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MYC amplification and overexpression in primary cutaneous angiosarcoma: a fluorescence *in-situ* hybridization and immunohistochemical study

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MYC*, a proto-oncogene located on chromosome 8q24, is involved in the control of cell proliferation and differentiation. Previous studies have documented high-level *MYC* gene amplification and MYC overexpression by immunohistochemistry (IHC) in post-irradiation angiosarcomas, but not in primary cutaneous angiosarcoma (AS-C) or in other radiation-associated vascular proliferations, such as atypical vascular lesions. Prompted by our recent finding of *MYC* amplification in a primary hepatic AS, we analyzed a large number of well-characterized AS-C for *MYC* amplification and protein overexpression. Formalin-fixed, paraffin-embedded blocks from 38 AS-C were retrieved from our archives and were examined by IHC analysis and fluorescence *in-situ* hybridization (FISH), using a commercially available antibody and probe. For FISH analysis, the number of copies of *MYC* was compared with the control gene, *CEN8* (*MYC/CEN8* ratio). All cases occurred on sun-exposed skin; no patient was known to have a history of therapeutic irradiation. Possible associations between survival and a wide variety of clinicopathological variables were evaluated using the log-rank test. By IHC analysis, MYC overexpression was present in 9/38 (24%) AS-C (2–3+: 6 cases, 16%; 1+: 3 cases, 8%). By FISH analysis, 2/5 (40%) informative cases with 2–3+ immunostaining showed high-level gene amplification. One additional case with 3+ immunostaining showed higher level aneusomy of chromosome 8 (5–8 *MYC* and *CEN8*). Two out of fourteen (14%) IHC-negative cases also carried *MYC* amplification (one high level and one lower level). Low copy number gain of chromosome 8 (3–5 *MYC* and *CEN8*) was observed in AS-C with or without MYC expression. *MYC* amplification and MYC protein overexpression were not correlated with clinical outcome. We have shown, for the first time, *MYC* gene amplification and protein overexpression in primary (non-radiation-associated) AS of the skin. MYC protein overexpression in cases lacking gene amplification likely reflects other mechanisms of MYC activation. The study of a larger number of AS-C showing *MYC* amplification may be necessary to determine whether the behavior of such cases differs from their more common non-amplified counterparts.
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The *MYC* proto-oncogene, a transcription factor located on chromosome 8q24, is thought to be involved in the regulation of cellular proliferation, differentiation, and apoptosis.[1] The mechanisms underlying *MYC* activation include gene amplification, activating mutations, and gene rearrangement, and appear to be different in different tumor types.[1] Relatively recently, *MYC* amplification has been shown to be a common feature in post-irradiation and chronic lymphedema-associated angiosarcomas (secondary angiosarcomas), where it is presumed to have a key oncogenic role.[2,3] In contrast, initial studies did not find *MYC* amplification in primary cutaneous or deep soft tissue angiosarcomas, suggesting pathogenetic differences between primary and secondary angiosarcomas.[2–4] However, two relatively small series have very recently identified high-copy number *MYC* amplification in primary cutaneous angiosarcomas,[5,6] and our own group has reported (in abstract form) *MYC* amplification in a

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subset of primary hepatic angiosarcomas.[7] In contrast, *MYC* amplification seems to be absent in a distinctive group of atypical vascular lesions following irradiation ('atypical vascular lesions'), a feature that helps to distinguish them from post-irradiation angiosarcomas.[3,8] At the protein level, MYC expression has been shown to be a feature of many post-irradiation angiosarcomas, but not of atypical vascular lesions or other benign cutaneous vascular proliferations.[3,8] Immunohistochemistry (IHC) analysis for MYC expression appears to correlate well with *MYC* amplification by fluorescence *in-situ* hybridization (FISH).[3]

We studied a large series of well-characterized primary cutaneous angiosarcomas for *MYC* amplification and MYC expression in order to more fully characterize the frequency of these events in these uncommon tumors. In addition, we correlated MYC status in these tumors with a variety of histopathological and clinical variables, including patient outcome.

Materials and methods

The Mayo Clinic Institutional Review Board granted approval for this study. We searched our institutional pathology archives for all cases coded as 'angiosarcoma' involving the skin for the period 1987–2007. Following identification of these cases, the medical records were re-reviewed and all cases thought to be post irradiation or lymphedema associated were excluded, leaving a final study population of 38 primary cutaneous angiosarcomas. Hematoxylin and eosin-stained slides were re-examined by two of the authors (WS and ALF) to confirm the original diagnosis, and a single representative tumor block was chosen for MYC immunostaining and interphase FISH analysis. The tumors were classified as 'vasoformative,' 'spindled,' 'epithelioid,' or 'mixed,' as previously described.[9] Follow-up information was obtained from our medical records.

For IHC analysis, standard whole sections were immunostained for MYC (clone EP121, 1:100; Epitomics, Burlingame, CA, USA) using heat-induced epitope retrieval and the Ventana Ultraview detection system (Ventana, Oro Valley, AZ, USA). Appropriate controls were employed. Only nuclear reactivity was considered positive. All cases were scored as 'negative' ($<5\%$ positive), '1+' (5–25% positive), '2+' (26–50% positive), or '3+' ($\geq 51\%$ positive).

FISH analysis for the quantitation of *MYC* and chromosome 8 centromere was performed on formalin-fixed, paraffin-embedded tissue sections cut at $5\,\mu$m using commercially available FISH probes for *MYC* (8q24) labeled with Spectrum Red (Abbott Molecular, Des Plaines, IL, USA) and CEP8 (eight centromere) probe labeled with Spectrum Green (Abbott Molecular). Standard laboratory protocols and quality control measures were followed for this study. In addition to the 38 angiosarcoma specimens tested, 20 normal skin specimens without significant vascular proliferation and 22 benign vascular lesions were also evaluated as control cases. In each case, 30 interphase nuclei were analyzed in a blinded manner by two technicians (60 total nuclei). An identical protocol is used at our institution for the evaluation of *Her2Neu* amplification in breast carcinoma, with respect to the number of counted nuclei. The expected normal pattern for these probes is two *MYC* and two CEP8 signals. Amplification of the *MYC* locus was defined as an increased number of *MYC* signals and a *MYC*/CEP8 ratio of ≥ 2. Polysomy was defined as a proportional gain of both *MYC* and CEP8 (ratio <2).

For statistical analysis, the median times to death and 5-year outcome estimates were estimated with the Kaplan–Meier method. The relationship between survival and MYC status, as well as other clinicopathological parameters (patient age, tumor size, and tumor morphology), was assessed by the

Table 1 Clinicopathological features (38 cases)

Sex	
M	25 (64%)
F	14 (36%)
Age at surgery (years)	
Median	73
Range	(27–89)
Site	
Head/neck	30 (78.9%)
Lower extremity	4 (10.5%)
Upper extremity	3 (7.9%)
Chest	1 (2.6%)
Morphology	
Vasoformative	22 (57.9%)
Mixed	12 (31.6%)
Spindled	3 (7.9%)
Epithelioid	1 (2.6%)
Local Recurrence	
No	7
Yes	14
5-year recurrence-free % (95% CI)	22.2% (0, 45.6%)
Metastasis	
No	6
Yes	17
5-year metastasis-free % (95% CI)	17.2% (0, 37.2%)
Death from disease	
No	8
Yes	26
Death from disease	11
Death from other disease	1
Death from unknown cause	14
5-year survival % (95% CI)	34.5% (18.3%, 50.7%)
Median survival, Kaplan–Meier (95% CI)	2.7 years (2.1–5.1 years)

Abbreviations: CI, confidence interval; F, female; M, male.
Note, recurrence status unknown for 17 cases, metastasis status unknown for 15 cases; final status (death/alive) unknown for 4 cases.

Table 2 FISH and IHC results

FISH result	*MYC expression by IHC (no. of cases)*				
	Negative	*1+*	*2+*	*3+*	*Total*
High-level amplification (>21 copies *MYC*; *MYC*:CEP8 ratio ⩾2)	1	0	0	2	3
Low-level amplification (*MYC*:CEP8 ratio ⩾2)	1	0	0	0	1
High-level polysomy chromosome 8 without *MYC* amplification (5–8 *MYC* and 5–8 CEN8)	0	0	0	1	1
Low-level polysomy chromosome 8 without *MYC* amplification (3–5 *MYC*, 3–5 CEN8)	7	2	0	1	10
Normal	6	0	2	0	8
Total	15	2	2	4	23

Abbreviations: FISH, fluorescence *in-situ* hybridization; IHC, immunohistochemistry.

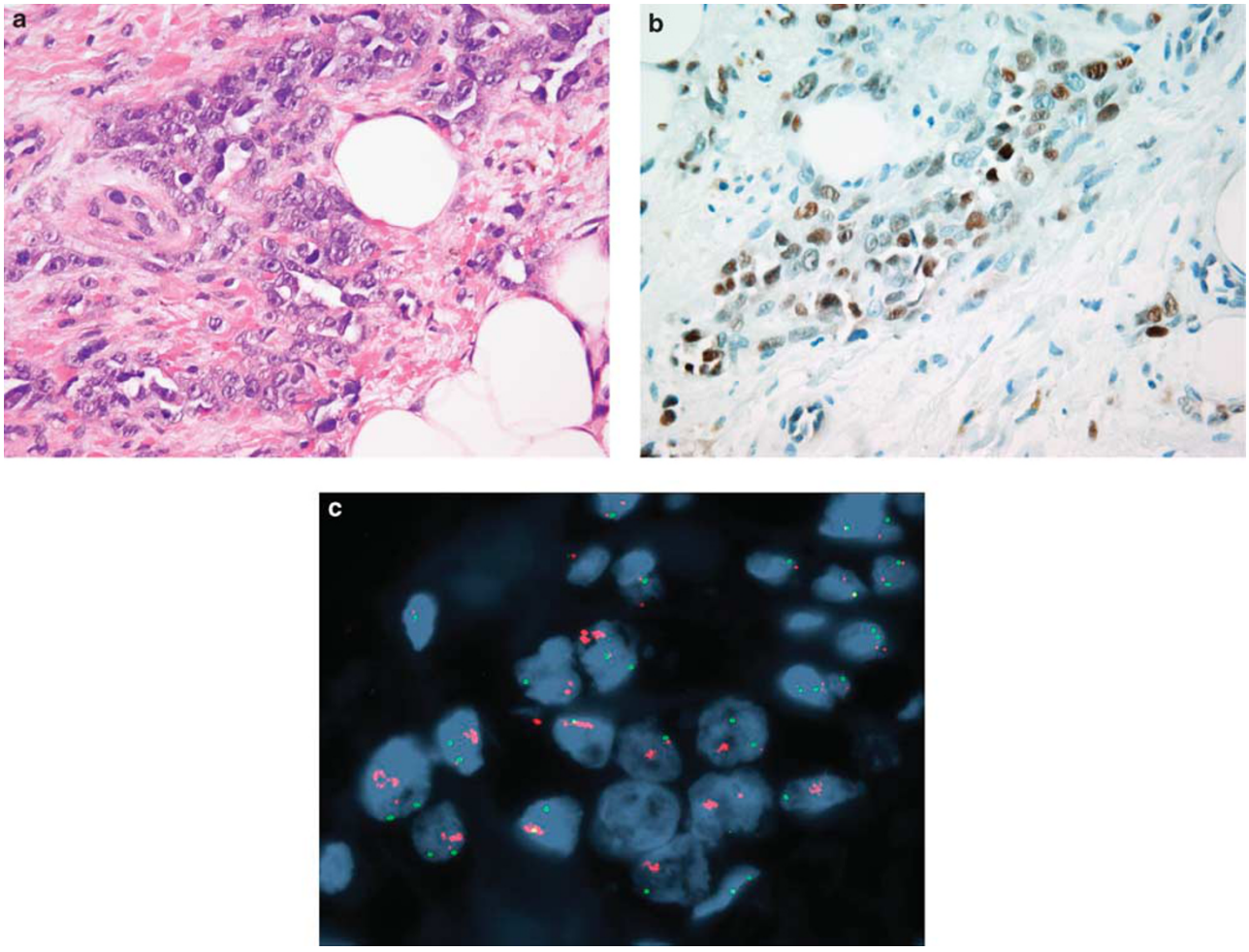

Figure 1 Primary cutaneous angiosarcoma with epithelioid morphology (**a**) showing nuclear MYC protein expression (**b**) and high-level *MYC* gene amplification (c); *MYC*: red and CEP8: green.

log-rank test. All analyses were performed in either SAS version 9 (Cary, NC, USA) or R (Foundation for Statistical Computing, Vienna, Austria).

Results

The clinicopathological findings are summarized in Table 1. The tumors occurred in 25 men and 14 women (mean age 73 years, range 27–89 years). Among the 38 with available clinicopathologic data, the tumors involved the skin of the head/neck (30 cases), leg (4 cases), arm (3 cases), and chest (1 case). The tumors ranged from 1.2 to 22 cm (median 7.2 cm) and were classified as vasoformative (22 cases, 58%), spindled (3 cases, 8%), epithelioid (1 case, 3%) and mixed (12 cases, 32%). Figures 1–4 illustrate selected cases of angiosarcoma showing

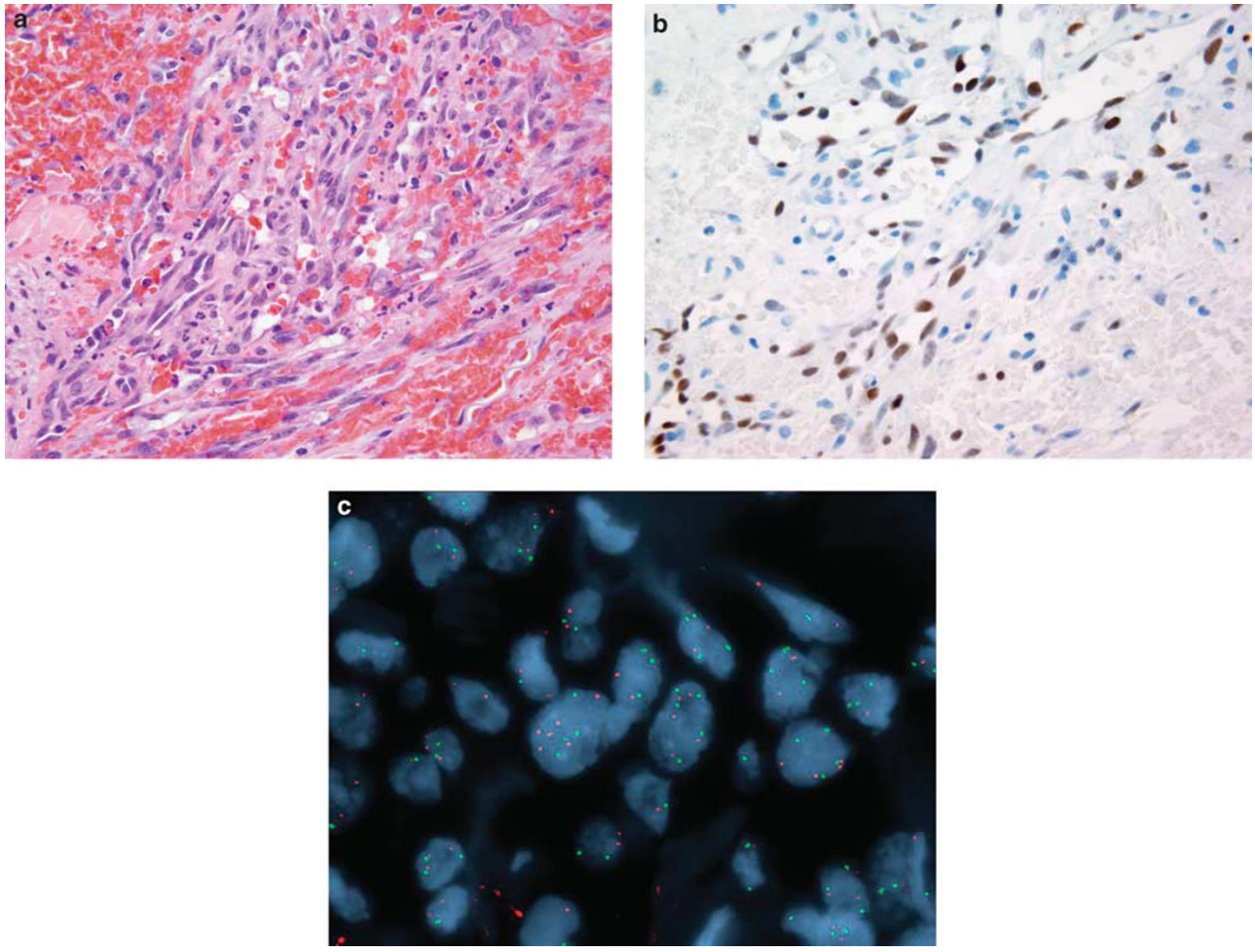

Figure 2 Another angiosarcoma (**a**) with strong MYC protein expression (**b**). By FISH, this same case showed polysomy of chromosome 8 with 5–8 red and green signals, so that the actual ratio of *MYC* to CEP8 is still equal or close to 1 in the tumor cells (**c**).

overexpression of MYC protein and/or *MYC* gene amplification by FISH. By IHC analysis, MYC overexpression was present in 9 of 38 (24%) cases (2–3+: 6 cases, 16%; 1+: 3 cases, 9%), including 6 cases arising in the head/neck region, 2 from the limbs, and 1 from the chest. Surrounding non-neoplastic endothelial cells were consistently MYC negative.

Interphase FISH analysis for *MYC* was successfully performed on 23 primary cutaneous angiosarcomas, including 8 IHC-positive and 15 IHC-negative cases. Fourteen additional cases failed to hybridize and were not scored. The FISH and IHC analyses results are detailed in Table 2. Of six cases that showed 2–3+ immunoreactivity, FISH analysis showed high-level amplification for *MYC* (>21 copies) in two cases (33%), both from the legs. One case with 3+ immunostaining showed polysomy of chromosome 8 without *MYC* amplification; the remaining three cases were found to be normal by FISH analysis. Of the 15 MYC IHC-negative cases, 2 (13.3%) showed *MYC* amplification, including 1 case from the head/neck and 1 from the chest. Polysomy of chromosome 8 was observed in angiosarcomas in both MYC-positive and -negative cases. Polysomic cases showed three to eight copies of MYC and CEP8. All of the control cases showed normal results by FISH analysis. The sensitivity and specificity of any positive MYC IHC (1–3+) was 66 and 70% for *MYC* amplification, respectively, and 47 and 66% for the presence *MYC* amplification or chromosome 8 copy gain, respectively. For cases showing MYC IHC of 2–3+, the sensitivity and specificity for *MYC* amplification was 66 and 79%, respectively, and 66 and 88% for *MYC* amplification or chromosome 8 copy gain, respectively.

Clinical follow-up data were available for 34 of 38 (90%) patients (median 2.7 years, range 2 months to 19 years). Of these 34 patients, 26 died: 11 died of disease, 14 died of unknown causes, 1 died of other cause, and 8 were alive without disease. The median time to death from disease was 2.7 years (95% confidence interval: 2.1–5.1 years), and the 5-year survival was 35% (95% confidence interval: 18%–51%).

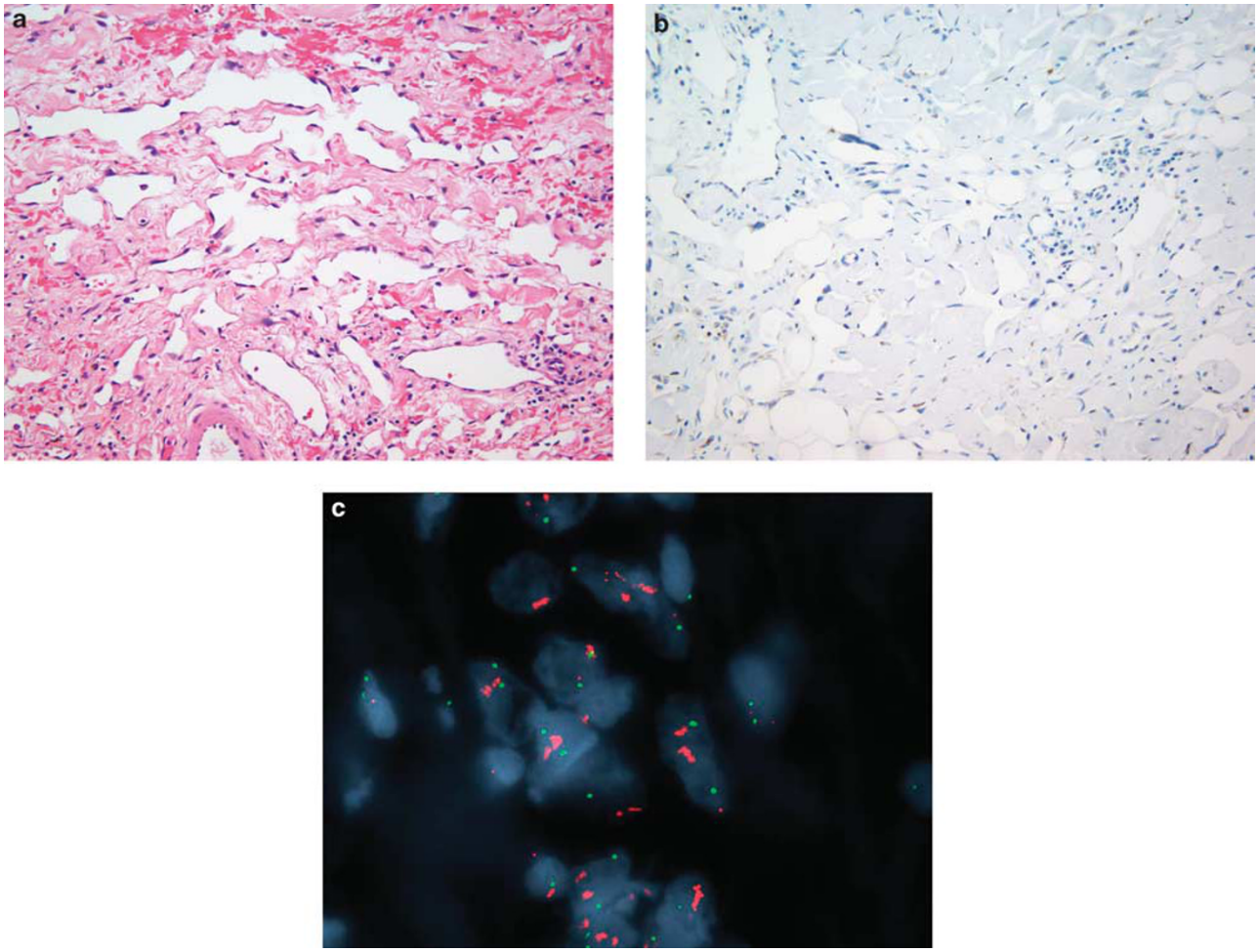

Figure 3 Angiosarcoma (**a**) lacking MYC protein expression (**b**) but showing high-level *MYC* gene amplification (**c**).

By univariate analysis, *MYC* amplification and MYC overexpression were not associated with overall survival (IHC, $P=0.94$; FISH, $P=0.81$) (Figure 5). Further, none of the evaluated clinicopathological parameters was associated with survival, including patient age ($P=0.84$), tumor size ($P=0.75$), and tumor morphology ($P=0.53$).

Discussion

The results of the present study, the largest to date of *MYC* in primary cutaneous angiosarcoma, confirm and extend upon the earlier results of Italiano *et al*[5] and Hadj-Hamou *et al*,[6] confirming the presence of both *MYC* amplification and MYC overexpression in a minority of such tumors. We have also identified relatively frequent chromosome 8 copy number gain without *MYC* amplification in a larger percentage of primary cutaneous angiosarcomas.

Regrettably, we were not able to demonstrate any relationship between *MYC* amplification and/or MYC overexpression, and any histopathological variables that have been previously associated with prognosis in cutaneous angiosarcoma, such as size or epithelioid *versus* vasoformative morphology.[9,10] Similarly, we were not able to detect any difference in the clinical behavior of angiosarcomas showing MYC abnormalities *versus* those without this finding. Similarly, Manner *et al*[4] were unable to find an association between *MYC* amplification and histopathological or prognosis in secondary (post-irradiation) angiosarcomas. These findings are somewhat disappointing, as several prior studies (reviewed in Nesbit and Tersak[11]) have shown an association between gene amplification and/or protein overexpression of MYC and advanced stage in a variety of non-angiosarcoma human malignancies. However, we were able to detect MYC abnormalities in only a small number of primary cutaneous angiosarcomas, and it is possible that analysis of much larger series might show different results.

Somewhat unexpectedly, we did not observe a tight correlation between *MYC* gene amplification and protein overexpression, with only two of three high-level *MYC*-amplified cases showing MYC overexpression and six of eight IHC-positive

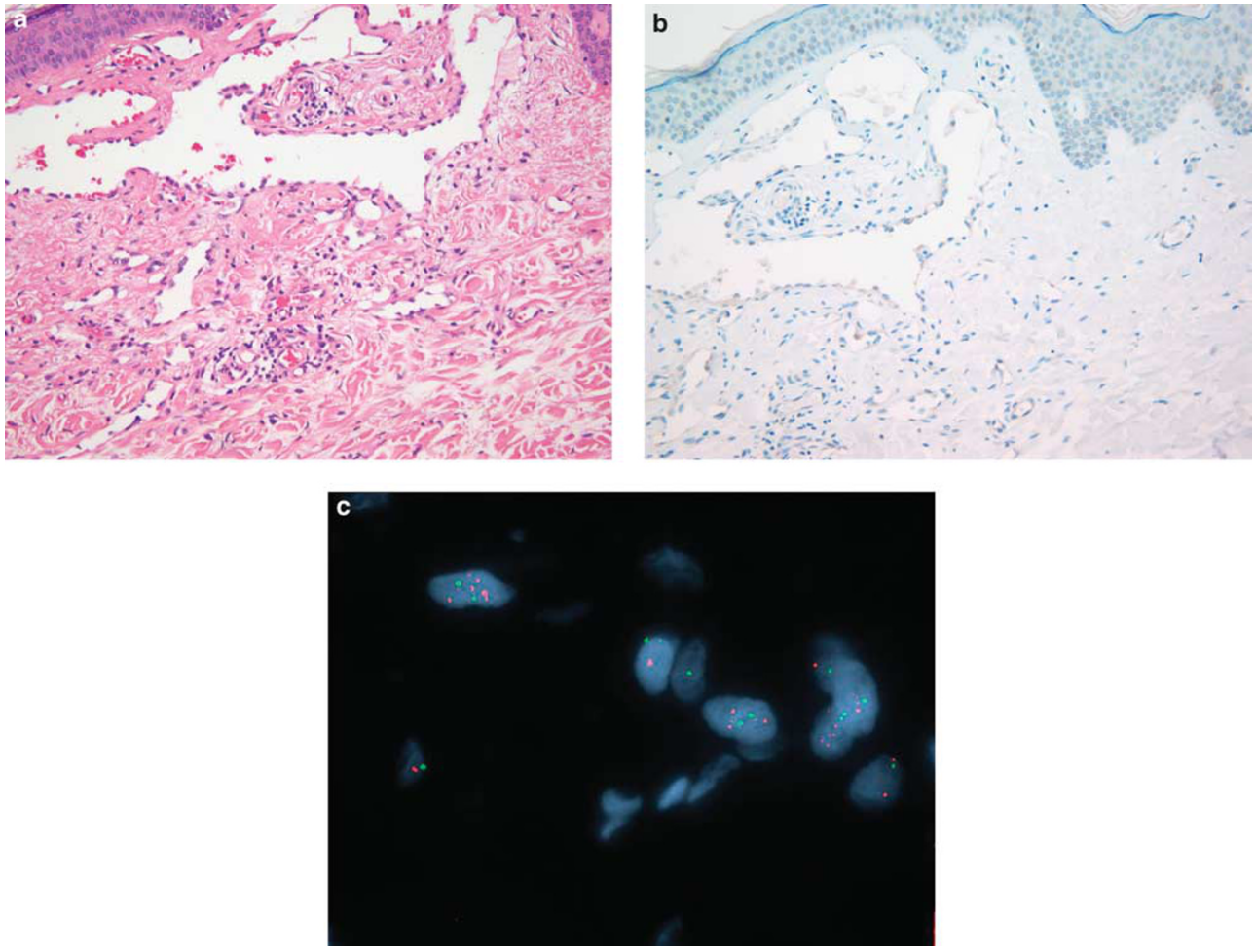

Figure 4 Angiosarcoma (**a**), negative for MYC expression by immunohistochemistry (**b**). Lower-level *MYC* amplification by FISH (**c**). Note the smaller number of red signals within the cells as compared to Figure 3.

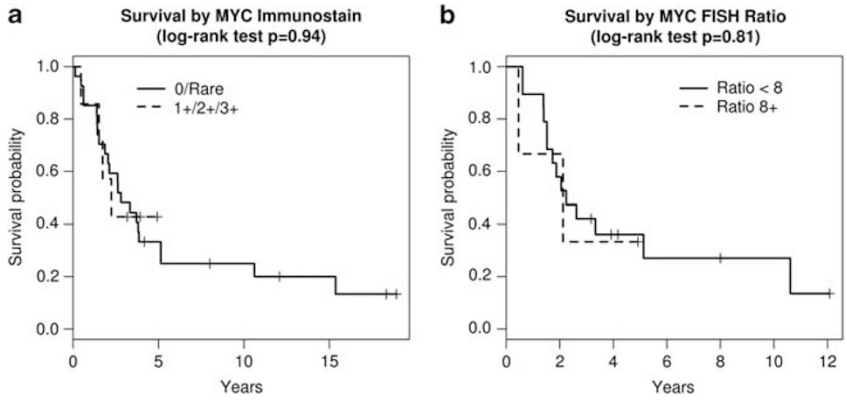

Figure 5 No differences in survival were seen in angiosarcoma patients whose tumors expressed MYC by immunohistochemistry (**a**) or showed high-level amplification by FISH (**b**) and those whose tumors did not.

cases lacking amplification by FISH analysis. The FISH-positive, IHC-negative case may be explained by limitations in the sensitivity of commercially available MYC antibodies or by the 5% threshold that we required in order to score cases as 'positive'. This arbitrary threshold level was established, because it has been our experience in the routine evaluation of post-irradiation cutaneous vascular proliferations that rare MYC IHC-positive cells may be seen in clearly non-angiosarcoma cases.

In addition, it is not known whether *MYC* amplification invariably results in MYC protein overexpression. Interestingly, four of the six IHC-positive cases lacking *MYC* amplification demonstrated chromosome 8 copy number gain, and it is possible that this may account for this finding. Alternatively, MYC protein expression may be regulated by other genetic or epigenetic mechanisms. For example, it is known that the neighboring gene *PVT1* (8q24) is a transcriptional activator of *MYC*.[12] In secondary (post-irradiation) angiosarcomas, Mentzel *et al*[3] have shown MYC overexpression in all but one *MYC*-amplified tumor.

Although the evaluation of MYC expression in other primary cutaneous vascular tumors that may enter the differential diagnosis of angiosarcoma was beyond the scope of the present study, it is difficult for us to see a potential role for these tests. This is largely because of the low frequency of *MYC* amplification in primary cutaneous angiosarcomas, but also a reflection of our discomfort with the lack of tight correlation between MYC FISH and IHC results. Certainly, we would be very reluctant to label a difficult primary vascular lesion as 'angiosarcoma' based only on IHC demonstration of overexpression. This is in contrast to post-irradiation lesions, where demonstration of MYC abnormalities seems to be of great value in the distinction of angiosarcoma from atypical vascular lesions.[3]

In summary, we have demonstrated *MYC* amplification and MYC protein overexpression in a subset of primary cutaneous angiosarcomas. The clinical significance of this finding is unclear, as MYC abnormalities do not seem to be related to histopathological or clinical variables. Study of larger numbers of additional cases may be necessary to determine the relative significance of gene amplification *versus* protein overexpression, especially as these do not always appear to be identical in all cases. In the future, identification of MYC-positive primary cutaneous angiosarcomas may prove to be of clinical significance, especially in the context of new therapeutic approaches targeting the *MYC* pathway.[1,13]

Disclosure/conflict of interest

The authors declare no conflict of interest.

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