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High cutaneous amphiregulin expression predicts fatal acute graft-versus-host disease

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Abstract

Background: Amphiregulin (AREG) is increased in circulation in acute graft-versus-host disease (aGVHD) and is associated with poor steroid response and lower survival. The expression of AREG in aGVHD target organs and its association with clinical outcomes are unknown.

Methods: We performed AREG immunohistochemical staining on skin specimens from 67 patients with aGVHD between the years 2010 and 2015. Two blinded reviewers assessed AREG expression and scored specimens with a semiquantitative scale ranging from 0 (absent) to 4 (most intense).

Results: Median AREG score of aGVHD cases was 3. Sixteen of 67 (23.9%) aGVHD cases had an AREG >3. High skin AREG expression (>3 vs. ≤3) was associated with increased overall clinical grade of aGVHD (52.9% vs. 33.4% clinical grade III-IV, $p = 0.02$), reduced 3-year overall survival (OS; 13% vs. 61%, $p < 0.01$), and increased 3-year non-relapse mortality (NRM; 56% vs. 20%, $p = 0.05$).

Conclusion: High skin AREG immunohistochemical expression is associated with high clinical grade aGVHD, poor OS, and increased NRM.

KEYWORDS
acute GVHD, allogeneic hematopoietic cell transplantation, amphiregulin, epidermal growth factor

1 | INTRODUCTION

Acute graft-versus-host disease (aGVHD) remains an important cause of morbidity and mortality after allogeneic hematopoietic cell transplant (HCT).[1] The skin is the most commonly affected organ.[1] Diagnosis, prognosis, and management of recurrent cutaneous aGVHD can be challenging.[2] An immunohistochemical (IHC) stain that aids in determining the risk of fatal aGVHD would be of clinical utility.

Tissue repair responses are important determinants of clinical outcomes after aGVHD. One such tissue repair factor, amphiregulin (AREG), an epidermal growth factor (EGF) receptor ligand, is increased in both serum and plasma in aGVHD and is associated with poor steroid response and lower survival[3–5]; EGF is often very low in plasma in steroid-refractory disease.[3] AREG expression is increased in inflammatory disorders including psoriasis,[6,7]

Brittney Schultz and Daniel D. Miller contributed equally to this study.

with important roles in tissue repair and immune response.[8] Gastrointestinal (GI) AREG expression has recently been shown to be variable in GI aGVHD.[9,10] The expression and role of AREG in human cutaneous aGVHD are unknown.

2 | MATERIALS AND METHODS

To define the association between AREG tissue expression and clinical outcomes, we analyzed serum samples and archived formalin-fixed, paraffin-embedded skin biopsy samples in post-HCT patients previously enrolled in the University of Minnesota clinical study "MT2009-22R: Monitoring of Immune Function and Minimal Residual Disease in Patients and Donors After Hematopoietic Cell Transplantation" (Principal Investigator: Michael Verneris, MD, USA). Patients were eligible for inclusion if they had a serum sample collected within 2 weeks prior to or following the onset of biopsy-confirmed cutaneous aGVHD ($n = 67$). Biopsy results from normal skin in non-HCT patients ($n = 10$) served as controls. Patients were selected by an expert reviewer (B. S.) following chart review of clinical history and dermatopathology reports. We deparaffinized and rehydrated unstained paraffin sections (4 μm) using standard methods. Subsequent steps were automated using an IHC staining platform (Nemesis, Biocare) using rabbit polyclonal anti-AREG (Biorbyt, San Francisco, CA, USA; 1:400) as the primary antibody. Slides were scored by two blinded expert reviewers (D. D. M. and B. S.) based on consensus score. The intensity of AREG nuclear expression in all cells (including keratinocytes, inflammatory cells) was determined with a semiquantitative score of 0 (absent) to 4 (most intense) with 0.5 gradations. Normal skin had no cases with AREG >3, and therefore we considered AREG above this level (scores 3.5 or 4.0) suggestive of pathology. Sera collected within 14 days of the skin biopsy of HCT cases were analyzed for concentrations of AREG (ELISA; R&D, Minneapolis, MN, USA) and EGF (multiplex bead array; Millipore, Billerica, MA, USA) according to the manufacturers' instructions. Sera were not available for non-HCT controls.

We determined the association of AREG both by IHC and in the sera with clinical endpoints including overall survival (OS) and non-relapse mortality (NRM) as well as EGF in the sera. OS and NRM were calculated from the time of the biopsy (aGVHD diagnosis). Organ staging and clinical aGVHD grading was performed using modified Consensus criteria.[1,11] Histopathologic grade of aGVHD was determined using modified Lerner criteria.[12] Risk stratification was performed using the refined MN aGVHD Risk Score.[1] Cox regression was used to examine the independent effect of AREG on OS. Fine and gray regression was used to examine the independent effect of AREG on NRM. Other factors considered in regression analyses were age, conditioning, donor type, Minnesota GVHD risk, comorbidity, and Karnofsky score. All reported p values were two-sided. All analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). This study was approved by the University of Minnesota Institutional Review Board.

TABLE 1 Acute graft-versus-host disease patient demographics

	All patients
N	67
Gender	
Male	39 (58%)
Female	28 (42%)
Age	
Median (range), (IQR)	50 (1–71), (33–59)
Donor type	
Matched sibling	20 (30%)
MM sibling	2 (3%)
Matched URD	10 (15%)
sUCB	5 (7%)
dUCB	30 (45%)
Conditioning	
Myeloablative	37 (55%)
Reduced intensity	30 (45%)
GVHD prophylaxis	
CSA/MMF	38 (57%)
CNI/MTX	17 (25%)
Siro/MMF	12 (18%)
Diagnosis	
Acute leukemia and myeloid malignancies	55 (82%)
Lymphoid malignancies	8 (12%)
Nonmalignant disorders	4 (6%)
MN GVHD risk	
Standard risk	59 (88%)
High risk	8 (12%)
Initial GVHD clinical grade	
I	25 (37%)
II	27 (40%)
III	14 (21%)
IV	1 (1%)
Skin initial staging	
1	8 (12%)
2	23 (34%)
3	35 (52%)
4	1 (1%)
Median day following HCT when skin biopsy was obtained	28.5 (interquartile range: Days 17–36)

Abbreviations: AREG, amphiregulin; CNI, calcineurin inhibitor; CSA, cyclosporine; dUCB, double umbilical cord blood; GVHD, graft-versus-host disease; MM, mismatched, MMF, mycophenolate mofetil; MN, Minnesota; MTX, methotrexate; Siro, sirolimus; sUCB, single umbilical cord blood; URD, unrelated donor.

3 | RESULTS

Demographics of the 67 patients with histopathologic diagnosis of aGVHD are detailed in Table 1. The median day following HCT when skin biopsy was obtained was day 28.5 (interquartile range, Days 17–36). There were only four patients with aGVHD skin biopsies

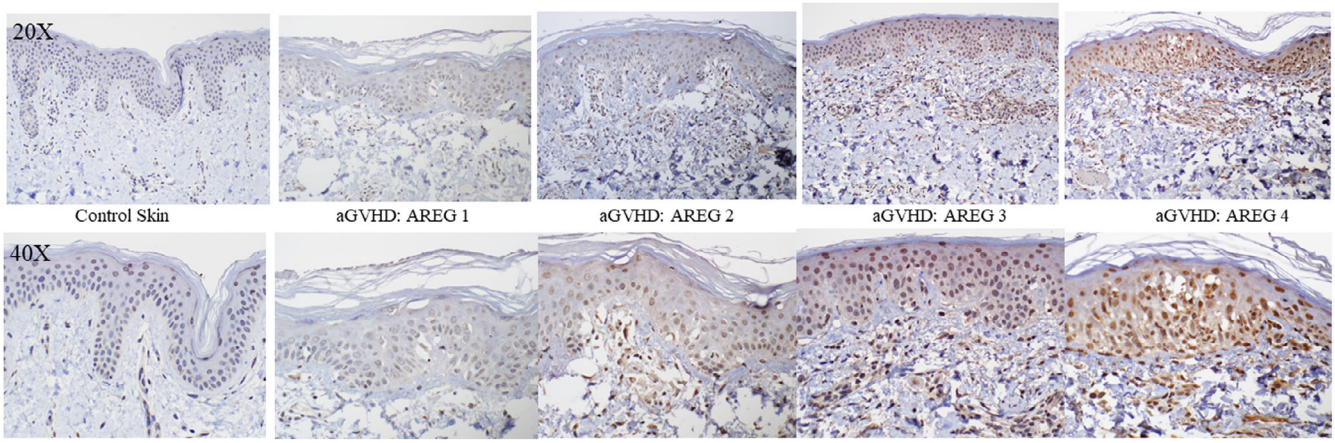

FIGURE 1 Cutaneous expression of amphiregulin. Representative staining of normal skin and skin in acute graft-versus-host disease, with a range of amphiregulin scores of 1–4 (×20 and ×40 magnification for each example)

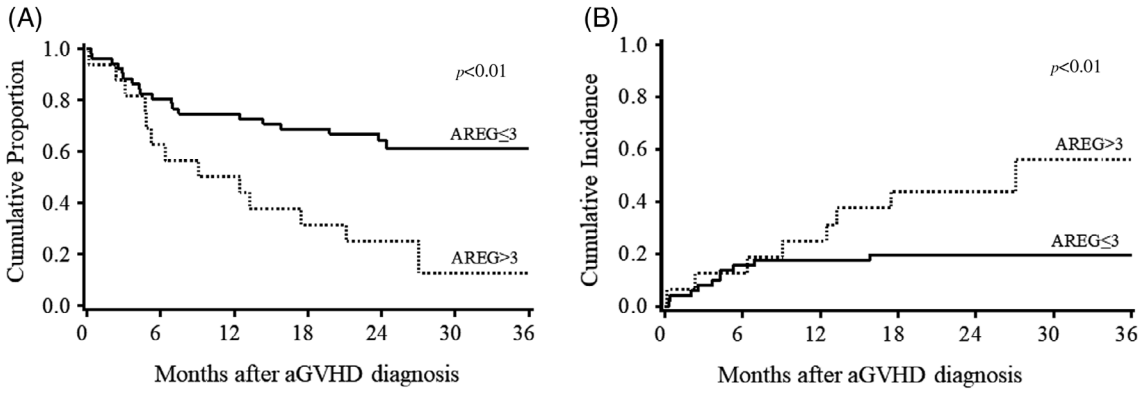

FIGURE 2 (A) Kaplan–Meier survival curve by immunohistochemical amphiregulin (AREG) score for patients with acute graft-versus-host disease (aGVHD), which shows reduced survival in patients with AREG expression >3 compared to ≤3. (B) Cumulative incidence of non-relapse mortality (NRM) by immunohistochemical skin AREG score for patients with aGVHD, which shows increased 3-year NRM in patients with AREG >3 compared to ≤3

beyond Day + 100 in this cohort; therefore this largely represents a classic aGVHD cohort. Figure 1 shows representative IHC stains. Median AREG score of aGVHD cases was 3. Sixteen of 67 (23.9%) aGVHD cases had an AREG >3. In all groups, expression of AREG was most prominent in keratinocytes and eccrine glands, although staining in lymphocytes was also observed.

High skin AREG expression (>3 vs. ≤3) was associated with increased overall aGVHD clinical grade (52.9% vs. 33.4% clinical grade III-IV, $p = 0.02$), reduced 3-year OS (13% vs. 61%, $p < 0.01$, Figure 2A), and increased 3-year NRM (56% vs. 20%, $p = 0.05$, Figure 2B). In multivariate analysis, patients with high skin AREG had a 3-fold increased risk of all-cause mortality (hazard ratio [HR] 3.0, 95% confidence interval [CI] 1.5–6.2, $p < 0.01$) and a 2.5-fold increased risk of NRM (HR 2.5, 95% CI 1.0-6.1, $p = 0.05$). High skin AREG did not correlate with Minnesota GVHD Risk ($p = 0.38$) or maximum skin aGVHD staging ($p = 0.68$). All patients except one had a serum AREG above the normal limit of 5 pg/ml (median 25.8 pg/ml, range 4.7–465.8 pg/ml). While there was no linear correlation between skin AREG staining and serum AREG in this cohort (Spearman's rho −0.06, $p = 0.7$), high skin AREG was associated with low serum EGF (Spearman's rho −0.34, $p = 0.006$), which is a negative prognostic marker of aGVHD.[3]

4 | DISCUSSION

In this analysis, we showed that high skin AREG IHC expression is indicative of pathology and associated with high-grade clinical aGVHD, low serum EGF, poor OS, and increased NRM. This adds valuable information to our previous work associating high circulating AREG with poor clinical outcomes.[4,5,13,14] This study also adds to our previous work detailing AREG expression in GI tissue in aGVHD. We previously found low AREG expression in GI mucosal epithelium and stoma in aGVHD biopsy samples compared to normal colon samples;

however, this study had largely non-severe GI aGVHD biopsy samples for analysis.[9] We subsequently found high AREG expression by mRNA in the GI tract from patients with fatal aGVHD.[10] Additionally, we found high circulating AREG protein and mRNA in peripheral blood mononuclear cells (PBMCs) in patients with fatal aGVHD.[14] Thus, we hypothesize that although AREG protein expression in the colon normally acts to maintain the integrity of the epithelial barrier, it is a negative prognostic factor if it is highly expressed in the skin (pathologic) or found in circulation either by ELISA or PBMC gene expression studies (pathologic). The data overall suggest that high expression of AREG could indicate unresolved damage and/or is produced in response to danger signals, acting as an indicator of high-risk aGVHD when pathologically expressed outside the GI tract.

In addition to its potential role as a prognostic biomarker for aGVHD, there may be a therapeutic role of AREG in aGVHD. AREG is involved in enhanced tissue repair after injury. Indeed, protective effects of AREG in the setting of aGVHD have previously been proven in murine models, where the blockade of AREG led to increased intestinal aGVHD severity and mortality.[15] AREG is also posited to have a role in immunity, as many cells in the innate and adaptive immune systems express AREG.[8,15] AREG has been shown to enhance the action of Tregs,[16] which may specifically have important consequences for aGVHD, as Tregs have been shown to prevent aGVHD.[17] Furthermore, AREG was protective in the development of dermatitis in mice who were undergoing bone marrow transplantation.[8,16]

Our study is strengthened by the blinded review and concomitant analysis of tissue and serum biomarkers. Although grading IHC staining remains is subjective, it is readily determined to be pathologically elevated if increased relative to normal skin.

In summary, our results suggest that high skin AREG expression is a high-risk feature of aGVHD. High AREG expression in epidermal skin relative to normal skin should be further studied in a larger cohort as a prognostic aid in cutaneous aGVHD. Elevated tissue and serum AREG may be a reflection of the underlying tissue damage mediated by aGVHD, a counter-regulatory response to inflammation and/or reflection of danger signal sensing. Mechanistic studies are needed to understand the host response underlying elevated skin AREG and poor outcomes after aGVHD.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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