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Array-CGH analysis identifies two distinct subgroups of primary angiosarcoma of bone

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Genes Chromosomes Cancer 2015. 54(2):72-81

Abstract

Molecular genetic studies on vascular tumors are rare. Recently, possible involvement of MYC and KDR has been documented in a subset of angiosarcomas of soft tissue. We performed a cytogenetic analysis of primary angiosarcomas of bone (n=13) and soft tissue (n=5) using high density array-Comparative Genomic Hybridization (array-CGH). Regions of interest were validated by Fluorescence *In Situ* Hybridization (FISH). Antibodies for candidate genes (SKI, MYC, KDR, and MAPK9) were selected and immunohistochemistry was performed. Six angiosarcomas of bone and four angiosarcomas of soft tissue showed chromosomal losses, gains and high level of amplifications. Cluster analysis identified two groups: a group with a complex genetic profile and a group with only few genetic aberrations. Five regions of interest were selected, which were located at chromosomes 1p36.23, 2q32-34, 5q35, 8q24 and 17q21.32-24.2. Interphase FISH confirmed the high-level of amplifications. Immunohistochemical analysis showed high expression of *MYC*, MAPK9 and SKI in 26%, 95% and 83%, of the tumors, respectively. There were no differences between the two groups with regards to location, immunohistochemical expression nor survival. In summary, we identified two subgroups of angiosarcomas: those with few or no gross aberrations and those which show numerous genetic aberrations consisting of chromosomal losses, gains and high level of amplifications or complex aberrations. The most common finding was amplification of 2q and 17q in both angiosarcoma of bone and soft tissue, suggesting overlap in tumorigenesis irrespective of their location. We show MYC amplification in primary angiosarcoma indicating this is not entirely specific for radiation induced angiosarcoma.

Introduction

Angiosarcoma is a rare malignant neoplasm composed of cells that demonstrate endothelial differentiation, accounting for less than 1% of all sarcomas (Fletcher et al., 2013; Weiss and Goldblum, 2008). The exact mechanism of tumorigenesis still remains unclear. However, one presumes that these tumors may arise from normal endothelium or at least cells with features of normal endothelium (Fletcher et al., 2013; Manner et al., 2010). Angiosarcoma occurs most frequently at the skin in the head and neck region, however it can arise at any anatomical site including the viscera and deep soft tissue (Fletcher et al., 2013; Weiss and Goldblum, 2008). Angiosarcoma primary of bone is extremely rare (Dorfman et al., 1971; Huvos, 1991; Mulder et al., 1993), and has a tendency to occur multifocal and has a more aggressive course(Verbeke et al., 2011; Vermaat et al., 2011). Thus, angiosarcomas are heterogeneous at the clinical and morphological level. Recent studies have suggested that this heterogeneity might be based on a different underlying molecular mechanism associated with the anatomical site of the tumor. *KDR* mutations are seen in angiosarcoma of the breast whereas high-levels of *MYC* amplification, with or without co-amplification of FLT4, are seen in secondary angiosarcoma after irradiation or chronic lymphedema (Antonescu et al., 2009; Guo et al., 2011; Manner et al., 2010).

Sarcomas are divided in two main categories based on their genetic abnormalities: i) tumors with a near-diploid karyotype and simple genetic alterations, such as specific translocations (e.g. synovial sarcoma) and mutations (e.g. gastro-intestinal stromal tumor sarcoma), which cause transcriptional deregulation or altered signaling and ii) tumors with complex and unbalanced karyotypes (e.g. osteosarcoma) (Borden et al., 2003; Helman and Meltzer, 2003; Taylor et al., 2011). To date, only a few number of angiosarcomas, mostly of soft tissue, have been genetically studied. To this point, only complex karyotypes and no recurrent chromosomal alterations have been described in angiosarcoma of soft tissue (Baumhoer et al., 2005; Cerilli et al., 1998; Fletcher et al., 1991; Gil-Benso et al., 1994; Kindblom et al., 1991; Schuborg et al., 1998;Van den Berg et al., 1994;Wong et al., 2001; Zu et al., 2001). It is therefore suggested that angiosarcomas belong to the group of sarcomas with a complex genetic profile (Helman and Meltzer, 2003). However, it may be that tumors lacking chromosomal genetic alterations were not reported, resulting in a biased representation of the literature. Dunlap and colleagues reported the first cytogenetic aberration in angiosarcoma of bone. They identified a unique clonal chromosomal rearrangement t(1;14)(p21;q24) in a primary angiosarcoma of the tibia, although a germline translocation was not excluded here (Dunlap et al., 2009). Here we report the first series of primary bone angiosarcomas with full molecular characterization. Our aim was to elucidate whether these tumors harbor recurrent genetic aberrations, or that these are tumors with complex karyotypes.

Material and Methods

Tumor tissue

Twenty five tumor samples (from 22 patients), either frozen or formalin-fixed paraffin-embedded (FFPE) material, with the diagnosis of angiosarcoma of bone were collected from the archives of the departments of Pathology of the Rizzoli Institute, Bologna, Italy (20 tumors, all FFPE material), University Hospitals Leuven, Leuven, Belgium (one tumor, frozen material), and Leiden University Medical Center, Leiden, the Netherlands (four tumor samples from one patient with multifocal disease, frozen material). Six tumor samples of angiosarcoma of soft tissue (from six patients) were collected from the archives of the department of Pathology of University Hospitals Leuven, Leuven, Belgium (three tumors, both frozen and FFPE material) and Leiden University Medical Center (three tumors, frozen or FFPE material) for comparison. The cases were originally diagnosed between 1964 and 2008. All clinical, radiodiagnostical and pathological data were reviewed, as described previously (Verbeke et al., 2011). All tumor samples were originally revised by 3 pathologists (Verbeke et al., 2011) and included in this study when the tumor had a clear-cut histology and tumor cells stained at least for one endothelial marker. Based on the histology (epithelioid hemangioma (n=1), epithelioid hemangioendothelioma (n=1), or other diagnosis (n=2) such as epithelioid sarcoma) and/or the presence of a disease specific genetic aberration (n=1; Ewing-like sarcoma with EWSR1-NFATc2 amplification) five tumor samples (from five patients) were excluded, leaving 20 tumor samples of angiosarcoma of bone (from 17 patients) and six tumor samples from angiosarcoma of soft tissue (from six patients). All specimens were handled according to the ethical guidelines described in "Code for Proper Secondary Use of Human Tissue in the Netherlands" of the Dutch Federation of Medical Scientific Societies.

Tissue microarray (TMA) construction

Four TMAs were assembled from formalin-fixed, paraffin embedded tissue using standard procedures (Verbeke et al., 2011) using a 2 mm-diameter punch (3DHistech Ltd., Budapest, Hungary; 3 TMAs) or a 0.6 mm-diameter punch (Beecher Instruments, Silver Spring, MD, USA; 1TMA) as previously extensively described (Verbeke et al., 2011; Verbeke et al., 2013) containing 42 angiosarcomas of bone and 19 angiosarcomas of soft tissue. Using a tape-transfer system (Instrumedics, Hackensack, NJ, USA), 4-μm sections were transferred to glass slides.

DNA isolation

DNA was isolated from 20 angiosarcomas of bone and six angiosarcomas of soft tissue, as described previously (de Jong D. et al., 2011). Four μm consecutive sections were cut, either from frozen tissue and/ or FFPE, and stained using standard hematoxylin and eosin (HE) to confirm a tumor content of at least 70%. DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer and quality was checked after electrophoresis separation using a 1% agarose gel stained with ethidium bromide.

Multicolor (COBRA)-FISH karyotyping

From case L2258, a multifocal angiosarcoma, life cells were available after surgical intervention (amputation). Tumor samples were collected from five different sites and samples subjected to short term culture followed by chromosome harvesting according to protocols described (Szuhai et al., 2009). Metaphase cells were obtained from three samples (annotated as L2258 A, D and E) and subjected to multicolor COBRA-FISH karyotyping analysis as described earlier (Szuhai and Tanke, 2006).

Array-CGH analysis

ULS labeling of DNA, derived from decalcified FFPE tissue sections, was performed as previously described (de Jong D. et al., 2011) using the Agilent Oligo array-CGH Labeling Kit for FFPE Samples (Agilent Technologies, Santa Clara, CA). Labeling of DNA, derived from frozen tissue sections, was done using the BioPrime Total Genomic Labeling System (Invitrogen Corporation**,** Carlsbad**,** CA). Labeling efficiency was calculated using a Nanodrop ND-1000 spectrophotometer measuring A_{260} (DNA), A_{550} (Cy3) and A_{649} (Cy5). As a reference, DNA from a commercial source (Promega Corporation, Madison, WI) was used. Labeled test and reference samples were mixed and hybridized as a gender mismatch to show dynamic range of hybridization on the X and Y chromosomes. Hybridization was performed on an Agilent 4x44k oligo array at 65°C for 40 hours. Slides were washed with Oligo array-CGH Wash Buffer 1 at room temperature for 5 min followed by a 1 min wash with Oligo array-CGH Wash Buffer 2 at 37°C. Finally, slides were dried without using the stabilization and drying solution. Slides were scanned using Agilent Scanner with 5μm scan resolution. Scan images were processed with the Feature Extraction Software and analyzed by using Genomic Workbench (Agilent Technologies, Santa Clara, CA) and data was deposited via CanGEM (Scheinin et al. (2008) Nucleic Acids Research 36:D830-D835). Altered regions were calculated using the array-CGH analysis tool in Chipster v1.4.6 (http://chipster.csc.fi/). The minimum number of probes per called segment was 5. To identify common regions the maximum amount of information loss allowed was 0.01, as described by van de Wiel et al (van de Wiel and Wieringen, 2007). Samples were divided into 2 groups, angiosarcoma of bone (black) and angiosarcoma of soft tissue (red) to see if these would cluster together in the wecca (weighted clustering of called array-CGH data) plot (Figure 1A).

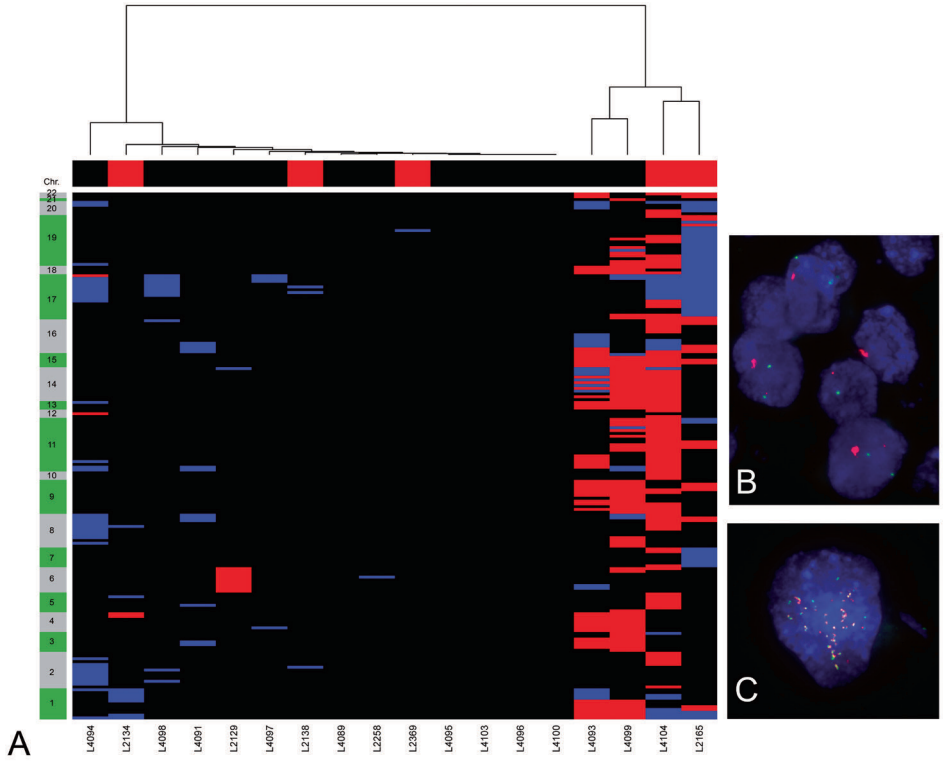

Figure 1A. Weighted clustering of called array-CGH data (WECCA) for the individual samples. The samples are divided into two groups: angiosarcoma of bone in black and angiosarcoma of soft tissue in red. On top the clustering is depicted of these groups. The green and grey bars on the left represent different chromosome segments with their called aberrations. Called losses are shown in red, gains in blue, and unaltered regions in black. Note that the size of a displayed chromosome is proportional to the size of the called region and not to the actual size; **1B.** Amplification of the *MYC* gene locus (detected in red) was confirmed by FISH on L2134. In green the centromeric region of chromosome 8 is detected; **1C.** High level amplification of chromosomes 2q31.1and 17q23.2 and a co-localization of the two probes was observed in L2138.

Confirmatory Fluorescence *In Situ* Hybridization (FISH)

Based on the array-CGH results, interphase FISH using region specific BAC clones on the tissue samples and the TMAs was performed to confirm the results. In short, slides were deparaffinized for 2 x 10 min in xylene, following dehydration and 30 min incubation in NaSCN at 90°C. After washing with PBS slides were treated for 30 min with 0.4% pepsin. Slides were washed with PBS, dehydrated and air-dried. To confirm some of the array-CGH results, two-color interphase FISH experiments were done. A BAC-clone (RP1-80K22) located at 8q24.21 covering the *MYC* gene locus (detected in red) in combination with an alpha satellite probe specific to the centromeric region of chromosome 8 (detected in green) was used as described earlier (Rossi et al., 2007). In order to validate the amplification of 2q31.1 and 17q23.2 in L2138, BAC-clones were used covering these regions. RP11-472A3 (detected in green) was located on 2q31.1 and

RP11-651H20 (detected in red) was located on 17q23.2. After validation of the amplification on whole sections from the two respective cases, these probes were also hybridized on the TMAs. All evaluable nuclei of the tissue cores were examined.

Immunohistochemistry

Based on the array-CGH results, antibodies for some candidate genes (SKI, MYC and MAPK9) were selected and immunohistochemistry was performed on the TMAs. Because *KDR* (*kinase insert domain receptor*, a.k.a. *VEGFR2*) mutations were described in primary angiosarcoma of the breast (Antonescu et al., 2009), the KDR immunoreactivity was also analyzed on the TMAs. Immunohistochemical reactions were performed according to standard laboratory methods (Pansuriya et al., 2011). For each antibody a positive and negative external control was included. The antibodies, their sources, antigen retrieval methods, dilutions, positive and negative external controls used are documented in Table 1. As negative control, sections were stained without adding the primary antibody. The intensity (0 = no staining, 1 = weak, 2 = moderate, 3 = strong) and percentage of positive neoplastic cells (0 = 0%, 1 = 1-24%, 2 = 25-49%, 3 = 50-74%, 4 = 75-100%) were evaluated. Lost tissue cores were excluded from the analysis. As decalcification could compromise the immunohistochemical result, we attempt to counteract this phenomenon by excluding the tissue samples in which an expected positive internal control was negative and tissue samples without a positive internal control that were negative for multiple antibodies. The sum of intensity and percentage was used for analysis.

Table 1. List of antibodies used for immunohistochemical analysis

Antibody	Clone	Dilution	AR	Blocking	Source	Positive control
c-MYC	Y69	1:8000	EDTA	-	Epitomics	Burkitt lymphoma
MAPK9		1:2000	Citrate	-	Abcam	Breast carcinoma
SKI		1:6000	Citraat	NGS 10%	Bioconnect	Kidney
KDR	55B11	1:125	EDTA	-	Cell Signaling	Tonsil

Results

Patient Characteristics

Patient characteristics of both angiosarcoma of bone and soft tissue are summarized in Table 2.

Table 2. Summary of clinicopathological characteristics of all angiosarcoma of bone and soft tissue

Sample ID	F/FFPE*	Age/sex	Diagnosis	Primary localisation
L2129	F	46/F	Multifocal epitheloid angiosarcoma	Bone - foot
L2258	F	39/F	Multifocal angiosarcoma	Bone - foot
L4089	FFPE	41/M	Angiosarcoma	Bone - metacarpal bone
L4091	FFPE	59/M	Angiosarcoma	Bone - femur
L4093	FFPE	72/M	Angiosarcoma	Bone - pubic bone
L4094	FFPE	56/M	Angiosarcoma	Bone - femur
L4095	FFPE	60/M	Angiosarcoma	Bone - femur
L4096	FFPE	35/M	Angiosarcoma	Bone - tibia
L4097	FFPE	74/F	Angiosarcoma	Bone - femur
L4098	FFPE	32/M	Angiosarcoma	Bone - sacrum
L4099	FFPE	54/F	Angiosarcoma	Bone - tibia
L4100	FFPE	54/M	Angiosarcoma	Bone - femur
L4103	FFPE	52/M	Angiosarcoma	Bone - femur
L2134	F/FFPE	79/F	Angiosarcoma	Soft tissue - leg
L2138	F/FFPE	67/M	Angiosarcoma	Soft tissue - retroperitoneum
L2165	F	37/M	Angiosarcoma	Soft tissue - heart
L2369	F	31/M	Angiosarcoma	Soft tissue - muscle arm
L4104	FFPE	42/F	Angiosarcoma	Soft tissue - spleen

F: frozen; FFPE: formalin-fixed paraffin-embedded

Array-CGH analysis

Twenty tumor samples of angiosarcoma of bone and six tumor samples of angiosarcoma of soft tissue were analyzed on a 44K oligonucleotide array chip (Agilent). However due to bad DNA quality the array-CGH failed in seven tumor samples of angiosarcoma of bone (from 5 patients) and in one tumor sample of angiosarcoma of soft tissue (from 1 patient). Alterations were observed in 6 out of 13 angiosarcomas of bone and 4 out of 5 angiosarcomas of soft tissue, varying from full complex genetic profiles with random gains and losses of whole chromosomes (2 angiosarcomas of bone: L4093 and L4099; and 2 angiosarcomas of soft tissue: L2165 and L4104) to tumors showing only smaller altered regions (2 angiosarcomas of bone: L4094 and L4098; and 2 angiosarcomas of soft tissue: L2134 and L2138). Two tumors (L2129 and L4097, both angiosarcoma of bone) only showed a single chromosome gain as aberration. Seven angiosarcomas of bone (L2258, L4089, L4091, L4095, L4096, L4100 and L4103) and one angiosarcoma of soft tissue (L2369) did not show any alterations, despite a percentage of tumor cells >70%. Cluster analysis demonstrated two distinct subgroups of angiosarcoma: one group of tumors with a complex genetic profile and a second group with only few genetic aberrations or a normal genetic profile (Figure 1A).

Alterations found in more than 25% of the cases were considered as recurrent genomic changes. However, none of the genomic alterations reached the 25% cut off. An overview of all genomic changes and wecca plots of all analyzed samples are shown in Figure 1A. Cluster analysis did not show a genetic difference between angiosarcoma of bone and soft tissue, indicating the absence of a genomic pattern specific for either bone or soft tissue angiosarcoma.

High level of amplifications/ homozygous deletions

High level of amplification was observed in two angiosarcomas of bone (L4094 and L4098) at 2q31.1 and at 17q23.2 (Table 3). Also in one angiosarcoma of soft tissue (L2138), amplicons located in these regions were observed (Table 3). The minimal common region on chromosome 2 includes several genes (listed in Table 3). The amplicon on chromosome 17 harbors even more genes (amongst others *USP32*, listed in Table 3). One angiosarcoma of soft tissue (L2134) showed high level amplification at region 5q35 (Table 3), which includes the *MAPK9* gene, and an amplicon at region 8q24.2, containing the *MYC* gene (amongst others). One angiosarcoma (L2165) of soft tissue showed an amplicon at region 1p36, containing the *SKI* gene (Figure 1A and Table 3). In two cases a homozygous deletion of the *CDKN2A/CDKN2B* locus was found (L4093 and L4094). Start and end position of the critical minimal region of amplification and homozygous deletion are provided in Table 3.

Multicolor (COBRA)-FISH karyotyping

In total 22 metaphase cells were analyzed, from L2258 A (12 metaphase cells), D (5 metaphase cells) and E (5 metaphase cells), respectively. All analyzed cells showed a seemingly normal karyotype, no balanced rearrangements were detected by this technique (data not shown). These results were in line with the array-CGH findings.

Confirmatory FISH

To confirm some of the array-CGH results, two-color interphase FISH experiments were done. The amplification of the *MYC* gene locus could be verified in case L2134 (Figure 1B), however, no other cases on the TMAs showed a *MYC* amplification. The amplification of 2q31.1 and 17q23.2 was verified in L2138. Also a clear co-localization was observed between the two probes used, suggesting a more complex rearrangement in this tumor (Figure 1C) that leads to co-amplification of the joined genomic regions. Interphase FISH with these probes on the TMAs did not reveal a second case with similar amplification and co-localization pattern of the probes. Amplification of both probes was confirmed in two tumor samples that were also analyzed with array-CGH (L4094, L4098) (Data not shown).

Immunohistochemistry

Irrespective of the interphase FISH results, 18.5% (5/27) of the angiosarcomas of bone showed expression of c-MYC (sumscore ≥ 3) as compared to one third (11/33) of the angiosarcomas of soft tissue. An overview of the number of positive tumors and percentages for the different antibodies used on the TMAs is given in in Table 4. The majority of angiosarcomas of bone showed expression for MAPK9 (sumscore ≥ 3; 87.5%; 28/32) and SKI (sumscore ≥ 4; 85.1%; 23/27). Also angiosarcoma of soft tissue showed a similar expression percentage for MAPK9 (94.6%; 35/37) and SKI (82.9%, 29/35).

Table 3. Overview of the tumor samples with an aberrant array-CGH result, either exhibiting homozygous deletions and/ or high level of amplifications, and the related genes of interest in angiosarcoma of bone and soft tissue

Sample ID	Overall aCGH result	Homozygous deletion	High level of amplification*	Genes of interest
L4093	Complex genomic pattern, Homozygous deletion	chr9		***CDKN2A/2B***
L4094	Homozygous deletion,	chr9		***CDKN2A/2B***
	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856
L4098	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856
L2134	High level of amplification on chr5 + chr8		chr5:178,611,827-180,846,638	CLK4, ZNF354A, BC024222, ZNF354B, ZFP2,
				ZNF454, GRM6, BX648737, ZNF354C,
				ADAMTS2, RUFY1, HNRPH1, CANX,
				MAML1, AB209135, LTC4S, MGAT4B,
				BC001874, SQSTM1, LOC51149, TBC1D9B,
				RNF130, RASGEF1C, **MAPK9**, GFPT2,
				CNOT6, FLT4, MGAT1, AK057194
			chr8:127,161,861-127,741,027	DQ515897, BC042052, M13930, ***MYC***
L2138	High level of amplification on chr2q + chr17q		chr2:171,979,233-173,263,789	HAT1, MAP1D, DLX1, DLX2, U61089,
				ITGA6, PDK1, RAPGEF4, ZAK
			chr17:60,222,436-63,379,832	USP32, C17orf64, APPBP2, PPM1D,
				BCAS3, TBX2, TBX4, BRIP1, INTS2,
				THRAP1, EFCAB3, METTL2A, TLK2,
				MRC2, RNF190, BC040294, AB046856

L2165	Complex genomic pattern, High level of amplification	chr1:1,569,923-3,759,635	SSU72, MIB2, CDC2L1, SLC35E2, NADK, AK096614, GNB1, PRKCZ, AK127994, AK126870, **SKI**, MORN1, RER1, PEX10, PLCH2, PANK4, TNFRSF14, C1orf93, MMEL1, AL713743, PRDM16, ARHGEF16, FAM79A, WDR8, TP73 , BC044603, CCDC27
L4104	Complex genomic pattern		
L4099	Complex genomic pattern		

*UCSC Genome Browser on Human Dec. 2013 (GRCh/hg38) Assembly

Table 4. Immunohistochemistry results of all analyzed angiosarcomas of bone and soft tissue present on the different TMAs.

	Angiosarcoma of bone		Angiosarcoma of soft tissue		*P*-value
	n	%	n	%	
c-MYC	5/27	18,5	11/33	33,3	ns
MAPK9	28/32	87,5	35/37	94,6	ns
SKI	23/27	85,1	29/35	82,9	ns
KDR	8/27	29,6	23/33	69,7	0,002

ns = not significant

As mutations in KDR were previously described in angiosarcomas of soft tissue, especially of the breast (Antonescu et al., 2009), we investigated whether KDR overexpression was also present in angiosarcoma of bone, and especially in the subgroup with relatively few genetic aberrations. The expression of KDR was significantly different ($p = 0.002$) between angiosarcoma of bone and soft tissue: Only 29.6% (8/27) of the angiosarcomas of bone showed overexpression of KDR (sumscore ≥ 6) as compared to more than two third (69.7%; 23/33) of the angiosarcomas of soft tissue. There was however no statistically significant difference in expression of KDR, nor of MYC, MAPK9, SKI, or any of the previously identified markers p53, p16, D2-40 and no difference in survival (performed previously (Verbeke et al., 2011;Verbeke et al., 2013)) between tumors with few genomic aberrations and tumors with complex genetic changes although numbers of cases in the different subgroups are very small (Table 5).

Table 5. Correlation of the cluster analysis, based on the array-CGH results, with present (SKI, KDR, MAPK9 and MYC) and previously (TP53, CDKN2A and D2-40) described immunohistochemical analysis and the relation with the corresponding survival data as published previously (Verbeke et al., 2011; Verbeke et al., 2013)

	Simple karyotype	Complex karyotype	*P*-value
Bone versus Soft Tissue	11 versus 3	2 versus 2	
Range median overall survival (months)	2 - 207	4 - 49	
TP53 overexpression	1/10 (10%)	0/3 (0%)	0,640
Lack of CDKN2A expression	4/10 (40%)	1/3 (33%)	0,793
D2-40 expression	6/8 (75%)	2/2 (100%)	0,429
MYC amplification	3/10 (30%)	1/3 (33%)	0,913
Overexpression of KDR	3/10 (30%)	1/3 (33%)	0,913
Expression of SKI	8/10 (80%)	2/3 (66%)	0,631
Expression of MAPK9	10/10 (100%)	3/3 (100%)	-

Discussion

We report the first large series of primary bone angiosarcomas with full molecular characterization, as cytogenetic studies of these tumors so far are limited to case reports (Dunlap et al., 2009). We previously demonstrated that the TGF-beta pathway is more active in angiosarcoma of bone compared to its soft tissue counterpart and that the PI3K/Akt pathway is involved in both angiosarcoma of bone and soft tissue, but through a different mechanism: decreased expression of PTEN in angiosarcoma of bone and overexpression of KIT in angiosarcoma of soft tissue (Verbeke et al., 2013). In the current study we show that there is no evident molecular genetic difference between angiosarcoma of bone and angiosarcoma occurring primarily in soft tissue (Figure 1A). This suggests that either alterations not detectable by array-CGH, or the microenvironment which differs between bone and soft tissue, may cause the previously detected differences in signaling pathways in angiosarcomas. Instead, we identified two different subgroups of primary angiosarcomas: one group of angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations (Figure 1A). Since sarcomas with complex karyotypes usually have a defective Retinoblastoma (Rb) and/or TP53 pathway (Helman and Meltzer, 2003; Perot et al., 2010), we questioned whether the angiosarcomas with complex changes also more often demonstrated alterations in the Rb and/or TP53 pathway, results which were available from our previous immunohistochemical study (Verbeke et al., 2013). We described that the Rb pathway was disrupted in 55% of angiosarcomas of bone which was mainly caused by lack of protein expression of CDKN2A. Interestingly, only two of the 18 cases showed a detectable involvement of the CDKN2A/2B region (homozygous deletion in L4093 and L4094, one with a complex and one with a simple genetic profile, respectively) indicating the involvement of other mechanisms leading to the loss of CDKN2A protein expression. Angiosarcomas of bone showing lack of expression of CDKN2A had also a significantly worse prognosis (Verbeke et al., 2013). In contrast to angiosarcoma of soft tissue, the TP53 pathway seemed not important in angiosarcoma of bone (Verbeke et al., 2013). Here we show that there is no statistically significant difference in lack of CDKN2A protein expression nor TP53 overexpression between angiosarcomas with complex genetic alterations and those that are genetically more simple (Table 5). Interestingly, neither was correlation found with survival, location in bone or soft tissue, nor with expression of D2-40, a marker of lymphangiogenic differentiation which previously showed to be an indicator of aggressive behavior and worse prognosis (Table 5) (Verbeke et al., 2011).

It is interesting that a subset of angiosarcomas lacks genetic aberrations detectable at array-CGH. Pre-analytic inaccuracies might be anticipated as we included tumor samples with a tumor percentage of 70% or more. As all cases were reviewed by three expert pathologists (Verbeke et al., 2011) and since both groups of tumors, with or without a complex genetic profile, showed a similar overall survival, misdiagnosis seems unlikely. Balanced genomic rearrangements, such as balanced translocations and inversions, and point mutations in the DNA are not detected by array-CGH. However, multicolor (COBRA)-FISH karyotyping did not detect any cytogenetically visible balanced rearrangements in case L2258 (A, D and E)

displaying multifocal lesions. Although this supports our array-CGH findings, the presence of cryptic structural rearrangements or point mutations cannot be excluded. We investigated the expression of KDR, which was shown to be mutated in angiosarcoma of soft tissue, especially the breast (Antonescu et al., 2009), and did not detect a higher number of cases with KDR overexpression in the angiosarcomas with simple karyotypes.

Previously, a clear genomic difference between primary angiosarcomas and angiosarcomas secondary to radiation or lymphedema was well documented (Guo et al., 2011). It was initially suggested that *MYC* amplification is a specific feature of secondary angiosarcomas (Guo et al., 2011). *MYC* amplification was shown to upregulate the miR-17–92 cluster, which subsequently downregulates thrombospondin-1 (THBS1), a potent endogenous inhibitor of angiogenesis, and thereby mediating the angiogenic phenotype of angiosarcoma (Italiano et al., 2012).

We here report the presence of *MYC* amplification in one angiosarcoma of soft tissue (L2134) without any history of radiation therapy or chronic lymphedema. This is in line with recent studies also demonstrating the presence of *MYC* amplification in a small subset of non-radiation induced angiosarcomas, primary cutaneous angiosarcomas and a subset of primary hepatic angiosarcomas(Ginter et al., 2014; Italiano et al., 2012; Shon et al., 2014). In addition to MYC, coamplification of *FLT4* (encoding *VEGFR3*) at 5q35 was identified in 25% of secondary angiosarcomas, and not in other types (Guo et al., 2011). Our case (L2134) with *MYC* amplification also revealed a high level of amplification at region 5q, containing the *FLT4* and the *MAPK9* (*JNK2*) gene. The protein JNK2 belongs to the JNK kinase family and in response to different stimuli, such as cytokines and growth factors, these proteins are activated by a series of phosphorylation events and subsequently activate several nuclear and non-nuclear proteins, including MYC, p53, and cell death regulators of the Bcl-2 family in the mitochondria (Bubici and Papa, 2014). Therefore, JNK signaling pathways do not only play a role in normal physiological processes, but are also involved in cancer pathogenesis (Bubici and Papa, 2014). Several studies have shown a potential role for JNK2 in tumor cell survival (Barbarulo et al., 2013; Raciti et al., 2012). We demonstrated high protein expression of MAPK9 (JNK2) in 87-94% of the angiosarcomas, irrespective of genomic amplification. This may be explained by the high expression of TGF-beta in angiosarcoma of bone that we previously identified (Verbeke et al., 2013), as TGF-beta can activate JNK2 (Galliher et al., 2006) thereby contributing to increased tumor cell survival. A potential role of JNK2 in angiogenesis has not been described yet.

Very recently, a next generation sequencing (NGS) approach revealed recurrent mutations in *PTPRB* in 26% of the analyzed cases with co-occurring *PLCG1* mutations in 3 of those cases (Behjati et al., 2014). Interestingly, these mutations only occurred in either known secondary angiosarcomas and/ or angiosarcomas with a *MYC* amplification. These data again confirm that secondary and/or *MYC* amplified angiosarcomas should be regarded as a separate subgroup. Our study included only one such case, as we selected primary angiosarcomas. It would be interesting to subject these cases to NGS, however, the archival formic acid decalcified FFPE material is not suited for NGS yet.

One angiosarcoma of soft tissue (L2165) showed an amplicon at region 1p36. Amongst many other genes, this region contains *SKI* (Sloan Kettering Institute proto-oncoprotein). The Ski proto-oncogene (alternative name Proto-oncogene c-Ski or c-Ski) is involved in many signaling pathways (Bonnon and Atanasoski, 2012) and its most well-known function is to negatively regulate TGF-beta signaling. In this study we demonstrate that both angiosarcoma of bone and soft tissue show a high expression of Ski. This finding is consistent with a previous report of Ski expression in hemangiomas (TM et al., 2009). Interestingly, our previous findings show that the TGF-beta pathway is highly active in angiosarcoma especially in angiosarcoma of bone (Verbeke et al., 2013). Thus, despite high expression of Ski, TGF-beta signaling is not suppressed. Although the precise mechanism is unclear one could speculate that other mechanisms are operable overruling the suppression of TGF-beta signaling by Ski in angiosarcoma of bone, and that Ski may contribute to tumorigenesis through other mechanisms. It has been demonstrated that Ski can promote cancer progression and is highly expressed in different human solid tumors, e.g. leukemia, gastro-intestinal cancers and melanoma (Bonnon and Atanasoski, 2012; Wang et al., 2013). It has been suggested that overexpression of Ski plays a role in tumor growth and angiogenesis in diffuse type gastric cancer (Kiyono et al., 2009), however its exact role in angiogenesis remains unclear.

Although the threshold for recurrent aberrations was not reached, three cases showed a high level of amplification of chromosome 2q and chromosome 17q (Table 3). By gene amplification cancer cells are allowed to promote expression of genes that are involved in tumor development and progression. High level amplification of 17q23 is described in many tumor types (Andersen et al., 2002; Parssinen et al., 2007) and especially in breast carcinomas it is associated with tumor progression and poor prognosis (Andersen et al., 2002; Barlund et al., 1997; Isola et al., 1995; Parssinen et al., 2007). So far, the high level amplification of chromosome 2q32-34 has not been described. Both amplified regions contain a large number of possible candidate genes (Table 3). To date, multiple genes have been proposed that could play a role in tumor development and progression (Parssinen et al., 2007). It has been hypothesized that not a single target gene is responsible for, or contributes to, tumor pathogenesis, but the simultaneous overexpression of multiple genes could lead to growth advantages of cancer cells (Parssinen et al., 2007).

In conclusion, we report the molecular genetic characterization of the first series of primary angiosarcoma of bone, in comparison to soft tissue. We identified two different subgroups: one group of angiosarcomas with a complex genetic profile and a second group of angiosarcomas with only few genetic aberrations: mainly high level amplifications. No evident molecular difference was found between both angiosarcoma of bone and soft tissue. We confirm that although *MYC* amplification first was described in radiation therapy and chronic lymphedema associated angiosarcomas, it can occur in a subset of primary angiosarcomas and is therefore not entirely specific for radiation induced angiosarcoma. Moreover, our data indicate that in addition to secondary/ *MYC* amplified angiosarcomas, also primary angiosarcomas are genetically heterogeneous.

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