Tumor Microenvironment and Its Clinicopathologic and Prognostic Association in Cutaneous and Noncutaneous Angiosarcomas

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ABSTRACT

Objectives: We explored features of the angiosarcoma (AS) tumor microenvironment to discover subtypes that may respond to immunotherapy.

Methods: Thirty-two ASs were included. Tumors were studied by histology, immunohistochemistry (IHC), and gene expression profile using the HTG EdgeSeq Precision Immuno-Oncology Assay.

Results: Comparing cutaneous and noncutaneous ASs, the second group showed 155 deregulated genes, and unsupervised hierarchical clustering (UHC) delineated two groups: the first mostly cutaneous AS and the second mainly noncutaneous AS. Cutaneous ASs showed a significantly higher proportion of T cells, natural killer cells, and naive B cells. ASs without MYC amplification revealed a higher immunoscore in comparison with ASs with MYC amplification. PD-L1 was significantly overexpressed in ASs without MYC amplification. UHC showed 135 deregulated genes differentially expressed when comparing ASs from the non–head and neck area with patients who had AS in the head and neck area. ASs from the head and neck area showed high immunoscore. PD1/PD-L1 content was significantly more highly expressed in ASs from the head and neck area. IHC and HTG gene expression profiling revealed a significant correlation between PD1, CD8, and CD20 protein expression but not PD-L1.

Conclusions: Our HTG analyses confirmed a high degree of tumor and microenvironment heterogeneity. Cutaneous ASs, ASs without MYC amplification, and ASs located in the head and neck area seem to be the most immunogenic subtypes in our series.

INTRODUCTION

Angiosarcomas (ASs) represent a heterogeneous group of vascular sarcomas that can arise in different organs with a variety of features. They occur more frequently in skin and soft tissue but appear also in the breast, bone, and viscera.1,7 Commonly, ASs are aggressive tumors with frequent local recurrence and the potential for lymph node and distant metastases.7,16 Primary and secondary ASs have been reported.1,4 The first represent the majority of cutaneous...
and soft tissue AS, and the latter occur associated with irradiation and/or chronic lymphedema (Stewart-Treves syndrome) and are mainly cutaneous.\textsuperscript{1-3} One special type of AS is located in the head and neck of the elderly (Wilson-Jones AS).\textsuperscript{1-3} Genetic analyses of ASs have revealed few currently actionable targets for therapeutic intervention.\textsuperscript{6-33} Amplification of MYC is a hallmark of secondary AS.\textsuperscript{1-8}

ASs are therapy refractory, and significant improvements in long-term survival with recent chemotherapy/radiotherapy regimens remain to be evaluated.\textsuperscript{1-19} Response rate is low, and median progression-free survival and overall survival (OS) are poor; therefore, alternative therapeutic options are urgently needed, particularly in patients with metastatic disease.\textsuperscript{7-20} The use of immune checkpoint inhibitors is a promising treatment modality yielding long-term clinical benefits in historically therapeutically refractory cancers.\textsuperscript{10-33} The immunologic tumor microenvironment (TME) in AS has not been systematically studied, with limited studies so far reporting controversial results.\textsuperscript{10-33} These findings suggest the need for a thoughtful and targeted approach to the use of immunotherapy in AS.\textsuperscript{32-33} ASs may have different TMEs depending on the tumor location (soft tissue, viscera, or cutaneous).\textsuperscript{1-8} Indeed, the stromal compartment is highly heterogeneous in AS and may influence the interrelationship between stroma, neoplastic cells, and immune cells.\textsuperscript{1-10} A high tumor mutation burden (TMB) characterizes a subset of AS, suggesting a possible response to immune checkpoint inhibitors.\textsuperscript{9-32}

\begin{table}
\centering
\caption{Clinical Parameters Available for Angiosarcomas (n = 32)}
\begin{tabular}{ll}
\hline
\textbf{Clinical Parameter} & \textbf{Value} \\
\hline
Sex & \\
Male & 13 (40.7) \\
Female & 19 (59.3) \\
Median age at diagnosis, y & 61.1 \\
Sample & \\
Primary tumor & 32 (80.0) \\
Metastasis and recurrences & 8 (20.0) \\
Primary site & \\
Cutaneous & 20 (62.5) \\
Noncutaneous soft tissue\textsuperscript{b} & 5 (15.6) \\
Noncutaneous visceral\textsuperscript{c} & 7 (21.8) \\
Cutaneous angiosarcoma & \\
Head and neck area & 6 (30.0) \\
Non–head and neck area & \\
Associated with previous radiotherapy (breast) & 12 (60.0) \\
Associated with lymphedema & 2 (10.0) \\
Surgery & \\
Yes & 28 (87.5) \\
No & 4 (12.5) \\
Chemotherapy treatment & \\
Yes & 11 (34.4) \\
No & 21 (65.6) \\
Radiotherapy treatment & \\
Yes & 2 (6.3) \\
No & 30 (93.7) \\
Outcome & \\
Died of disease & 21 (65.6) \\
Alive & 11 (34.4) \\
Median survival, mo & 25.3 \\
\hline
\end{tabular}
\footnotesize{\textsuperscript{a}Values are presented as number (%) unless otherwise indicated. \\
\textsuperscript{b}Thigh (2), pelvis (1), groin (1), and thorax (1). \\
\textsuperscript{c}Ovary (1), liver (2), duodenum (1), testis (1), pleura (1), and brain (1).}
\end{table}

\begin{table}
\centering
\caption{Pathological Parameters}
\begin{tabular}{ll}
\hline
\textbf{Histologic and Immunohistochemical Profile} & \textbf{Value} \\
\hline
Median size, cm & 10.1 \\
Cellular type & \\
Epithelioid & 24 (75.0) \\
Spindle & 5 (15.6) \\
Epithelioid and spindle & 2 (6.2) \\
Round or pleomorphic and anaplastic & 1 (3.1) \\
Necrosis & \\
Yes & 17 (53.1) \\
No & 15 (46.9) \\
Mitoses $\times$ 2 mm\textsuperscript{2} & \\
0-5 & 3 (9.4) \\
>5-10 & 10 (31.2) \\
>10 & 19 (60.3) \\
Lymphocyte infiltration & \\
Immune desert (no lymphoid infiltration) & 3 (9.3) \\
Immune excluded (only peritumoral infiltration) & 6 (18.7) \\
Immune infiltrated (intratumoral and peritumoral infiltration) & 23 (71.8) \\
Surgical margins & \\
Positive & 8 (25.0) \\
Free & 24 (75.0) \\
CD31 & \\
Positive & 32 (100.0) \\
Negative & 0 (0.0) \\
ERG & \\
Positive & 32 (100.0) \\
Negative & 0 (0.0) \\
Podoplanin & \\
Yes & 27 (84.3) \\
No & 5 (15.7) \\
Ki-67 & \\
0-10 & 16 (50.0) \\
>10 & 16 (50.0) \\
MYC, immunohistochemistry & \\
0-40\% & 10 (31.2) \\
>40\% & 7 (21.8) \\
NP & 15 (46.8) \\
MYC FISH & \\
Nonamplified & 10 (31.2) \\
Amplified\textsuperscript{a} & 7 (21.8) \\
NP & 15 (46.8) \\
\hline
\end{tabular}
\footnotesize{\textsuperscript{a}Values are presented as number (%) unless otherwise indicated. \\
\textsuperscript{b}Postradiation breast cutaneous angiosarcoma.}
\end{table}
FIGURE 1  

**A.** Head and neck angiosarcoma.  

**B.** Lymphedema-associated angiosarcoma.  

**C.** Postradiotherapy angiosarcoma.  

**D.** Epithelioid cutaneous angiosarcoma (H&E, ×20).  

**E.** Angiosarcoma with spindle cells (H&E, ×20).  

**F.** Mitoses in high-grade angiosarcoma (H&E, ×40).
FIGURE 1 (cont)  

G, Intratumoral lymphoid infiltrate in cutaneous angiosarcoma (H&E, ×10).  
H, Strong and diffuse cytoplasmic and membranous CD31 expression in angiosarcoma (×40).  
I, Strong and diffuse nuclear anti-ERG immunoreactivity in angiosarcoma (×40).  
J, Strong and diffuse cytoplasmic and membranous podoplanin/D2-40 expression in angiosarcoma (×40).  
K, Strong and patchy nuclear MYC expression in cutaneous angiosarcoma (×40).  
L, Fluorescence in situ hybridization in cutaneous postradiotherapy angiosarcoma with MYC amplification (red signals, arrows).
The HTG EdgeSeq Precision Immuno-Oncology Assay (HTG Molecular Diagnostics) characterizes the immune response in TMEs and enables the expression of hundreds of genes with minimal sample input to be measured. In a recent observation from our working group, we noticed a heterogeneous lymphoid infiltration in cutaneous ASs and believed it would be interesting to explore whether the immunologic component in AS may provide additional prognostic information or be associated with an alternative therapeutic option. Immunotherapy holds substantial promise for tumors that are refractory to standard therapies and for which disease recurrence is a major clinical problem. In this study, we explored features of the TME by histology, immunohistochemistry, and HTG to define subtypes of AS that may respond to different forms of immunotherapy.

MATERIALS AND METHODS

Patient Selection
Formalin-fixed, paraffin-embedded (FFPE) tissue of cutaneous and noncutaneous AS (soft tissue and visceral) samples, collected from patients with AS diagnosed between 2000 and 2015, were retrieved from the Pathology Department from various hospitals in our region. The following clinical and follow-up information was obtained from medical records: patient age, sex, tumor size, clinical history, tumor location, treatment (surgery, chemotherapy, radiotherapy), type of surgery (for tumor removal), and clinical outcome (died of disease or alive). The present study was approved by the institutional review board in accordance with the ethical standards established by the investigating institution in accordance with the Declaration of Helsinki of 1975, as revised in 2008.

Histopathology and Fluorescence In Situ Hybridization Analysis
The following histopathologic parameters (H&E) were assessed by three pathologists (I.M., F.G., and A.L.-B.) in noncutaneous and cutaneous ASs: tumor size, predominant tumor cells (epithelioid, round, spindle), necrosis, mitoses × 2 mm², and margins. Lymphoid infiltration was assessed by the same pathologists and classified as previously reported as “immune desert” in the complete absence of lymphoid cells.

FIGURE 2 Overall survival (OS) Kaplan-Meier curves comparing noncutaneous (n = 12) and cutaneous (n = 20) angiosarcomas. Log rank P = .00039.

FIGURE 3 Differential expression (DE) analysis comparing cutaneous (red; n = 20) and noncutaneous (blue; n = 12) Angiosarcomas. Unsupervised hierarchical clustering. DE genes cutaneous vs noncutaneous (155) (P = .1).
of lymphoid infiltration, “immune excluded” if lymphoid infiltration was present exclusively at the tumor-stroma border, and “immune infiltrated” if intratumoral lymphocytes were present.

Fluorescence in situ hybridization (FISH) analysis for the quantitation of MYC was performed on FFPE tissue sections cut at 5 mm using a commercially available break-apart FISH probe set for the MYC locus (8q24), with the 5’ probe labeled with Spectrum Red and the 3’ probe labeled with SpectrumGreen (Vysis FISH; Abbott Molecular). With this probe set, a yellow fusion signal is produced by the juxtaposition of the 5’ and the 3’ probe when the MYC locus has a normal configuration. Standard laboratory protocols and quality control measures were followed for this study. In each case, 100

<table>
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<th>Cell type</th>
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<th>Cutaneous</th>
<th>p-value</th>
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<tr>
<td>CD4 T cells</td>
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<td>Macrophages M2</td>
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<td>Regulatory T cells</td>
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FIGURE 4 A. Heatmap scores in noncutaneous (n = 12) and cutaneous (n = 20) angiosarcomas. B. Lymphoid and inflammatory population distribution in noncutaneous and cutaneous tumors. NK, natural killer.
interphase nuclei were analyzed in a blinded manner by two biologists (200 total nuclei). Amplification of the MYC locus was defined as (1) the presence of an increased number of MYC signals (more than 6) in tumor cells or (2) an increased number of 5’ MYC signals (more than 4 per cell) in at least 40% in tumor cells. A clear high level of MYC amplification was seen in large areas, with an average of more than 20 signals per cell.

**Immunom-Oncology Panel (HTG EdgeSeq Precision Immuno-Oncology Assay)**

Gene expression analysis was performed with the HTG EdgeSeq Precision Immuno-Oncology Assay (HTG Molecular Diagnostics), which characterizes the immune response in TMEs.\(^{35-38}\) This is a novel technique consisting of a prehybridization with specific probes using a quantitative nuclease protection assay, followed by a standard RNA sequencing (RNA-seq) protocol in the Nextseq sequencer (Illumina). The main advantage of this technique is its sensitivity, since it requires very little sample input (5-µm FFPE section). Tissue is microdissected in order to analyze only tumor tissue, and instead of analyzing the entire transcriptome, it focuses on a panel of 1,392 messenger RNAs (mRNAs) related with immune response (Precision Immuno-Oncology). The analysis includes the main genes involved in almost all the major tumor types and implicated in the immune system.

Results are further standardized using the technology and maximize the biologic variation measured by the statistical tests, we used median normalization, as originally suggested by Anders and Huber.\(^{39}\) Differential expression between groups in normalized data was inferred adjusting the data to a binomial negative distribution (DESeq2)\(^{8}\) implemented in R v.4.1.2.

Baseline samples were analyzed to identify genes differentially expressed between patients in the different clinical groups. Gene set analysis was performed using the gage (Generally Applicable Gene-set/Pathway Analysis) package in R.\(^{40}\) A visualization of the Kyoto Encyclopedia of Genes and Genomes pathways was performed with the pathview package, which maps and renders inferred data on pathway graphs.\(^{41}\) All these analyses were performed with R v.4.1.2 and revealed differential pathway regulation between the groups.

**Immunohistochemistry Validation of Gene Expression Profile**

PD-L1, PD1, CD8, and CD20 expression was examined by immunohistochemistry using the anti-PD-L1 (clone 22C3, KIT

![Figure 5](https://academic.oup.com/ajcp/advance-article/doi/10.1093/ajcp/aqad003/7074437)
ready to use; Dako), PD1 (clone PA32543NAT/105, dilution 1/150; Thermo Fisher Scientific), CD8 (clone C8/144B, ready to use; Dako), and CD20 (clone L26, ready to use; Dako). Tissue samples were evaluated by two pathologists (I.M. and A.L.-B.) for expression of these four antibodies using tissue microarrays (TMAs) with three tumor cores for each tumor. The threshold for positive PD-L1 expression was greater than 1%. The tumor-infiltrating lymphocyte (TIL) score was determined by examining the immunostained TMAs. The immunohistochemistry TIL score was assigned as follows: 3 (>20 positive lymphocyte cells per high-power field), 2 (11-20 positive lymphocyte cells per high-power field), 1 (1-10 positive lymphocyte cells per high-power field), and 0 (<1 or 0 positive lymphocyte cells per high-power field). Samples with score 0 or 1 were categorized as “low immune infiltrated” and score 2 or 3 as “high immune infiltrated,” as previously described. 7

Statistical Analysis

OS curves were calculated using the log-rank test, and plots were constructed using the Kaplan-Meier method. All the statistical analyses were performed using R v.4.1.2. The OS was defined as time from diagnosis (first biopsy) to death by any cause or until the most recent follow-up. The level of significance was defined as $P < .05$, and all the tests were two-tailed.

A nonparametric Mann-Whitney test was employed to compare protein expression by immunohistochemistry (PD-L1, PD1, CD8, and CD20) and gene expression by HTG.

RESULTS

Patient Characteristics

Thirty-two patients with AS were included. These patients were diagnosed (20 cutaneous and 12 noncutaneous) between 2000 and 2015. Patient characteristics are described in TABLE 1. The present study included 32 primary tumors (cutaneous and noncutaneous) as well as 8 recurrence/metastasis samples. Histological, immunohistochemical, and FISH results are summarized in TABLE 2. Clinical, histological, immunohistochemical, and FISH results are depicted in FIGURE 1.

Overall, we noted significantly better survival in patients with cutaneous ASs than noncutaneous AS, $P = .00039$ FIGURE 2.
Transcriptomic Characterization at the Immune Level

In order to study the main clinical and biological characteristics of AS, clinical annotation was used for differential expression analysis consisting of the following dichotomous groups: (1) cutaneous and noncutaneous ASs, (2) ASs with and without MYC amplification, and (3) ASs located in the head and neck area vs other anatomic sites.

**FIGURE 6**

A. Heatmap scores comparing angiosarcomas with and without MYC amplification. B. Immune score \( (P = .016) \). C. Stroma score \( (P = .14) \). D. Tumor microenvironment (TME) score \( (P = .17) \). PIO, Precision Immuno-Oncology.
Comparison of Cutaneous (n = 20) and Noncutaneous ASs (n = 12)

Comparing cutaneous and noncutaneous ASs, the second group showed 155 deregulated genes (102 upregulated, 53 downregulated) (Figure 3). This genomic profile may be related to better prognosis of cutaneous subtypes likely associated with a dominant mutational signature of UV light. Gene set enrichment analysis identified phagosome, focal adhesion, extracellular matrix-receptor interaction, and complement and coagulation cascade signaling pathways as upregulated in patients with noncutaneous ASs, while the cell cycle pathway was downregulated (Supplementary Table 2).

Microenvironment cell populations were compared in the context of tumor immune and nonimmune stromal cell populations in cutaneous and noncutaneous ASs. Regarding stromal score, immunoscore, and TME score, no significant differences were found between the two groups for the three scores analyzed (Supplementary Figure 1). Nevertheless, cutaneous ASs showed a significantly higher proportion of T cells, natural killer cells, and naïve B cells than noncutaneous ASs. Furthermore, CTLA4 was significantly more overexpressed in cutaneous ASs than in noncutaneous ASs, while PD1 and PD-L1 expression levels were similar in both cutaneous ASs and noncutaneous ASs (Supplementary Graphic 1).

AS Without MYC Amplification (n = 10) vs AS With MYC Amplification (n = 7)

Comparing AS tumor samples with and without MYC amplification, 148 genes were deregulated (73 upregulated, 75 downregulated) (Figure 5A). Unsupervised hierarchical clustering distinguished two distinct clusters, mainly with a more reduced number of genes (47 genes, P = .024) (Figure 5B). Gene set enrichment analysis identified the MAPK signaling and cell cycle pathway to be upregulated in patients with ASs with MYC amplification (Supplementary Table 3). Gene expression of MYC correlates with MYC genomic amplification measured by FISH (Supplementary Graphic 2).
Heatmaps depicting scores from both groups are summarized in \textbf{FIGURE 6}. ASs without MYC amplification revealed a higher immunoscore than ASs with MYC amplification. In addition, ASs without MYC amplification showed an enriched profile of CD4 and CD8 T cells as well as B cells (Supplementary Table 4). PD-L1 was significantly overexpressed in ASs without MYC amplification, whereas CTLA4 was significantly overexpressed in ASs with MYC amplification (Supplementary Graphic 3). Notably, all MYC-amplified/positive tumors were cutaneous ASs associated with previous radiotherapy in the setting of breast carcinoma.

Cutaneous ASs From the Head and Neck Area (n = 6) vs ASs From the Non–Head and Neck Area (n = 14)
Comparing tumor samples from the head and neck area with the non–head and neck area, 135 genes (84 upregulated, 51 downregulated) were found to be differentially expressed \textbf{(FIGURE 7)} (Supplementary Table 5). There were two groups in the unsupervised analysis \textbf{(FIGURE 7)}. ASs from the head and neck area showed a higher immunoscore than ASs from other sites \textbf{(FIGURE 8)}. In addition, ASs from the head and neck area showed a significantly higher proportion of CD8 T cells and B cells than ASs from the non–head and neck area (Supplementary Table 6). Furthermore, PD1 and PD-L1 content was significantly more highly expressed in ASs from the head and neck region (Supplementary Graphic 4).

No correlation was found between the histopathologic assessment of intratumoral lymphocytes and immunoscore, stromal score, and TME score (Supplementary Graphic 5).

Comparison Between Primary Tumors and Metastasis or Recurrence
Comparing 32 samples from primary tumors with 8 recurrence/metastasis samples, we found five differentially expressed genes (EGR1, FOS, ID3, KRT5, and HNF1B). There were no significant differences in scores between primary and recurrence/metastasis samples, but the last group showed an enriched profile of CD4 memory T cells, CD8 T cells, CD8 central memory T cells, macrophages, and macrophages M1.

Immunohistochemistry Validation of Gene Expression Profile
\textbf{FIGURE 9} and \textbf{FIGURE 10} describe protein expression in comparative groups: cutaneous vs noncutaneous AS, AS with or without MYC amplification, and AS from the head and neck area vs AS from the non–head and neck area. \textbf{FIGURE 11} shows a comparison between protein expression by immunohistochemistry and the corresponding gene expression profile by HTG. A significant correlation was observed with PD1, CD8, and CD20 but not with PD-L1.

\textbf{FIGURE 7} (cont) B. 5 genes (P = .000001).
**DISCUSSION**

AS is a rare histologic sarcoma subtype with significant clinical and molecular heterogeneity and poor survival.\(^1\) Commonly, cutaneous ASs have a different clinical course and prognosis compared with noncutaneous ASs.\(^1\)-\(^5\) MYC-amplified AS are mostly secondary to radiotherapy or lymphedema, and recent reports have described that...
AS originating in the head and neck area appears to have a better prognosis than other ASs, possibly related to the mutational profile, TMB, and the immunologic microenvironment.1-7,9-15,17-33 In terms of OS, the results in the present series are fairly similar to those previously reported.1-7,9-33 We have observed that cutaneous ASs had better OS than noncutaneous ASs. These findings are comparable to those found in other sarcoma series or studies, where the superficial location confers a better prognosis, probably related to the opportunity of complete resection with tumor-free margins, earlier diagnosis, less likelihood of distant dissemination, and perhaps a different immunologic microenvironment.1-7

In the present series, we profiled some of the most relevant characteristics of ASs at the immunogenomic level. To do this, we first compared the profile of differentially expressed genes in cutaneous and noncutaneous ASs. These findings are comparable to those found in other sarcoma series or studies, where the superficial location confers a better prognosis, probably related to the opportunity of complete resection with tumor-free margins, earlier diagnosis, less likelihood of distant dissemination, and perhaps a different immunologic microenvironment.1-7

In the present series, we profiled some of the most relevant characteristics of ASs at the immunogenomic level. To do this, we first compared the profile of differentially expressed genes in cutaneous and noncutaneous ASs, finding a signature of 155 genes. Of particular interest among these genes, FOS has been described as deregulated in radiation-associated AS of the breast.33 Similarly, there was a profile of differentially expressed genes when comparing ASs with and without MYC amplification; the two groups showed evident differences, displaying a profile of 148 deregulated genes. Moreover, we also found a profile of differentially expressed genes when comparing ASs located in the head and neck area with ASs arising in other locations. The top overexpressed genes are two keratins (KRT16 and KRT17) with fold changes of 44.82 and 24.35, respectively (q value of 1.03 * 10e-4 and 1.23 * 10e-9). These last findings are in line with the results obtained in a recent study showing that ASs located in the head and neck area showed a high TMB with a different prognosis and a better response to immunotherapy,7 probably related to a more immune TME.

Cutaneous ASs, ASs without MYC amplification, and ASs from the head and neck area showed a higher proportion of CD4 or CD8 T cells in the HTG analysis. Furthermore, ASs without MYC amplification and those located in the head and neck area showed a significantly higher immunoscore than ASs with MYC amplification and ASs arising outside the head and neck area. In addition, HTG showed significantly higher expression of PD-L1 in ASs located in the head and neck area, as well as in ASs without MYC amplification. These findings are concordant with previously published results suggesting that ASs from the head and neck area appear to be more susceptible to immunotherapy, probably related to a different immunologic profile.7 Moreover, larger series have reported varying levels of PD-L1 expression, with a recent study reporting that 6 of 24 AS samples of different origins, including bone, skin, breast, soft tissue, and visceral primary tumors, had at least some membrane expression of PD-L1.31-33 Furthermore, cutaneous ASs are known to be infiltrated with lymphocytes and to express PDL1 and PD-L1. In addition, cutaneous ASs of the head and neck area may have a high TMB, likely related to a dominant mutational signature of UV
light, which is hypothesized to explain some of the exceptional responses to immunotherapy.\textsuperscript{1,3,7} Fujii et al\textsuperscript{29} reported that patients with a diagnosis of cutaneous AS have higher levels of CD8-positive T lymphocytes and demonstrated that tumors with a higher density of TILs tended to have a longer metastasis-free interval.

Our results suggest that cutaneous ASs, ASs without MYC amplification, and those located in the head and neck area probably have a different TME, which could justify the different prognosis of these tumors and the probability that they are more susceptible to immunotherapy. However, additional studies with larger series are needed to confirm these results.

The present study also demonstrates a profile of five genes (EGR1, FOS, ID3, KRT5, and HNF1B) differentially expressed between primary and recurrence/metastasis samples, the latter being enriched with CD4 memory T cells, CD8 T cells, CD8 central memory T cells, macrophages, and macrophages MI. These findings may prompt us to suggest a relatively high susceptibility to immunotherapy in recurrence/metastasis samples, although further studies with larger series of AS with various paired primary and recurrence/metastasis samples are needed to confirm these results.

The profile of differentially expressed genes suggests the implication of certain metabolic pathways in AS as observed in the current study, but these are not necessarily the only mechanisms that can influence the biological behavior of these neoplasms.\textsuperscript{1,3,4,7,10-33} The present study analyzes a transcriptome enriched in immune-related genes, and thus a pathway analysis should be biased toward immune-related pathways. Targeted exome sequencing has shown AS to be a highly heterogeneous disease, not easily classifiable at the molecular level with frequent mutations in the MAP kinase pathway\textsuperscript{2,13,32,34} supported by one such altered pathway found in the present series. The higher expression of MYC obtained in MYC-amplified ASs on RNA-seq analysis confirmed our FISH results for MYC.

The lack of correlation between the results of the histologic assessment of lymphoid infiltrate achieved with H&E by the pathologists and the results of the immune profile obtained by HTG can be explained by the tissue fragments with the highest proportion of tumor being selected for the HTG analysis regardless of the lymphoid infiltrate. Additionally, the peritumoral lymphoid infiltrate, which is usually higher than the intratumoral lymphoid infiltrate, was not necessarily included in the material selected for HTG.

One of the drawbacks of this study is the limited number of tissue samples available for correlative analysis; therefore, the present series may not necessarily be representative of the biological behavior of AS. However, the results obtained in our study do provide an initial step for new studies integrating the mutational

**FIGURE 10 A-E.** Cutaneous angiosarcoma from the non–head and neck area with low immune profile by HTG. A, H&E, x40. B, PD1 positive in isolated lymphocytes, x40. C, PD-L1 negative, x40. D, CD8 negative, x40. E, CD20 negative, x40. F-J, Cutaneous angiosarcoma from the head and neck area with high immune profile by HTG. F, H&E, x40. G, PD1 expression in tumor-infiltrating lymphocytes (TILs), x40. H, Lack of PD-L1 expression, x40. I, CD8 positivity in TILs, x40. J, CD20 positivity in B-cell lymphocytes with nodular and intratumoral pattern, x40. K-O, Cutaneous angiosarcoma from the head and neck area and high immune profile. K, H&E, x40. L, PD1 expression in TILs, x40. M, PD-L1 positivity in TILs, x40. N, CD8 immunoreactivity in TILs, x40. O, CD20 strong positivity in B-cell lymphocytes, x40.
profile with the immunologic profile in AS, aiming to discover which types of AS could really benefit from immunotherapy and which would not.

The implementation of a rapid and practical immunohistochemical-based screening tool as a surrogate for gene expression profiling could be helpful to identify patients with a

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specific TME that may confer a more favorable response to treatment. In fact, the agreement between gene and protein expression may be influenced by several factors and includes multiple processes related to posttranscriptional regulation of gene information. The concordance in dichotomous terms for measuring the presence/absence of mRNA and protein expression ranged between 46% using microarrays and 68% using immunohistochemistry. In the present series, there was an acceptable correlation between protein expression by immunohistochemistry and gene expression in most cases, the most significant being for CD20 (P = .00049), although it was poor for PD-L1. CD20 protein expression is easier to assess by immunohistochemistry, and PD-L1 protein expression may have been influenced by several preanalytical factors; in addition, the assessment may not be as easy as with other antibodies. A combination of an immunologic genomic profile study and immunohistochemistry assessment may provide additional information in cases of inconclusive or doubtful results.

Our HTG analyses confirmed a high degree of heterogeneity, even within the tumor immune and stromal cell compartments of this small AS series. Additional research and prospective studies with larger cohorts are needed to better understand the immune context of AS and to differentiate between the different primary sites of disease.

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REFERENCES


