RESEARCH LETTER

blood advances

TO THE EDITOR:

High BM plasma S100A8/A9 is associated with a perturbed microenvironment and poor prognosis in myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) represent a heterogeneous group of malignant hematopoietic stem cell (HSC) disorders.[1-3] Recent advances in the immunome of the bone marrow (BM) microenvironment identified aberrant immune activation and proinflammatory signaling as vital drivers in MDS pathogenesis.[4-6] Among these complex networks, the S100A8/A9-toll–like receptor axis is a critical MDS phenotypes definer.[7]

The inflammatory proteins S100A8 and S100A9 often exist as a heterodimer under physiological conditions.[8] In MDS, S100A8/A9 is synthesized and secreted by, among others, myeloid-derived suppressor cells (MDSCs), which play a central role in pathogenesis.[9] The ligation of S100A8/A9 to TLR4 leads to NF-κB-mediated transcription and subsequent production of proinflammatory cytokines, and the induction of NLRP3 inflammasome,[5,10] which consequently drives pyroptosis of HSCs and an inflammatory milieu in the BM.[11,12] Although S100A8/A9 serves as biomarkers in various diseases,[13-15] its clinical implication in MDS is not fully deciphered.[16]

To investigate the clinical and microenvironmental relevance of S100A8/A9 in patients with MDS, we recruited 215 patients with MDS at the National Taiwan University Hospital and quantified BM plasma S100A8/A9 dimer levels by enzyme-linked immunosorbent assay (supplemental Method). Genomic DNA and mRNA were extracted from BM mononuclear cells and sequenced as previously described.[17,18] Methods for bioinformatic and statistical analysis are detailed in supplemental Methods. Patient characteristics are summarized in supplemental Table 1. The median age of the patients was 67.5 years. Over a median follow-up duration of 39.7 months, 64 patients (29.8%) progressed to acute myeloid leukemia (AML), and 93 patients succumbed to the disease. The National Taiwan University Hospital Research Ethics Committee approved the study (#201709072RINC). Informed consent was obtained in accordance with the Helsinki Declaration.

Correlation analysis revealed that the BM plasma S100A8/A9 protein levels significantly correlated with the mRNA expression in whole BM cell RNA sequencing ($r^2 = 0.32$ and $r^2 = 0.31$, respectively; supplemental Figure 1). We then explored the distribution of S100A8/A9 levels (supplemental Figure 2A) across disease subgroups. Patients with MDS/AML (MDS with 10%-19% blasts in the BM or peripheral blood) and concurrently mutated *TP53* had higher S100A8/A9 levels than others (supplemental Figure 2B). Meanwhile, there was no difference in S100A8/A9 levels among patients in the International Prognostic Scoring System (IPSS) or the Revised IPSS (IPSS-R)[3] subgroups (supplemental Figure 2C-F).

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RNAseq data reported in this article have been deposited in the Gene Expression Omnibus database (accession number GSE223305).

Data are available on request from the corresponding authors, Chien-Chin Lin (lincc@ntu.edu.tw) and Hwei-Fang Tien (hftien@ntu.edu.tw).

The full-text version of this article contains a data supplement.

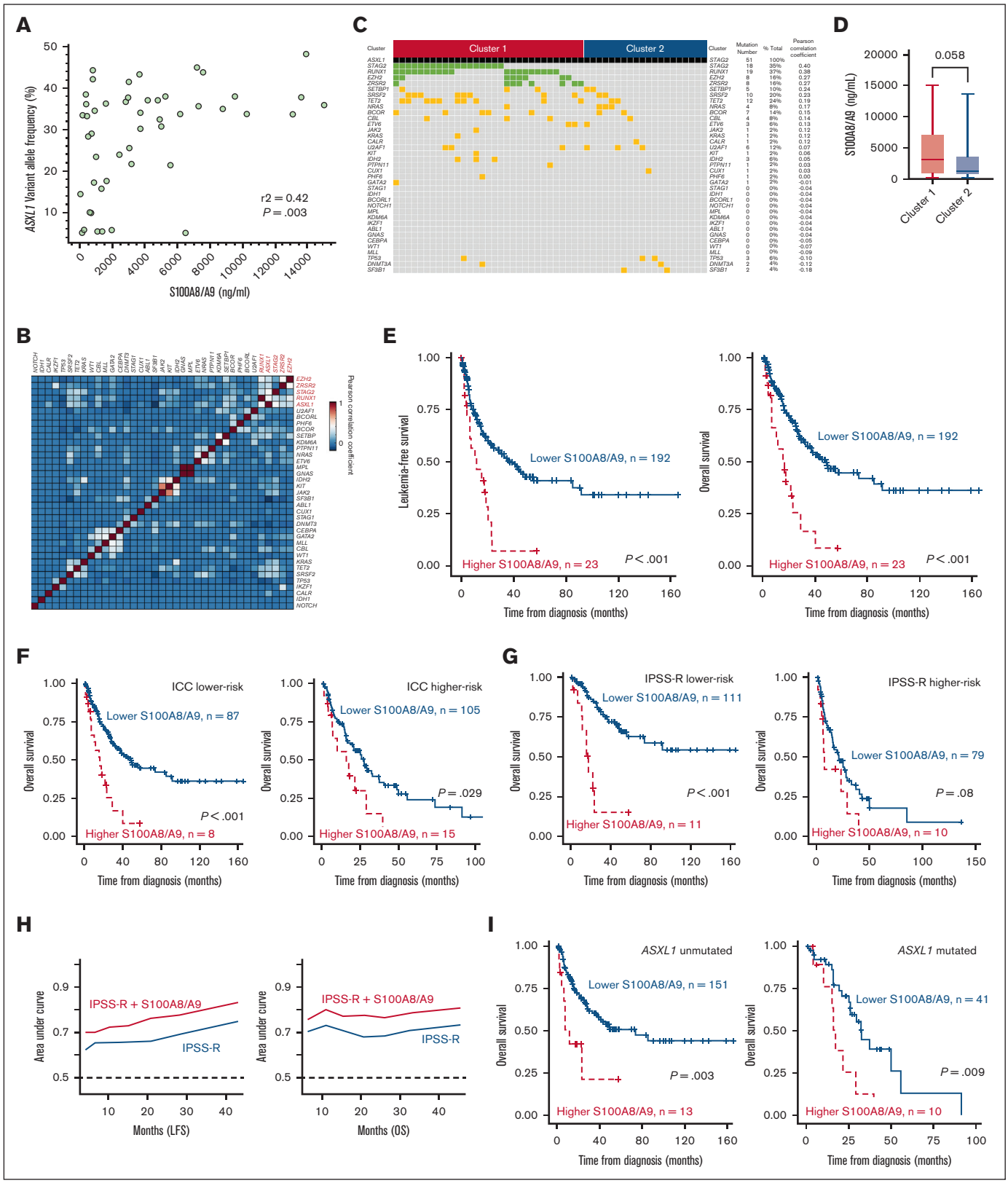


Figure 1. Distinct clinical and biological characteristics of patients with MDS with higher bone marrow plasma S100A8/A9. (A) Scatter plots shows a moderate correlation between *ASXL1* VAF and S100A8/A9 levels. (B) Heatmap of correlations among mutations. (C) Clustering 51 *ASXL1*-mutated patients based on concurrent mutations of *STAG2*, *RUNX1*, *EZH2*, and *ZRSR2*. (D) Cluster 1 had a trend of higher S100A8/A9 levels than cluster 2. (E) Higher S100A8/A9 conferred inferior LFS and OS of the 215 patients with MDS.

Because previous studies demonstrated the impact of genetic events on innate immune and inflammasome-signaling,[5] such as the NF-kB pathway,[19,20] pyroptosis and β-catenin signaling,[21] and NLRP3 inflammatory pathways,[7] we examined whether S100A8/A9 concentrations differ across patients with various genotypes. Patients with *ASXL1*-mutation had significantly higher S100A8/A9 than those with unmutated *ASXL1* (supplemental Figure 2G), whereas no difference was detected between patients with or without other epigenetic or splicing gene mutations (supplemental Figure 2G-H).

Next, we sought to investigate the relationship between mutant *ASXL1* and S100A8/A9 levels. *ASLX1* variant allele frequencies (VAF) significantly correlated with S100A8/A9 levels in 49 patients with *ASXL1*-mutation with available VAFs (Figure 1A). Hierarchical clustering suggested close associations among mutations in *ASXL1, STAG2*, *RUNX1*, *EZH2*, and *ZRSR2* (Figure 1B). Interestingly, patients with *ASXL1*-mutation with concurrent above-mentioned mutations (cluster 1) had a trend of higher S100A8/A9 than those without (cluster 2) (Figure 1C,D).

The 215 patients with MDS were subsequently divided into higher- and lower-S100A8/A9 groups with cutoff point of 7093 ng/mL determined by maximally selected rank statistics. There were no significant differences in the distribution of disease subgroups according to the International Classification Consensus (ICC), IPSS-R, and karyotypes between the 2 groups; but high-S100A8/A9 patients had a higher *ASXL1* mutation rate (43.5% vs 21.4%) (supplemental Tables 1-3).

We then examined the impact of S100A8/A9 levels on patients' survival. Higher-S100A8/A9 was associated with significantly inferior leukemia-free survival (LFS) and overall survival (OS) not only in the total cohort (Figure 1E), but also in the lower- and higher-risk subgroups based on the ICC and IPSS-R (Figure 1F-G; supplemental Figures 3-4). Time-dependent receiver operating characteristic curve analysis also suggested the potential for S100A8/A9 to supplement IPSS-R (Figure 1H). Moreover, despite having higher frequencies of mutant *ASXL1*, high-S100A8/A9 patients had poorer survival than those of the lower-S100A8/A9 group irrespective of *ASXL1* mutation statuses (Figure 1I; supplemental Figure 5).

The prognostic implications of S100A8/A9 levels on survival were also demonstrated in patients carrying different karyotypes (supplemental Figure 6) or receiving different treatments (supplemental Figure 7). Remarkably, in 50 patients who received hypomethylating agent monotherapy, higher-S100A8/A9 retained strong discriminatory prognostic impact on LFS and OS (supplemental Figure 8). In multivariable analysis, we included parameters with a P value $<.05$ in the univariate analysis (supplemental Table 4), and hazard ratios were adjusted with treatments that well-stratified survivals (supplemental Figure 9). Higher-S100A8/A9 remained an independent adverse prognostic factor for LFS and OS (Figure 2A).

Considering that RNAseq was performed on whole BM MNCs, we referenced the single-cell data set of healthy controls[22] to identify which cell types contributed to the differentially expressed genes. Curiously, 18 of the 20 most down-regulated genes (supplemental Table 5) in high-S100A8/A9 BM were regularly expressed by lymphocytes (supplemental Figure 10), implying different composition of lymphocytes in higher- and lower-S100A8/A9 BMs. We further adopted CIBERSORTx,[18,23] which infers the landscape of infiltrating immunocytes in the BM from gene expression profiles. Higher-S100A8/A9 was associated with significantly lower fractions of CD8 T-cells and activated natural killer (NK) cells (Figure 2B; supplemental Table 6).

Weighted gene coexpression network analysis revealed that the turquoise and blue modules were closely associated with lower- and higher-S100A8/A9, respectively (supplemental Figure 11). Corresponding to CIBERSORTx analysis, the 914 genes in the turquoise module were enriched in pathways involving NK- and T-cell functions, whereas the 509 genes in the blue module were enriched in pathways involving MDSCs in cancer immune escape and altered metabolism (Figures 2C,D).

To the best of our knowledge, this is the first study to significantly stratify the survival of patients with MDS based on S100A8/A9 levels. We also observed that patients with *ASXL1*-mutation had higher S100A8/A9 concentrations than their unmutated counterparts, corresponding with the increase in NADPH oxidase and ROS, TLR4 activation and pyroptosis.[7] Fundamentally, the upregulation of S100A8/A9 can exert genotoxic stress in HSCs, thereby advancing the risk for AML transformation,[24] in accordance with our finding that higher-S100A8/A9 group had a shorter LFS. In a homogeneously treated lower-risk MDS cohort, S100A8/A9 expression in mesenchymal stem cells was correlated with p53 and TLR4 upregulation.[24] Furthermore, high S100A8/A9 concentrations doubled the risk of leukemic transformation and significantly reduced the time for AML transformation.

Comparisons of the transcriptomic data highlighted differences in functions and properties of immune cells between higher- and lower-S100A8/A9 BM and suggested a high-risk subentity that was not considered in current risk stratification in this heterogenous disease and required more attention. However, the lack of external validation, biological validation, and the assessment of the impact of other inflammasome components such as cytokines or chemokines is a major issue. Meanwhile, the demographics of our MDS population are more similar to Korean patients,[25] with a younger and higher-risk skewing when compared with that of western cohorts, suggesting that the extrapolation of our data may be compromised. Treatment heterogeneity could also potentially confound our analysis. Serial follow-up data after treatment will strengthen the prognostic power of S100A8/A9 and provide more insight into the changes in the BM microenvironment. In addition, our findings could be more granular and justified if cytometry by time of flight or single-cell multiomics approaches were adopted.

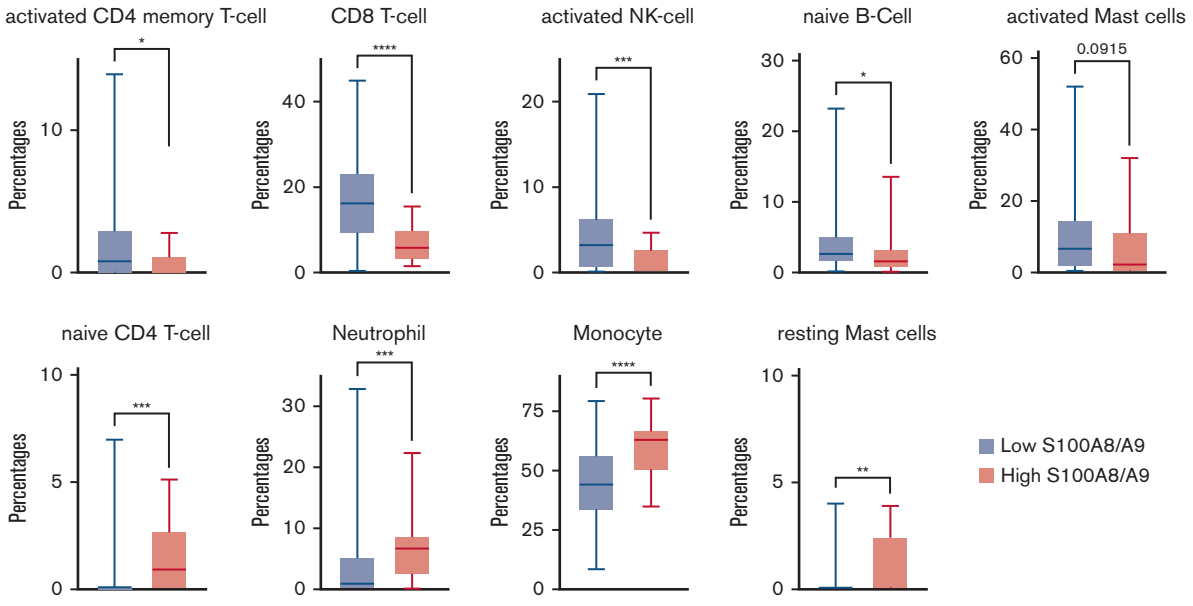
Despite above limitations, this study clearly showed that S100A8/A9 level was an independent poor prognostic factor in MDS, and

Figure 1 (continued) (F) Higher S100A8/A9 conferred significantly worse OS in ICC lower-risk group and higher-risk group. Higher-risk: MDS with excess blast and MDS/AML; and lower-risk: others. (G) Higher S100A8/A9 conferred significantly shorter OS in IPSS-R lower-risk (very low, low, and intermediate) group and a trend of worse OS in IPSS-R higher-risk (IPSS-R high and very high) group. (H) Time-dependent ROC curve analyses demonstrate that S100A8/A9 levels can be complementary to IPSS-R, increasing area under curves when incorporated. (I) Patients with higher S100A8/A9 had significantly inferior OS irrespective of their *ASXL1* mutation statuses. ROC, receiver operating characteristic.

A

	LFS				OS			
		95% CI				95% CI		
Variable	HR§	Lower	Upper	*P*	HR§	Lower	Upper	*P*
Age*	1.020	1.005	1.034	.007	1.029	1.013	1.046	< .001
IPSS-R†	1.425	1.153	1.761	.001	1.616	1.282	2.037	< .001
RUNX1	1.433	0.819	2.509	.208	1.492	0.824	2.701	.187
SF3B1	0.654	0.346	1.236	.191	0.667	0.335	1.330	.250
STAG2	0.832	0.449	1.542	.559	0.912	0.485	1.717	.776
TP53	3.552	2.000	6.310	< .001	6.244	3.290	11.849	< .001
Higher S100A8/A9‡	2.405	1.346	4.299	.003	2.279	1.248	4.162	.007

B



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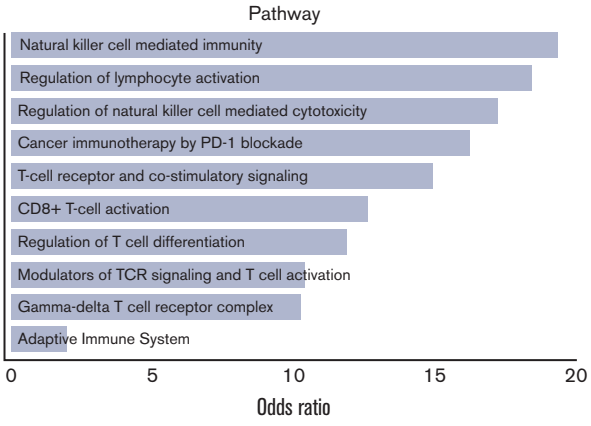

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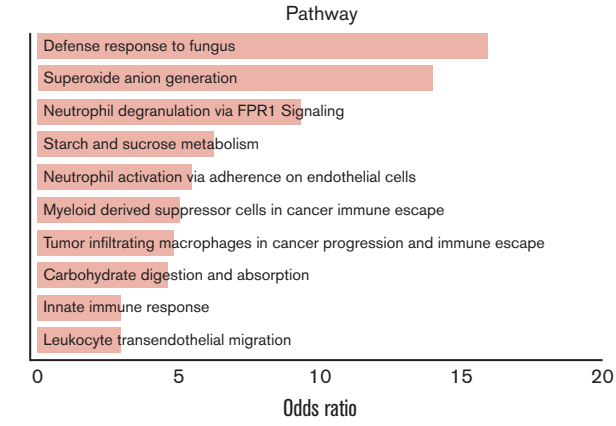

Figure 2. Multivariable analysis for LFS and OS and transcriptomic implication of high vs low bone marrow plasma S100A8/A9. (A) Multivariable analysis for LFS and OS. Statistically significant if $P \leq .007$ (adjusted by Bonferroni correction). *As continuous variable analysis. †IPSS-R risk groups: very low, low, intermediate, high, very high.

higher S100A8/A9 in the BM intimated a perturbed microenvironment with enhanced MDSC signal and impairment in the functions and quantities of $CD8^+$ T cells and NK cells. We propose that S100A8/A9 can be incorporated to the current risk stratification systems and prospectively assessed in clinical trials.

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Contribution: Y.-H.W. was responsible for data collection and management, statistical analysis, interpretation, visualization, literature research, and manuscript writing; F.M.R.A. and C.-Y.Y. assisted in bioinformatic analysis and visualization; C.-J.K, S.-C.Y., and P.-T.S. helped with sample preparation and processing; H.-A.H. and W.-C.C. were responsible for data collection and management; and C.-C.L. and H.-F.T. conceived and coordinated the study and revised the manuscript.

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Figure 2 (continued) ‡High vs low S100A8/A9. §adjusted with different treatments: supportive care only, HSCT with or without any other treatment, hypomethylating agent with or without other chemotherapies but HSCT, and all other treatments. Note: only variables with *P* value ≤.05 in univariate analysis were incorporated into the multivariable Cox proportional hazard regression analysis. (B) CIBERSORTx analysis revealing significant differences in the fractions of specific cell types between higher- and lower-S100A8/A9 BM. There were higher proportions of activated CD4 T-cells, CD8 T-cells, NK cells, and naïve B-cells in the lower-S100A8/A9 BM, whereas the fractions of naïve CD4 T-cell, resting mast cells, monocytes, and neutrophils were higher in the higher-S100A8/A9 BM. (C,D) Bar charts showed 10 robustly enriched functional pathways in lower-S100A8/A9 (panel C) and higher-S100A8/A9 (panel D) BMs, respectively. CI, confidence interval; HR, hazard ratios; HSCT, hematopoietic stem cell transplant.

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