequencing. The results are remarkable with identification of similar *CIC* gene rearrangements co-existing with *CIC* mutations in both cases. The extended screening further identifies a renal AS in a young patient with *CIC* gene rearrangements by FISH, as

well as additional 6 cases with missense mutations. In total, *CIC* alterations were detected in 9% of AS cases showing a predilection for young patients and lacking *MYC/FLT4* amplifications. *CIC*, a human homolog of the *Drosophila* gene *capicua*, belongs to the HMG-box family.²⁹ *CIC* binding to target promoters and enhancers suppresses transcription in the absence of signaling. The activation of *CIC* protein through phosphorylation results in either attenuation of *CIC* DNA binding activity or prevents its nuclear import, relieving the default inhibitory ability of *CIC*.^{30,31,32} *CIC* gene abnormalities have been described in a variety of cancers, such as oligodendroglioma, medulloblastoma, breast cancer, and small blue round cell tumors (SBRCT).^{20,25,33–35} *CIC*-related fusions account for two-thirds of *EWSR1*-negative SBRCTs, with the *CIC-DUX4* fusion exhibiting high chimeric *CIC* protein expression and an enhanced transcriptional activity of *CIC* downstream targets, including the *PEA3* family genes, such as *ETV1*, *ETV4*, and *ETV5*.^{25,35} Remarkably, the 2 index AS harboring *CIC* rearrangements, shared the gene signature of *CIC*-fusion positive SBRCTs, with up-regulation of *ETV1*, *ETV4*, *ETV5* genes. Moreover, the *CIC* fusion partners, *LEUTX* and *DUX4*, belong to the same class of paired (PRD) homeobox genes³⁶, and *KAT6A-LEUTX* fusion has been reported in a therapy-related acute myeloid leukemia.³⁷

A high frequency (69–78%) of homozygous *CIC* mutations has been reported in oligodendrogliomas with 1p/19q deletion.^{20,38,39} The biallelic *CIC* inactivation caused by mutation and deletion also results in up-regulation of the *CIC*-targeted genes (*ETV1*, *ETV4*, *ETV5*, *CCND1*).²⁷ Most *CIC* mutations reported in oligodendroglioma occur in the HMG-domain or are protein-truncating mutations. In contrast, *CIC* mutations identified in AS clustered in exons 18–20 (single, double or homozygous mutations), targeting the nuclear localization and repressor activity of the *CIC* protein.²⁹

These findings bring forward an underappreciated disconnect between tumor differentiation and pathogenesis. On one hand the 2 index AS cases expressed all markers of endothelial lineage (CD31, ERG), while on the other hand displayed the gene signature characteristic of its driving genetic abnormality, the *CIC*-rearrangement, with up-regulation of *PEA3* transcription factors. A somewhat similar example in a different vascular tumor type is the *TFE3*-fusion positive epithelioid hemangioendothelioma, where the endothelial lineage markers are expressed despite the oncogenic activation of *TFE3* gene signature⁴⁰. As these concepts evolve, it remains to be determined if the therapy of these *CIC*-rearranged AS should target the line of differentiation, i.e. endothelial receptors using cytotoxic therapy or VEGFR-inhibitors typically used for conventional AS, or target the *PEA3*-transcriptional signature, thus being managed with Ewing sarcoma type protocols. The latter approach seems to be more in keeping with today's 'basket trials' strategy, enrolling patients based on their specific genetic alterations rather than the histologic type.

In a recent study, Behjati et al⁸ identified *PTPRB* and *PLCG1* in 26% of AS, being restricted to radiation-associated/*MYC*-amplified AS. The 3 *PLCG1* mutations (p.R707Q) co-existed with *PTPRB* mutations and occurred in 9% of AS tested. The same *PLCG1* hot spot mutation was subsequently identified in 3 out of 10 cardiac AS tested.⁹ A different *PLCG1* missense mutation (p.S345F) was also more recently reported in cutaneous (19%) and nodal (13%) peripheral T-cell lymphoma.^{21,41} Our study screened a large cohort of AS cases for

both hot spots and found 11 (9.5%) *PLCG1*-mutated cases, confirming that p.R707Q mutation occurs in both primary (breast, heart) and secondary AS. Our results also show *PLCG1* p.S345F in 2 primary AS (breast, spleen), a previously unreported finding. *PLCG1* encodes phospholipase C γ 1 (PLC γ 1), a member of the phosphatidylinositol-specific phospholipase-C (PI-PLC) family, which generates inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), triggering Ca²⁺-dependent signaling and protein kinase C (PKC) activation, respectively. The p.S345F mutation alters the PLC γ protein catalytic domain, thus activating the downstream calmodulin/calcineurin signaling through facilitating the interaction with lipids and its phosphodiesterase activity.²¹ In contrast, the codon 707 mutation maps at the c-terminal Src homology 2 (cSH2) domain^{8,9}; mutations at this site preventing the intramolecular interaction of phosphorylated PLC γ 1 and triggering constitutive activation of DAG and IP-dependent signaling.

In our previous study, we reported activating *KDR* mutations in 10% of AS, being only identified in primary breast or secondary AS after breast cancer.⁶ Kunze et al reported two cases of cardiac AS harboring *KDR* mutation each in the extracellular immunoglobulin domain (R720W) and transmembrane domain (T771K).⁹ The present investigation identifies 4 new cases (7% in total), including one case arising in the vertebral bone. The most common hot spot was *KDR* codon 771 in exon 16 (transmembrane domain, 5 cases), followed by *KDR* codons 717 and 731 in exon 15 (extracellular domain Ig-like C2 type 7, 2 cases) and *KDR* exon 24 codon 1065 (kinase domain, 1 case). Our results also show that *KDR* and *PLCG1* mutations, accounting for 17% of AS cases, are mutually exclusive and evenly distributed among primary and secondary AS, mainly affecting breast and only occasionally present in bone or viscera, with no impact on morphology or outcome. Both *KDR* and *PLCG1* mutations share a common VEGFR2/PLC γ 1 signaling pathway. *KDR*, also known as *VEGFR2* (vascular endothelial growth factor receptor 2), is a member of human VEGFR family of receptor tyrosine kinases (VEGFR1-3), with critical regulatory functions in vasculogenesis and angiogenesis upon binding to various VEGFs.^{42,43} Although activating *KDR* mutations are relatively rare in AS, *KDR* is universally overexpressed in AS⁶ through alternative yet undefined mechanisms. Therapy with anti-VEGFR agents, such as sorafenib, which targets VEGFR2 and Ras kinase⁴³, have shown limited antitumor activity.⁴⁴ An alternative mechanism of VEGFR activation in secondary AS is the presence of *FLT4* (a.k.a. *VEGFR3*) amplification, which mostly occurs in association with *MYC* amplification.^{11,14} *FLT4* amplification was identified in 13% (5/39) of *MYC*-amplified AS, mutually exclusive from *KDR/PLCG1* mutations, and associated with a poor outcome. All except one *FLT4*-amplified secondary AS occurred in the breast/chest wall area, being co-amplified with *MYC*. Only one case of a primary scalp AS had *FLT4* amplification in the absence of *MYC* abnormalities by FISH. A recent study suggested that diffuse and strong cytoplasmic FLT4 immunostaining is a potential surrogate ancillary test for *FLT4* gene amplification by FISH.¹⁴

MYC deregulation is a well-known oncogenic event involving a wide array of human cancers, lying at the crossroads of diverse signal transduction pathways, including cell growth, metabolism, differentiation, transformation, cell cycle, and angiogenesis.⁴⁵ High level *MYC* amplification at the 8q24.21 locus was first shown by Manner and colleagues in 55% of secondary AS using array-comparative genomic hybridization¹⁰. Subsequently,

larger studies showed that *MYC* amplification is the hallmark of radiation-induced AS, occurring in the majority of secondary AS, but not in other radiation-related sarcomas or AVLs.^{11,13} However, more recent studies identified *MYC* amplification in rare cases of primary AS, including breast, bone, visceral and skin.^{4,12,15,46} Compared to FISH assay, the immunohistochemical analysis detects a higher number of *MYC* overexpression in primary AS.^{12,46} Our findings support prior results of *MYC* amplification in a small subset (7%) of primary AS, encompassing breast and somatic soft tissue. In contrast *MYC* amplification occurs in 91% of secondary AS related to breast cancer radiation or lymphedema.

In conclusion, this is the largest molecular investigation to date of a diverse cohort of 120 AS patients. We describe recurrent, novel, *CIC* gene rearrangements in a subset of young adults with primary soft tissue and visceral AS, with or without concurrent *CIC* mutations, associated with up-regulation of *CIC*-target genes, such as *ETV1*, *ETV4*, and *ETV5*. An additional subset of AS harbored only missense *CIC* mutations, occurring with predilection in younger patients with primary AS, and being associated with a highly aggressive clinical course. The mutually exclusive *KDR/PLCG1* mutations were present in both primary and secondary AS, but restricted to breast, bone and viscera, with no histologic or prognostic correlates. Our results show that in total, the *CIC*, *PLCG1*, *KDR*, *MYC* and *FLT4* genetic abnormalities account for around 50% of our study cohort. Albeit our study did not examine abnormalities in *PTPRB*, *TP53*, *RAS*, *PIK3CA*, etc, described in other studies^{7–9}. However, the molecular alterations of a significant number of cases remain to be elucidated. The divergent genetic abnormalities emerging in different AS subsets promise both challenges and opportunities in designing future clinical trials of targeted therapy in this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(A) The schematic diagram of chromosome 19q13 locus, encompassing *CIC*, *LEUTX*, and *PEPD* genes (arrows, direction of transcription; color dots indicate the BAC location used for 3-color FISH). (B) RT-PCR confirmed the chimeric *CIC* exon 20–*LEUTX* exon 3 transcript. (C) 3-color FISH confirmed the *CIC* and *LEUTX* break-apart with a complex and heterogeneous pattern in AS1: 3 different cells showing a variable pattern of rearrangement, with smaller and bigger gaps between the 3 BACs used (O, orange, *CIC* centromeric; G, green, *CIC* telomeric; R, red, *PEPD*; arrows indicate possible breaks) (D) *CIC* intra-chromosomal rearrangement in AS2 by FISH, with consistent gaps between red, orange and green BAC probes. Spectrum of *CIC* mutations in AS including heterozygous exon 15

mutations (p.G1190R, AS1)(E); synchronous exon 19 mutations (p.S1461F and p.R1465S, AS6)(F), and homozygous exon 19 mutations (p.Y1460H, AS7)(G).

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Figure 2. Pathologic features of *CIC*-rearranged AS

Solid sheets of monotonous epithelioid cells, with moderate to abundant eosinophilic cytoplasm, and vesicular nuclei (A,B; AS1). Small epithelioid to round cells with scant, light eosinophilic cytoplasm and vesicular nuclei with inconspicuous nucleoli (C; AS2).

Distinctive rhabdoid cells arranged in vague pseudoalveolar and nested pattern (D; AS3) All 3 cases expressed diffuse and strong CD31 (E) and ERG (F) immunoreactivity.

Figure 3. The morphologic spectrum of *CIC*-mutated AS

A solid growth is noted in AS6 harboring double *CIC* mutations (A) and AS7 bearing *CIC* homozygous mutations (B); pleomorphic tumor cells and inconspicuous vascular formation in AS8 with single *CIC* mutation (C); well-formed anastomosing vascular channels in AS4 with concurrent *CIC* and *PLCG1* mutations (D).

Figure 4.

(A) High *ETV1*, *ETV4*, *ETV5* overexpression in *CIC*-rearranged AS (CIC-AS) and SBRCTs, but not in classic AS (AS25, AS32, AS110) (RPKM, Reads Per Kilobase per Million mapped reads). (B) *CIC* overexpression (160 kDa) by western blotting in AS1-2 and *CIC-DUX4*-positive SBRCTs (SBRCT1-2), compared to *CIC*-negative AS (AS25, AS32) (control actin 43 kDa, lower part).

Clinicopathologic features of *CIC*-mutated or rearranged angiosarcomas.

Table 1

Case	<i>CIC</i> Mutation	<i>CIC</i> Translocation	Age/ Sex	Location	Etiology	Cytomorphology	Vascular Formation	Size (cm)	Initial Stage	Recur (month)	Follow-up (month)	Final Status
1 [*]	p.G1190R (exon 15)	<i>CIC-LEUTX</i>	26/F	Thigh (st)	Primary	Round/epithelioid	UD	7.5	T2bN0M0	10	33	DOD
2 [*]	p.S1422W (exon 18)	Positive	47/F	Back (st)	Primary	Round	UD	8.5	T2bN1M0	-	13	NED
3	Wild type	Positive	21/F	Kidney	Primary	Round/rhabdoid	UD	3.5	T1bN1M0	21	23	AWD
4	p.T1161S (exon 15)	Negative	40/F	Breast	Primary	Flattened	WD	9	T2N0M0	17	28	DOD
5	p.E1222G (exon 15)	Negative	41/F	Spleen	Secondary	Pleomorphic	MD	18	T2bN0M0	5	10	AWD
6	p.S1461F, p.R1465S (exon 19)	Negative	21/M	Neck (st)	Primary	Spindled/round	PD	2.5	T1bN0M0	3	12	AWD
7	p.Y1460H (exon 19)	Negative	77/M	Scalp (skin)	Primary	Epithelioid/spindled	PD	0.6	T1aN0M0	12	55	DOD
8	p.V1505I (exon 20)	Negative	34/M	Pelvis (st)	Primary	Pleomorphic	PD	9	T2bN1M0	2	2	AWD
9	p.P1549L (exon 20)	Negative	60/M	Scalp (skin)	Primary	Epithelioid	MD	1.6	T1aN0M0	-	4	NED

^{*}Tested by RNA sequencing;

st: soft tissue, UD: undifferentiated, PD: poorly differentiated, MD: moderately differentiated, WD: well differentiated, DOD: dead of disease, NED: no evidence of disease, AWD: alive with disease