Evaluation of SOX4 levels in multiple myeloma patients

Multiple myeloma (MM) is a complex hematologic cancer in which many factors interplay to promote cancer progression. MM is characterized by clonal proliferation of malignant plasma cells within the bone marrow which, in most cases, leads to abnormal expansion of a monoclonal component. In several cases, MM is preceded by a monoclonal gammopathy of undetermined significance (MGUS) or by smoldering multiple myeloma (SMM). MGUS and SMM are asymptomatic conditions characterized by the presence of monoclonal cells. SMM can be distinguished from MGUS by a higher risk of progression to MM (10% *vs.* 1%, respectively, in the first 5 years).[1]

MM invariably relapses, even after the administration of new advanced therapeutic regimens (*https://seer.cancer.gov/statfacts/html/mulmy.html*). The tumor microenvironment can play a crucial role in both MM development and progression: MM tumor cells interact with stromal cells and with the extracellular matrix in an altered inflammatory milieu.[2] The release of inflammatory cytokines triggers physical adhesion and settling of MM cells in the bone marrow.[3] MM-specific bone marrow mesenchymal stromal cells, together with inflammation, play a pivotal role in fostering tumor resistance, as well as survival and proliferation of MM cells.[2,3]

Transcription factors, deregulating the expression of pivotal genes,[4] are of paramount importance in the development and progression of MM. In particular, the transcription factor SOX4 is involved in cell proliferation, cell survival, inhibition of apoptosis and general tumor progression.[4] Its pro-oncogenic activity has been demonstrated in many solid tumors.[5,6] SOX4 is also expressed in MM cell lines and its downregulation is linked to cell toxicity.[7] In solid cancers such as oral squamous cell carcinoma, SOX4 contributes to trigger inflammation. SOX4 expression is enhanced by transforming growth factor beta 1 (TGFβ1), which is often dysregulated in hematologic malignancies.[8] TGFβ1 drives the expression and transcriptional activity of SOX4 during epithelial to mesenchymal transition of breast and gastric cancer.[4,9] Previous studies showed that both MM cells and bone marrow stromal cells produce high levels of TGFβ1,

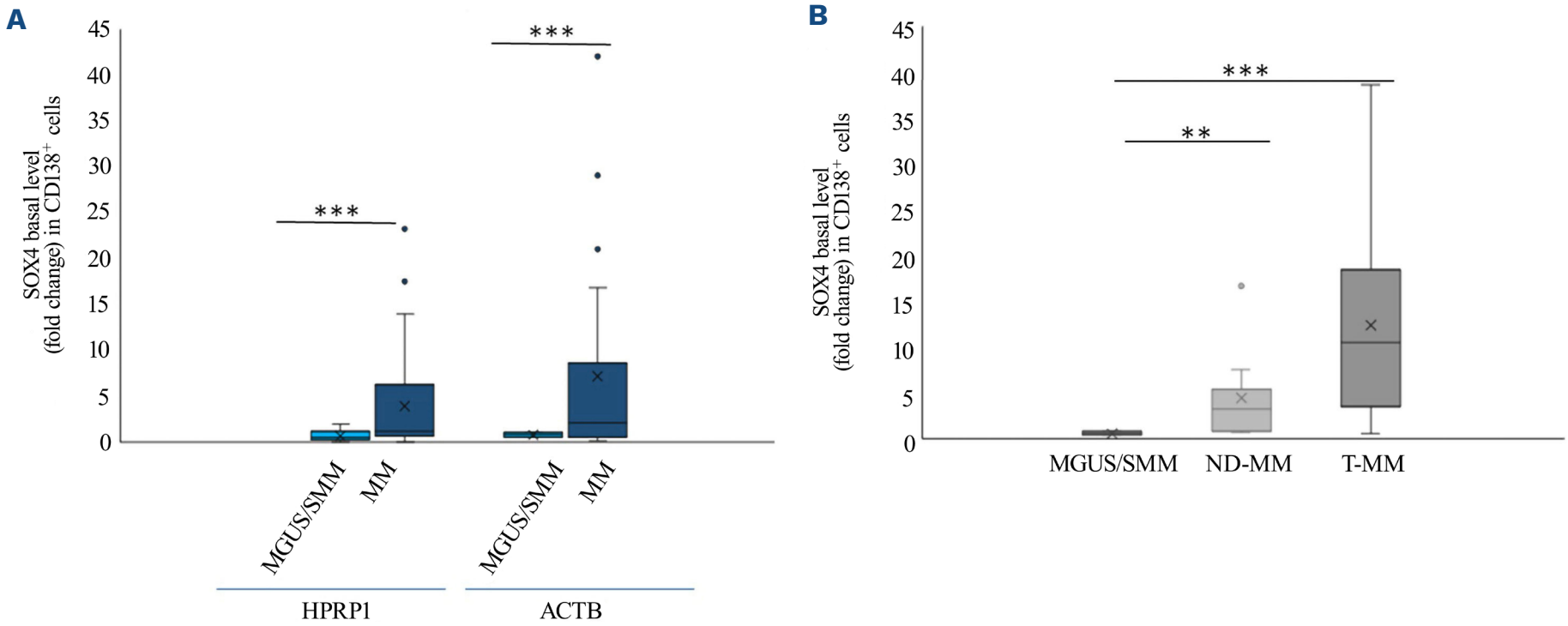

Figure 1. Constitutive expression of SOX4 in patients' samples. (A) Analysis of SOX4 basal levels in CD138+ plasma cells, performed by reverse transcriptase polymerase chain reaction (RT-PCR), on nine samples from patients with monoclonal gammopathy of undefined significance/smoldering multiple myeloma (MGUS/SMM) and on 74 specimens from patients with overt multiple myeloma (MM). The MM group comprises samples derived from newly diagnosed (ND-MM) and treated (T-MM) patients. Total RNA extraction was performed using Trizol RNA Isolation Reagents (Thermo Fisher Scientific, Carlsbad, CA, USA). SOX4 (Hs.PT.58.24974948.g, IDT, San Diego, CA, USA) expression level was normalized to the levels of the housekeeping genes, HPRP1 (Hs.PT.58v.4545621572, IDT, San Diego, CA, USA) and ACTB (actin B,Hs.PT.39a.22214847, IDT, San Diego, CA, USA), using the -ΔΔCt relative quantification method. The solid line within the box plot represents the mean value, the x denotes the median value. The statistical analysis was performed by a Student's *t* test for unpaired data. ***P<0.001. (B) SOX4 basal expression, detected by RT-PCR and using ACTB (Hs.PT.39a.22214847, IDT, San Diego, CA, USA) as a housekeeping gene, in CD138+ patients' plasma cells. The assay was performed on nine MGUS/SMM samples (used as control), 31 ND-MM samples and 43 T-MM samples. The solid line within the box plot represents the mean value, the x denotes the median value. The statistical analysis was performed by one-way analysis of variance for independent samples with Tukey's honestly significant difference as a *post hoc* test. **P<0.01, ***P<0.001.

leading to tumor progression.[10]

SOX4 regulates the expression of membrane molecules that play a crucial role in tumor-niche interactions, such as CD56, a membrane glycoprotein expressed in neural tissues, natural killer cells and T lymphocytes.[11] Multiple studies have indicated that CD56 is expressed in 55-85% of patients with MM, but not in normal plasma cells.[12] Its expression is inversely correlated with the number of circulating plasma cells and with the extent of bone marrow infiltration.[13] High CD56 expression is associated with the presence of lytic bone lesions.[13] Iqbal *et al*. demonstrated that overexpression of SOX4 upregulates the expression of CD56[14] and, in turn, SOX4 silencing leads to downregulation of CD56 and cell apoptosis. In hematologic malignancies, the inflammatory state in the tumor microenvironment is also maintained by progranulin (PGRN),[15] a cytokine that drives tumor growth, survival, and drug resistance in many types of tumors. In the bone marrow microenvironment PGRN modulates the function of cancer-associated fibroblasts and immune cells, in turn promoting cancer progression.[15] Previously, we reported on the role of SOX4 in a panel of MM cell lines.[7] Given the importance of the bone marrow niche in both the development and growth of MM, we here extended our analysis to an investigation of SOX4, CD56 and PGRN in plasma cells from therapy-resistant MM patients compared to MGUS/SMM patients. Our aim was to analyze SOX4 in plasma cells derived from a pathological context, taking into account the role of the tumor microenvironment niche and inflammation on its expression. To the best of our knowledge this is the first analysis of SOX4, tumor microenvironmental factors and CD56 in samples from MM patients over the course of the evolution of their disease.

Our study, approved by the Ethics Committee (code: CRO-2021-31) of the Centro di Riferimento Oncologico di Aviano (CRO-IRCCS), and performed in accordance with the Declaration of Helsinki, involved 67 patients. Each patient signed informed consent. Our samples comprised pre-MM (MGUS) and not active MM (SMM) (included in the same control group [N=9]), active, newly diagnosed MM (ND-MM) (N=31) and treated MM (T-MM) (N=43) (*Online Supplementary Table S1*). The discrepancy between the number of samples and the number of patients is due to the fact that some patients underwent multiple bone morrow biopsies. The T-MM group included patients who underwent pharmacological therapy with or without autologous stem cell transplantation. The MGUS/SMM samples were considered as the baseline reference, while ND-MM samples were considered to have come from treatment-naïve patients.

We evaluated basal SOX4 expression in $CD138^+$ plasma cells and found it to be significantly increased (3.5-fold mean value) in pooled samples from ND-MM and T-MM patients compared to MGUS/SMM patients (Figure 1A). Based on SOX4 expression levels, MGUS/SMM samples were classified as low and MM as high expressors. When compared to

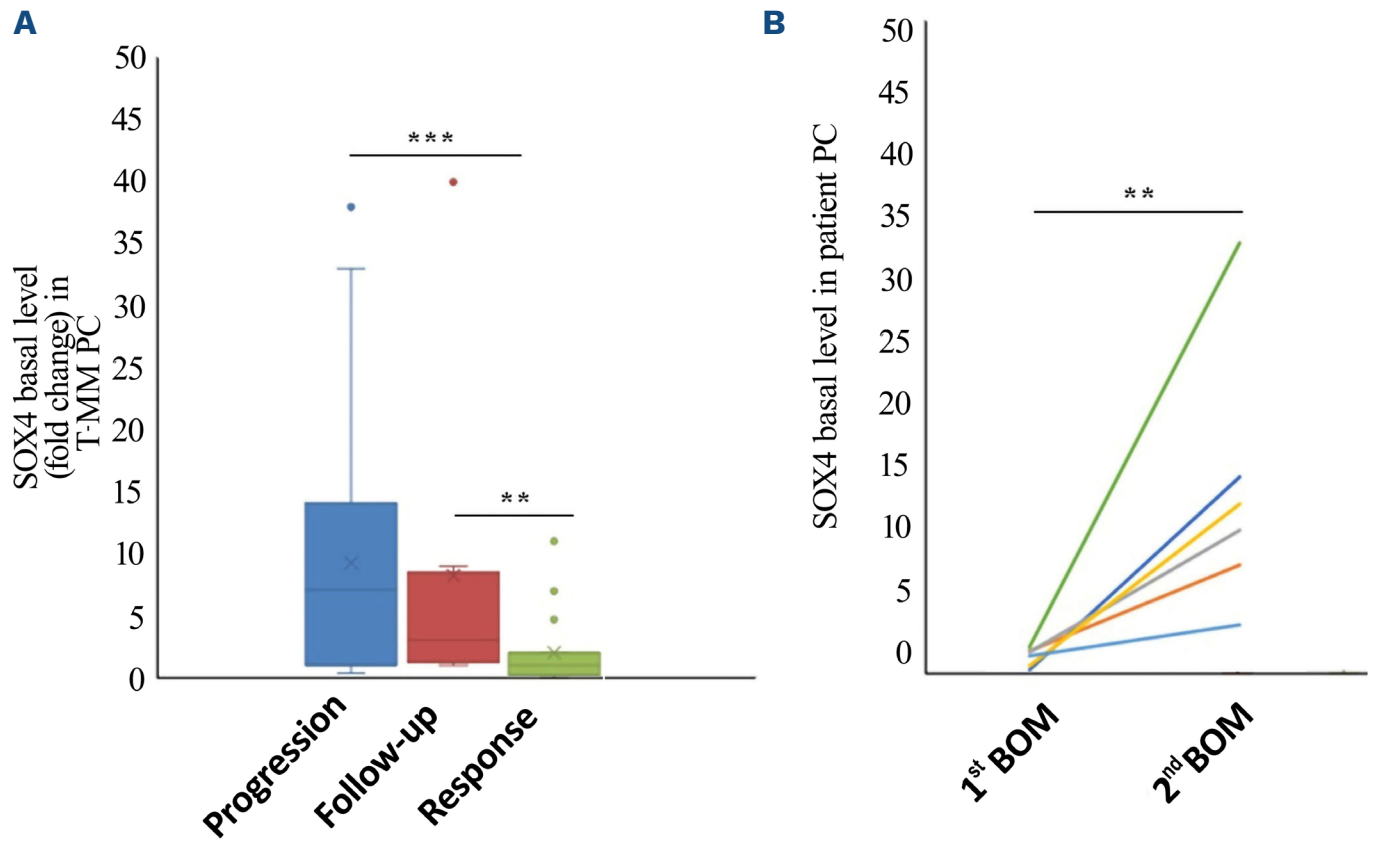

Figure 2. SOX4 expression in biopsies from patients with treated multiple myeloma. SOX4 (Hs.PT.58.24974948.g, IDT, San Diego, CA, USA) expression levels, normalized using ACTB (actin B, Hs.PT.39a.22214847, IDT, San Diego, CA, USA) as a housekeeping gene. (A) Gene levels in patients with treated multiple myeloma (T-MM) based on disease progression. The solid line within the box plot represents the mean value, the x denotes the median value. The statistical analysis was performed by one-way analysis of variance for independent samples with Tukey's honestly significant difference as a *post hoc* test. $^{**}P<0.01$, $^{***}P<0.001$. (B) Gene expression level in first and second bone marrow biopsies in samples from patients with recurrent T-MM. The statistical analysis was performed by a Student's *t* test for unpaired data. $^{**}P<0.01$. PC: plasma cells; BOM: bone marrow biopsy.

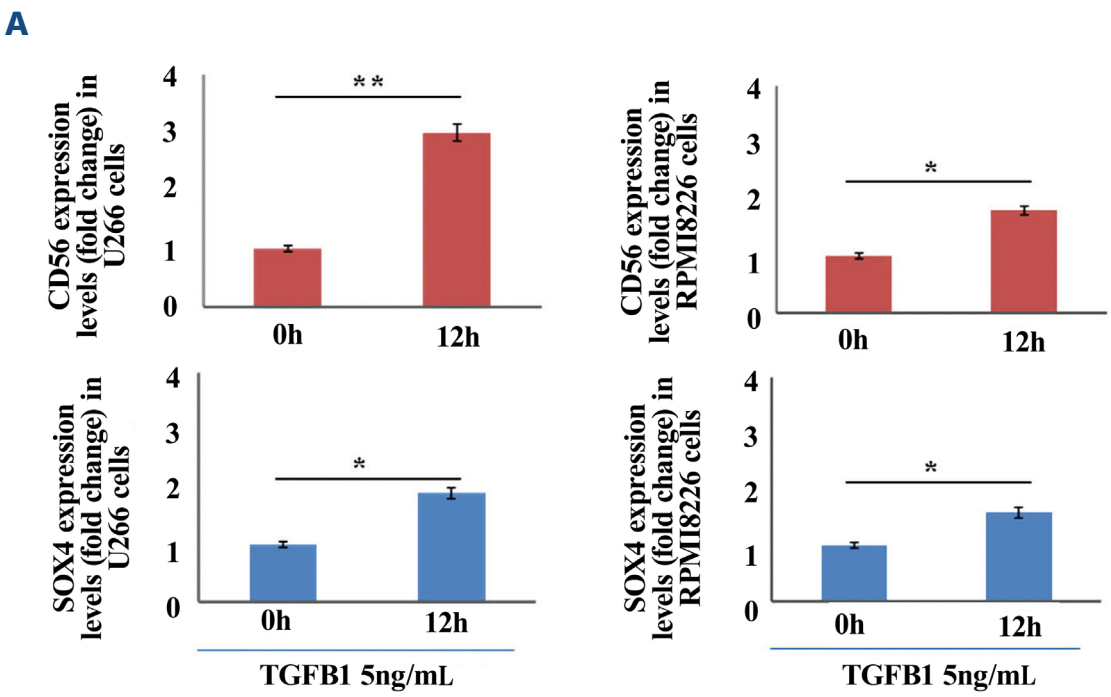

Figure 3. Correlation between CD56 expression on the surface of plasma cells, SOX4 expression and inflammation state in bone marrow niche. Transforming growth factor beta 1 (TGFβ1; 5 ng/mL for 12 hours in whole RMPI medium) upregulates SOX4 and CD56 expression in commercial U266 (ATCC® TIB-196™, Manassas, VA, USA) and RPMI-8226 (ATCC® CCL-155™, Manassas, VA, USA) cell lines. (A) SOX4 (lower panels) and CD56 (upper panels) were analyzed using IDT PrimeTime® Predesigned qPCR Assays (Hs.PT.58.24974948.g for SOX4 and Hs.PT.58.39694135 for CD56; IDT, San Diego, CA, USA). The histogram values represent the mean of three independent experiments. Bars represent the standard deviation. The statistical analysis was performed by a Student's *t* test for unpaired data. **P*<0.05, ***P*<0.01. (B) CD56 expression on the surfaces of U266 and RPMI cells, as evaluated by fluorescence-activated cell sorting (FACS) analysis at 0 and 12 hours after incubation with TGFβ1 (NCAM1/CD56-APC BD Biosciences, #341027). (C) There was a significant, direct correlation (R^2=0.49, *P*=0.02) between SOX4 (x axis) and CD56 (y axis) expression, as determined by IDT PrimeTime® Predesigned qPCR Assays (Hs.PT.58.24974948.g for SOX4 and Hs.PT.58.39694135 for CD56; IDT, San Diego, CA, USA). Data are shown as fold change values in CD138-positive cells from 20 patients' samples using actin B as the housekeeping gene. Data were analyzed by standard regression analysis. (D, E) TGFβ1 and progranulin (PGRN) concentrations in bone marrow CD56-negative (N=12) and CD56-positive (N=20) samples. The presence of CD56 was detected by FACS assay (NCAM1/CD56-APC BD Biosciences, #341027), intended for the diagnosis and follow-up of multiple myeloma. The concentrations of active TGFβ1 (D) and PRGN (E) in bone marrow plasma collected from enrolled patients were determined using a Quantikine ELISA Immunoassay (R&D systems, Bio-Techne Ltd., UK Cat. N. DY240) and human PGRN ELISA kit (AdipoGen Inc. Cat. N. AG-45A-0018YEK-KI01) according to the manufacturers' instructions. The optical density of each sample was determined using a microplate reader (Tecan INFINITE M200) set at 450 nm, with wavelength correction set at 540 nm. The concentrations of TGFβ1 and PGRN were calculated using standard curves, considering the dilution factors, and healthy donor peripheral serum samples were used as control. The solid line within the box plot represents the mean value, the x denotes the median value. The statistical analysis was performed by a Student's *t* test for unpaired data. **P*<0.05.

levels in ND-MM, SOX4 only tended (*P*=0.07) to increase in T-MM (without distinction based on therapeutic response), probably due to the low number of samples and the heterogeneity of MM (Figure 1B).

In T-MM samples, SOX4 expression was compared to tumor progression. SOX4 expression was high in samples from patients in clinical progression and, conversely, was low in samples from patients in clinical remission (Figure 2A). A longitudinal survey in T-MM patients over a 6- to 12-month interval showed that SOX4 basal levels were significantly higher at the time of the second biopsy than at the time of the first biopsy (*P*<0.01) (Figure 2B). This suggests that SOX4 is upregulated in the transition from premalignant and inactive MM to overt malignant MM, as observed in solid tumors,[5,6] and that its levels of expression reflect tumor progression.

To assess the impact of inflammation on SOX4 and the adhesion molecule CD56, we treated MM cell lines with TGFβ1 (5 ng/mL). We found that, within 12 hours, TGFβ1 upregulated the transcription levels of both SOX4 and CD56 (Figure 3A) and CD56 expression (Figure 3B). Next, we sought a link between SOX4 and CD56 in plasma cells derived from MM patients. By regression analysis, we found a direct and significant (*P*=0.02) correlation between SOX4 and CD56 transcription levels in bone marrow CD56-positive samples based on routine fluorescence-activated cell sorting analysis (Figure 3C). Samples defined as CD56-negative were also tested by reverse transcriptase polymerase chain reaction analysis, which failed to detect CD56 mRNA expression (*data not shown*), and were not included in this analysis.

Samples characterized by high expression of CD56 showed enhanced expression of TGFβ1 (*P*<0.05), explaining the persistence of high SOX4 levels in T-MM patients as a consequence of a TGFβ1-rich milieu. In MM patients, TGFβ1 indirectly upregulates the adhesion molecule CD56, providing plasma cells with greater binding to the tumor niche and potentially contributing to cancer cell survival.[14] CD56-positive samples also had significantly increased levels of PGRN (*P*<0.01) compared with CD56-negative samples (Figure 3D, E). PGRN was increased in the bone marrow of CD56-positive patients, suggesting its role in triggering the activation of inflammatory pathways in the presence of plasma cells in MM. PGRN is involved in PD-L1 upregulation on macrophages, thus leading to cancer immune escape.[11] Therefore, PGRN released in the MM bone niche acts as an autocrine growth factor promoting the survival of cancerous plasma cells.[11]

In conclusion, we report high levels of SOX4 in full-blown MM which persist and increase over time regardless of therapy, suggesting a possible diagnostic use of SOX4 as a marker of disease progression. Our data confirm that SOX4 expression is enhanced by TGFβ1,[7,9] a cytokine enriched in the MM cancer niche[8] which increases SOX4 and CD56, reducing apoptosis.[8] We also found increased levels of PGNR in CD56-positive samples, suggesting that TGFβ1 could synergize with PGNR to create a tumor-protective microenvironment, promoting immune suppression and therapy resistance. Since chemotherapy *per se* induces systemic and localized tumor microenvironment inflammation, the observed SOX4 upregulation during MM evolution may be a consequence of chemotherapy itself. A persistent upregulation of SOX4 is associated with cell proliferation, a key feature of MM as an incurable disease. Our data suggest that SOX4 could be considered as a new therapeutic target to delay disease relapse in MM.

Authors

Elisabetta Lombardi,[1] Kinga Eva Kowal,[1] Gonzalo Almanza,[2] Francesco Agostini,[1] Francesco Da Ros,[1] Marco Valvasori,[1] Carla Vicinanza,[1] Daniel Alzetta,[3] Barbara Montante,[3] Miriam Marangon,[1] Silvia Crestani,[3] Giuseppe Lamon,[3] Maurizio Rupolo,[3] Mario Mazzucato,[1] Cristina Durante,[1] Maurizio Zanetti[2] and Mariagrazia Michieli[3]

[1]Stem Cell Unit, Department of Research and Advanced Cancer Diagnostic, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Aviano, Italy; [2]The Laboratory of Immunology, Department of Medicine and Moores Cancer Center, University of California San Diego, La Jolla, CA, USA and [3]Oncohematology and Cell Therapy Unit, Department of Medical Oncology, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Aviano, Italy

Correspondence:
E. LOMBARDI - elombardi@cro.it

https://doi.org/10.3324/haematol.2024.286794

Received: November 19, 2024.
Accepted: April 29, 2025.
Early view: May 8, 2025.

Disclosures

MZ is a cofounder of FutuRNA Pharmaceuticals, a company formed on intellectual property licensed from the University of California.

Contributions

EL, KEK, MMaz, MMi and MZ conceived the study. EL, FA, CV, MMaz and MMi curated the data. EL, KEK, MV and FDR performed the formal analysis. MMaz and MMi acquired funding. EL, KEK, MV and FDR performed the investigations. EL, MM, MM, MZ and GA were responsible for the methodology. EL was the project administrator. DA, BM, SC, Mar, MR, CD and GL obtained resources. EL, KEK, FDR and AF were responsible for software. MMaz, MMi and MZ provided supervision. CV, CD, FA, MZ, MMaz and MMi were responsible for validation and visualization. EL, MMaz, MMi and MZ wrote the

original draft. CV, CD, FA, MZ, MMaz and MMi wrote, reviewed and edited the manuscript.

Acknowledgments
Special thanks to Prof. Vincenzo Canzonieri and the Pathological Anatomy Unit of CRO-Aviano.

Funding
This work was supported by a grant received from the association "Finchè ci siete voi ci sono anch'io" (code I31I170007), by the organization "Associazione Italiana contro Leucemie-linfomi e mielomi-AIL", by the Ministry of Health Ricerca Corrente, Alleanza Contro il Cancro ACC (code J34I20000600001) and by the Foundation "Ottone Zanolin e Elena Dametto" ONLUS.

Data-sharing statement
Data are available on request to the corresponding author.

References

1. Rajkumar SV, Landgren O, Mateos M-V. Smoldering multiple myeloma. Blood. 2015;125(20):3069-3075.
2. Melaccio A, Reale A, Saltarella I, et al. Pathways of angiogenic and inflammatory cytokines in multiple myeloma: role in plasma cell clonal expansion and drug resistance. J Clin Med. 2022;11(21):6491.
3. García-Ortiz A, Rodríguez-García Y, Encinas J, et al. The role of tumor microenvironment in multiple myeloma development and progression. Cancers (Basel). 2021;13(2):217.
4. Vervoort SJ, van Boxtel R, Coffer PJ. The role of SRY-related HMG box transcription factor 4 (SOX4) in tumorigenesis and metastasis: friend or foe? Oncogene. 2013;32(29):3397-3409.
5. Tiwari N, Tiwari VK, Waldmeier L, et al. Sox4 is a master regulator of epithelial-mesenchymal transition by controlling Ezh2 expression and epigenetic reprogramming. Cancer Cell. 2013;23(6):768-783.
6. Hur W, Rhim H, Jung CK, et al. SOX4 overexpression regulates the p53-mediated apoptosis in hepatocellular carcinoma: clinical implication and functional analysis in vitro. Carcinogenesis. 2010;31(7):1298-1307.
7. Lombardi E, Almanza G, Kowal K, et al. miR-335-laden B cell-derived extracellular vesicles promote SOX4-dependent apoptosis in human multiple myeloma cells. J Pers Med. 2021;11(12):1240.
8. Dong M, Blobe GC. Role of transforming growth factor-β in hematologic malignancies. Blood. 2006;107(12):4589-4596.
9. Peng X, Liu G, Peng H, Chen A, Zha L, Wang Z. SOX4 contributes to TGF-β-induced epithelial–mesenchymal transition and stem cell characteristics of gastric cancer cells. Genes Dis. 2017;5(1):49-61.
10. Urashima M, Ogata A, Chauhan D, et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. Blood. 1996;87(5):1928-1938.
11. Lanier LL, Testi R, Bindl J, Phillips JH. Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J Exp Med. 1989;169(6):2233-2238.
12. Geng C, Zhou H, Wang H, et al. Newly diagnosed multiple myeloma patients with CD56 expression benefit more from autologous stem cell transplantation. BMC Cancer. 2022;22(1):1349.
13. Ely SA, Knowles DM. Expression of CD56/neural cell adhesion molecule correlates with the presence of lytic bone lesions in multiple myeloma and distinguishes myeloma from monoclonal gammopathy of undetermined significance and lymphomas with plasmacytoid differentiation. Am J Pathol. 2002;160(4):1293-1299.
14. Iqbal MS, Otsuyama K, Shamsasenjan K, Asaoku H, Kawano MM. CD56 expression in human myeloma cells derived from the neurogenic gene expression: possible role of the SRY-HMG box gene, SOX4. Int J Hematol. 2010;91(2):267-275.
15. Göbel M, Eisele L, Möllmann M, et al. Progranulin is a novel independent predictor of disease progression and overall survival in chronic lymphocytic leukemia. PLoS One. 2013;8(8):e72107.