Short Communication

MYC High Level Gene Amplification Is a Distinctive Feature of Angiosarcomas after Irradiation or Chronic Lymphedema

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Angiosarcomas (AS) are rare vascular malignancies that arise either *de novo* as primary tumors or secondary to irradiation or chronic lymphedema. The cytogenetics of angiosarcomas are poorly characterized. We applied array-comparative genomic hybridization as a screening method to identify recurrent alterations in 22 cases. Recurrent genetic alterations were identified only in secondary but not in primary AS. The most frequent recurrent alterations were high level amplifications on chromosome 8q24.21 (50%), followed by 10p12.33 (33%) and 5q35.3 (11%). Fluorescence in situ hybridization analysis in 28 primary and 33 secondary angiosarcomas (31 tumors second-

ary to irradiation, 2 tumors secondary to chronic lymphedema) confirmed high level amplification of *MYC* on chromosome 8q24.21 as a recurrent genetic alteration found exclusively in 55% of AS secondary to irradiation or chronic lymphedema, but not in primary AS. Amplification of *MYC* did not predispose to high grade morphology or increased cell turnover. In conclusion, despite their identical morphology, secondary AS are genetically different from primary AS and are characterized by a high frequency of high level amplifications of *MYC*. This finding may have implications both for the diagnosis and treatment of these tumors. (Am J Pathol 2010, 176:34–39; DOI: 10.2353/ajpath.2010.090637)

Angiosarcomas (AS) are rare sarcomas with morphological and functional properties of endothelial cells.¹ AS represent <1% of all sarcomas.² Roughly 35% of cases arise in the skin, 25% in soft tissue and the rest in various other locations including breast, liver, spleen, and bone.¹ The prognosis of AS has generally been considered to be poor with unpredictable clinical behavior. However, several publications clearly showed that prognosis depends on the primary site with a particularly poor prognosis for tumors arising in liver, spleen, heart, and bone with a 5-year-survival rate of 0%, as compared with approximately 50% for skin and soft tissue AS.^{3,4} Other factors

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with an adverse impact on prognosis are older age, tumor necrosis, and epitheloid features.⁵ Tumors can arise either *de novo* (primary AS, pAS) or as secondary conditions in patients with long standing lymphedema^{6,7} or after irradiation⁸ (secondary AS; sAS), especially in female patients irradiated for breast cancer. In fact, sAS of the breast is by far the most frequent radiation-induced sarcoma in women treated with radiation therapy as part of their initial treatment,⁹ with a more than 1000-fold increased relative risk¹⁰ as compared with the general population. It has been calculated that the standardized incidence ratio in irradiated breast cancer patients to develop AS is 26 compared with 3.8 to develop any other sarcoma⁹ over a latency period of 5 to 10 years. Of note, the latency period of sAS has been reported to be much shorter than in other radiation-induced sarcomas, which is generally >10 years.^{11–14} Relatively little is known about the genetic changes in postradiation sarcomas in general^{15–18} and in sAS in particular, where published genetic studies are limited to case reports or small case collections.^{19–28} Probably due to the small case numbers, available data have so far failed to show consistent recurrent chromosomal abnormalities. In this report, we studied clinicopathologic and molecular genetic features in 61 primary and secondary AS from multiple international institutions.

Materials and Methods

The clinicopathologic data of studied cases are shown in Table 1. Paraffin blocks of histologically proven angiosarcomas were retrospectively collected from the collaborating institutions in Germany, France and Belgium. All tumors were stratified by histological grade according to the scoring system of the Fédération Nationale des Centres de Lutte Contre le Cancer²⁹ by two of the authors (A.M. and Ph.S.).

Immunohistochemistry

Immunohistochemistry was performed according to standard protocols.³⁰ Primary antibodies used were CD34 (1:500; Beckman Coulter, Krefeld, Germany) and CD31 (1:500, Dako, Hamburg, Germany) and ki67 (1:800, Dako). Proliferation was quantified under a light microscope by counting ki-67 stained nuclei per 100 tumor cells in randomly chosen high power fields. Apoptosis was measured using the commercially available terminal deoxynucleotidyl transferase dUTP nick-end labeling kit (In Situ Cell Death Detection Kit, Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions and quantitated under a Zeiss Axiophot fluorescence microscope by counting fluorescein isothiocyanate-stained nuclei per 100 tumor cell nuclei counterstained with 4',6-diamidin-2'-phenylindol-dihydrochloride (Linaris, Wertheim, Germany).

Array-Based Comparative Genomic Hybridization

Array-comparative genomic hybridization was performed as previously described,^{31–33} using genomic DNA isolated from fresh frozen tumor material on microarrays with more than 8000 large insert clones (8-k array). The array contained the Sanger Center 1-Mb clone set covering the genome at an average resolution of approximately 1Mb, 3000 gene- and region-specific RCPI (Roswell Park Cancer Institute database) (RZPD, Berlin, Germany) and CalTech (Invitrogen, Carlsbad, CA) bacterial artificial chromosome clones. Arrays were hybridized with Cy3-labeled test DNA and Cy5-labeled reference DNA. Reference DNA pools were generated from ten healthy women and from ten healthy men. Array-comparative genomic hybridization data were processed using the ChipYard framework (Heidelberg, Germany). Diagnostic thresholds were defined by extensive positive and negative control experiments as ± 7 SD of the mean.

Bicolour Fluorescence in Situ Hybridization Analyses

Fluorescence *in situ* hybridization was performed as previously described.³⁴ Hybridization was performed using a P1 probe for MYC labeled with digoxigenin by nick translation (RMC08P001; provided by the Cancer Center, University of San Francisco, San Francisco, CA) and an α -satellite probe for the centromeric region of chromosome 8 (Vysis Inc., Downers Grove, IL). For detection of the MYC probe, the tyramide signal amplification system from NEN Life Science GmbH (Köln, Germany) was applied according to the manufacturer's recommendations. In brief, after a 30-minute blocking step, slides were treated with peroxidase-conjugated anti-digoxigenin antibody (1:50 in tyramide signal amplification blocking solution; Roche Diagnostics GmbH) for 90 minutes at 37°C. For visualization of the probe, slides were incubated for 7

Table 1. Summary of Clinicopathological Findings in Primary and Secondary Angiosarcomas

	pAS (n = 28)		sAS (n = 33)	
	n =	%	n =	%
Male	15	54	5	15
Female	13	46	28	85
Tumor site				
Breast	2	7	20	61
Skin	8	29	7*	21
Soft tissue	3	11	2	6
Bone	3	11	1	3
Heart	4	14	0	0
Spleen	3	11	0	0
Liver	1	4	0	0
Other	4	14	3	10
FNCLCC grade				
1	9	32	10	30
2	11	39	8	24
3	8	36	15	39

pAS, primary angiosarcomas; sAS, secondary angiosarcomas.
*Including 2 cases associated with chronic lymphedema.

Table 2. Chromosome Loci Displaying Recurrent Copy-Number Amplifications or Homozygous Deletions

Mapping	Start [Mbp]	End [Mbp]	Size [Mbp]	Type	pAS (n = 8)	sAS (n = 14)
5q35.3	178.69	180.15	1.46	Amp	0	2
8q24.21	128.04	129.04	1.00	Amp	0	8
10p12.33	17.84	18.94	1.10	Amp	0	6

pAS, primary angiosarcomas; sAS, secondary angiosarcomas; Del, deletion; Amp, amplification.

minutes with tyramide-Cy3 (1:50 in tyramide signal amplification solution). Interphase nuclei were counterstained with 0.5 $\mu\text{g}/\mu\text{L}$ of 4,6-diamidino-2-phenylindole in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). At least 200 nuclei per sample were assessed and scored semiquantitatively. Amplification was assumed if more than two times as many gene signals compared with centromere signals were detected in >25% of tumor cells. Detection of >9 gene signals or tight clusters of at least 10 gene signals was considered high level amplification.

Statistical Analyses

Statistical differences between categoric variables were determined using Yates corrected Chi-Square test. Comparisons between groups were analyzed using Mann-Whitney-U-test, as provided by the SPSS software (Version 2000, SPSS Inc., Chicago, IL). For all analyses, a *P* value <0.05 was considered significant, unless otherwise stated.

Results

Clinicopathologic Features

Cases were retrospectively collected in reference sarcoma centers. A summary of the clinicopathologic data are shown in Table 1. pAS and sAS occurred in the same age groups (pAS: age median 69 years [range, 32 to 85]; sAS: age median 73 years [range, 34 to 88]). The majority (61%) of sAS were observed in the breast of females with a history of irradiation for mammary carcinomas, followed by skin (21%) and soft tissues (6%); two cases arose in the setting of chronic lymphedema. There were no AS associated with a history of thorotrast exposure in this series. In contrast to the marked female preponderance among sAS, males and females were affected with equal

frequency in the pAS group. Skin tumors made up roughly one third of the cases, the remaining cases were located in soft tissues and internal organs. The relative distribution of histological tumor grades 1 to 3 was identical among pAS and sAS. Tumors with epitheloid features were slightly more frequent in the pAS (six cases) than in the sAS group (two cases).

Recurrent Chromosomal Alterations in Angiosarcomas

Twenty-two cases (8 pAS; 14 sAS) were analyzed by array-comparative genomic hybridization. Of these, 10 sAS (nine irradiated cases and one case associated with chronic lymphedema) showed a total of 16 recurrent alterations (Table 2 + 3, Figure 1; full data available online under in Supplemental Table S1 at <http://ajp.amjpathol.org>), while no recurrent alterations were observed in pAS. The most frequent alterations in sAS were high level amplifications on chromosome 8q24.21 (eight cases), followed by amplifications on chromosome 10p12.33 (six cases) and amplifications on chromosome 5q35.3 (two cases). Amplifications on chromosome 8q24.21 were frequently observed in combination with other amplifications, most often on chromosome 10p12.33 (six cases), but also chromosome 5q35.3 (two cases).

High Level Amplifications of the MYC Gene Are a Characteristic Feature of Secondary Angiosarcomas

Since the most likely candidate gene for high level amplification in the chromosomal region 8q24.21 was *MYC*, we next applied fluorescence *in situ* hybridization to verify this assumption (Figure 2, A–C). In an analysis of 61 cases (28 pAS, 33 sAS), a total of 18 cases with high level gene amplifications of *MYC* were observed. Of these, all

Table 3. Clinicopathologic Characteristics of Angiosarcomas with Chromosomal Alterations

Case no.	Etiology	Localization	Grade	Detected chromosomal aberrations
HA 96	Radiation	Breast	3	Amp 5q35.3; amp 8q24.21
BO 5315	Radiation	Breast	2	Amp 5q35.3; amp 8q24.21; amp 10p12.33; amp 19q13.32
BO 3108	Radiation	Breast	3	Amp 8q24.21; amp 10p12.33
K 6911	Radiation	Breast	1	Amp 8q24.21; amp 10p12.33
K 7334	Radiation	Breast	2	Amp 8q24.21; amp 10p12.33
BO 4592	Radiation	Breast	1	Amp 8q24.21
K 12803	Radiation	Soft tissue	2	Amp 8q24.21
GN 516	Radiation	Breast	2	Amp 10p12.33
EF 73	Chronic LE	Soft tissue	3	Amp 8q24.21; amp 10p12.33

Chronic LE, chronic lymphedema.

Figure 1. DNA-copy number profiles of two representative angiosarcoma cases. Log₂ copy number ratios of tumor versus an opposite sex diploid control genome were plotted on the y axis against 10,000 chromosome fragments in chromosomal order on the x axis. Log₂-ratios above 0 indicate copy-number gains, below zero copy number losses. **Upper panel:** primary angiosarcoma showing several small low-level copy number gains and losses. **Lower panel:** radiation induced angiosarcoma displaying partial amplification of chromosome band 5q35.3 and of the MYC locus at 8q.24.21.

18 (100%) were sAS ($P < 0.001$) (Table 4). Of note, apart from 16 radiation-induced AS, also both of the AS associated with chronic lymphedema showed MYC amplifications. By morphology, sAS with MYC high level amplifi-

Table 4. Statistical Comparison of Primary and Secondary Angiosarcomas

	MYC high level	MYC nc.	
pAS	0 (0%)	28 (100%)	28
sAS	18 (55%)	15 (45%)	33
	18	43	61
$P < 0.0001$			
	MYC high level	MYC nc.	
G3	5 (45%)	6 (55%)	11
Other	11 (55%)	9 (45%)	20
	16	15	31
$P > 0.05$			
	MYC high level	MYC nc.	
Ki67 (%)	45 $\pm$ 18.0	39 $\pm$ 13.4	$P > 0.05$
TUNEL (%)	4 $\pm$ 4.0	7 $\pm$ 10.4	$P > 0.05$

pAS, primary angiosarcomas; sAS, secondary angiosarcomas; nc, no change.

High frequency of MYC high level gene amplifications among sAS, but not pAS (Chi-Square test). High tumor grade was as frequent in sAS with MYC high level gene amplifications as in sAS without (Chi-Square test). Proliferation (ki67-positive cells per 100 tumor cells) and apoptosis rates (terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells per 100 tumor cells) were identical among pAS and sAS (Mann-Whitney-U test).

cations were not different from either sAS or pAS without MYC amplification. Cases with high histological grade (G3) were observed with the same frequency in both groups ($P > 0.05$) (Table 4). Since MYC plays an important role in cell cycle regulation, we also analyzed proliferation and apoptosis by immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick-end labeling assay, respectively, but found no differences between AS with and without MYC gene amplifications ($P > 0.05$) (Table 4).

Discussion

In this comprehensive analysis, we show for the first time that primary and secondary AS are genetically different entities, although these tumors are undistinguishable by morphology. Irradiation and chronic lymphedema are by far the most important risk factors for the development of sAS. We here demonstrate that the genetic alterations in these two different risk groups are partially overlapping, if not identical. Our observation that a) high-level amplifications of MYC are virtually 100% specific for sAS associated with irradiation or lymphedema, and b) are present in more than 50% of cases now places this subgroup of secondary sarcomas among other sarcomas defined by recurrent high level gene amplifications, such as well-differentiated liposarcoma or neuroblastoma. Since sAS are most often observed in the setting of irradiated breast carcinomas, with a more than 1000-fold increased relative risk,¹⁰ as compared with the general population, our series of sAS was heavily biased toward women and sAS of the breast. However, MYC amplifications occurred at the same relative frequency in sAS of other localizations, so we believe that MYC amplifications are a feature of sAS in general and not specific for breast sarcomas.

Figure 2. Morphology and fluorescence *in situ* hybridization analysis in primary and secondary angiosarcomas. **A:** Representative image of an angiosarcoma (FNCLCC grade 2) showing endothelial tumor cells growing in anastomosing, dissecting channels (primary retroperitoneal angiosarcoma). Primary and secondary angiosarcomas were morphologically undistinguishable. **B:** Inter- and metaphase nuclei (blue) of a primary angiosarcoma without MYC amplification. **C:** Interphase nuclei (blue) of a radiation-induced angiosarcoma with high level amplification of MYC (magenta signals) compared with the probe for the centromeric region of chromosome 8 (green signals).

Somewhat surprisingly, the amplification of *MYC* appeared not to have a major impact on tumor cell morphology or tumor cell turnover, hence the functional consequences of the *MYC* amplification remain yet to be established. In this multicenter retrospective study, available clinical data were insufficient to decide whether sAS with and without *MYC* amplification (or any other of the observed genetic changes) behaved differently in terms of prognosis. Further studies will be needed to address this relevant question. A presumably important role of *MYC* is not unique to sAS and has been reported also for other sarcomas, usually associated with high histological tumor grade. Interestingly, the Kaposi's sarcoma-associated herpesvirus LANA protein has been reported to stabilize and activate *MYC*.³⁵ Increased *MYC* copy numbers are frequent in malignant fibrous histiocytoma of bone,³⁶ high grade chondrosarcoma³⁷ and proximal type epitheloid sarcoma³⁸ and may be involved in the progression of myxoid liposarcomas.³⁹ *MYC* overexpression is also frequent in osteosarcomas⁴⁰ where it confers resistance to methotrexate.⁴¹ Moreover, inactivation of *MYC* reverses the malignant phenotype of rhabdomyosarcomas⁴² and even brief inactivation of *MYC* has been reported to result in sustained regression in osteosarcoma cells.⁴³ These observations raise the possibility that specific manipulation of *MYC* may be an effective therapeutic strategy for selected sarcomas including sAS.

Candidate genes for the other recurrent gene amplifications in sAS are yet to be identified. Locus 10p12.33 contains the so far uncharacterized micro-RNA mir-511-2. One of the most interesting candidate genes in locus 5q35.3 is *FLT4*, the gene encoding the receptor tyrosine kinase vascular endothelial growth factor receptor 3.⁴⁴ Vascular endothelial growth factor receptor 3 plays an important role both in vasculogenesis⁴⁵ and angiogenesis.⁴⁶ *FLT4* has been frequently found mutated in hereditary lymphedema^{47,48} and vascular endothelial growth factor receptor 3 has been found to be expressed by the vast majority of AS.⁴⁹

It is of interest that the pAS did not show recurrent genetic abnormalities, at least with the techniques applied here. Available data^{19–28} (reviewed in Ref. 21) suggest that pAS may also harbor recurrent aberrations, including gains on chromosome 20p (5 of 13 published cases),^{19,21,23,25} polysomy 8 (4/13 cases),^{23,25} chromosome 7 (4/13 cases),^{21,23,25} and 1q (3/13 cases),^{21,24} as well as losses on chromosome 5q and 16q (two cases each).²¹ Remarkably, these alterations do not overlap with the two published sAS cases and our own series. Another, more technical consideration may come from one study of a cultured angiosarcoma reported marked tumor cell heterogeneity with a diploid karyotype in about 40% of tumor cells, while the remaining cells showed a multitude of nonclonal structural and numeric alterations,²⁷ which may be an obstacle to detect alterations in whole tissue extracts. It will be important to determine whether different technical approaches (eg, single nucleotide polymorphism arrays or analyses using microdissected tumor tissues) will reveal recurrent genetic alterations also in primary AS.

In summary, we identified high level amplifications of *MYC* at locus 8q24.21 as a specific alteration of second-

ary but not primary angiosarcomas. This observation may have importance both for the molecular classification and therapy of these tumors.

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