Leukemia Research Reports 14 (2020) 100215

Contents lists available at ScienceDirect

Leukemia Research Reports

journal homepage: www.elsevier.com/locate/lrr

Immune checkpoints bone marrow expression as the predictor of clinical outcome in myelodysplastic syndrome

Nikolai Tcvetkov*, Artem Gusak, Elena Morozova, Ivan Moiseev, Vadim Baykov, Maria Barabanshikova, Kirill Lepik, Evgenyi Bakin, Julia Vlasova, Anna Osipova, Ludmila Zubarovskaya, Boris Afanasyev

R.M. Gorbacheva Memorial Institute of Children Oncology, Hematology and Transplantation, Pavlov First Saint-Petersburg State Medical University, Saint-Petersburg, Russian Federation

ARTICLE INFO

Keywords:
Myelodysplastic syndrome
Immune checkpoints
Prognosis

ABSTRACT

Aims: In our single-center retrospective study we evaluated whether level of different checkpoint molecules in bone marrow biopsies at diagnosis affect the clinical course of patients with myelodysplastic syndrome (MDS).
Methods and results: A consecutive cohort of 55 MDS patients treated in our center from 2003 to 2018 with available bone marrow biopsies at time of diagnosis was studied. We used a technique able to detect the expression of the following antigens: PD-1, PD-L1, PD-L2, LAG-3, Gal-9, TIM-3, CD80. The association between expression level and 3-year overall and relapse-free survival and time-to-progression was analyzed. Intensive expression of TIM-3 was observed in 100% of cases. Also, in most cases, moderate Gal-9 expression was observed. With 3-year follow-up disease progression was seen in 72.9% of patients with high CD80 level and 52.1% of patients with low CD80 level ($p = 0.04$). PD-1, CTLA4 and TIM-3 ligands were co-expressed in the majority of patients. General checkpoint ligand expression level also was associated with increased 3-year incidence of progression: 67.2% of patients with high level of checkpoint ligands progressed, while in the group with low checkpoint ligand expression level progression was observed only in 33.3% of cases ($p = 0.059$). There was an association between the expression of checkpoint molecules CD80, PD-L2, TIM3, the number of bone marrow blasts and risk according to IPSS and IPSS-R scales.
Conclusions: Our preliminary study underlined heterogeneous immune checkpoint molecules expression in MDS and warrants further studies to define the role of this heterogeneity and develop optimal treatment approaches.

Introduction

Myelodysplastic syndrome (MDS) is heterogeneous group of hematological malignancies affecting different ages but mostly elderly people over 70 years [1]. Its heterogeneity implies wide range of clinical presentations at the moment of diagnosis: from mild to moderate cytopenia of one or more lineages to rapidly progressing condition with high tumor load and aggressive course. The most popular and effective risk stratification tools in MDS so far are prognostic scores IPSS [2], WPSS [3], IPSS-R [4], and some others. While in benign cases watchful waiting is favored, allogeneic hematopoietic stem cell transplantation is the mainstay of therapy for high risk population and the only curative option [5]. The existing prognostic tools are not comprehensive though with quiet limited predictive power. Especially, it is related to patients in the intermediate risk group along with patients having normal karyotype, comprising up to 50% of MDS population and considered more and more heterogeneous as new data emerge [6, 7]. Considering elderly patient population with high rate of comorbidities and donor search issues the percent of transplant eligible patients in high risk population is far from majority. For those who still need treatment and are not transplant candidates few options are now available. Two hypomethylating agents (HMAs, 5-azacytidine [8] and decitabine [9]) along with immunomodulating drug lenalidomide [10], used predominantly for MDS with 5q deletion subtype, are now approved. These drugs possess moderate activity with high rates of resistance and intolerance and dismal prognosis for non-responders [11, 12].

One of the promising groups of agents for MDS is checkpoint inhibitors, which has already revolutionized treatment landscape in other malignancies including melanoma, lung cancer, Hodgkin's disease and several others. The results of in vitro and animal studies indicate that early in MDS development inflammatory microenvironment is established in bone marrow [13] leading to pyroptosis of healthy

* Corresponding author.
E-mail address: nikolai.tcvetkov@yandex.ru (N. Tcvetkov).

https://doi.org/10.1016/j.lrr.2020.100215
Received 27 January 2020; Received in revised form 21 June 2020; Accepted 25 June 2020
Available online 28 June 2020

hematopoietic progenitors, activation of myeloid-derived suppressor cells and specific cytokine milieu, which results in overexpression of checkpoint molecules including PD-1/PD-L1 [14]. Nevertheless, the trials of Nivolumab as single agent in MDS did not provide encouraging results [15]. Other targets including CTLA-4 [16] and TIM-3 [17] are under investigation, but further studies are needed. So far few studies addressed the role of immune checkpoints expression in defining MDS clinical course though this information could be implemented to fine-tune the existing prognostic models and outline the population with potential indication for checkpoints-involving approaches.

In our study we carried out retrospective single-center analysis of MDS patients with different treatment course by correlating the expression level of different checkpoint molecules in bone marrow biopsies with clinical outcome.

Materials and methods

The study was approved by local ethics committee. The bone marrow biopsies were obtained at time of diagnosis before the start of any treatment with the patient's consent that it can be used for scientific purposes. We included consecutive adult patients with confirmed MDS diagnosis admitted to our center in the period from 2003 to 2018 for whom trephine biopsy specimens at diagnosis could be retrieved. Information on 55 adult MDS patients was collected. Male-to-female ratio was 29:26, median age was 51 years. Most of patients were MDS with excess of blasts-I and -II – 15 and 25, respectively. Twenty three patients undergone allogeneic bone marrow transplantation, the others received conservative treatment. Twenty seven patients were high or very high risk according to IPSS-R score. Median follow-up period was 900 days. Basic characteristics of study group are summarized in Table 1.

On histological material of the lymph nodes of patients with Hodgkin's lymphoma and bone marrow of patients with MDS a technique was developed to detect the expression of the following antigens: PD-1 (ab52587), PD-L1 (ab205921), PD-L2 (ab200377), LAG-3 (ab40465), Gal-9 (ab69630), TIM-3 (ab185703), CD80 (ab64116). We used monoclonal antibodies produced by Abcam (1 Kendall Square, Suite B2304 Cambridge, MA 02139-1517 USA) and BOND-III Fully Automated IHC and ISH Stainer produced by Leica Biosystems (1700, Leider Lane, Buffalo Grove, IL 60089 USA). In the process of testing the technique, various modes of antigen unmasking, use of a peroxidase block, antibody concentration, duration of incubation with antibodies, incubation with a chromogen, and hematoxylin staining were studied. As a result of the tests, the modes were selected that allowed obtaining the most satisfactory staining. Expression was evaluated semiquantitatively by a 4-point scale: 0.5 points - single cells in separate fields; 1 point - single cells in each field; 1.5 points - moderate number of positive cells in some fields; 2 points - moderate number of positive cells, diffuse distribution; 2.5 points - the number of positive cells is more than 50% in some fields; 3 points - the number of positive cells is more than 50%, cells form clusters of 5 or more; 4 points - antigen expression on more than 90% of cells. The reaction was considered positive if membrane staining was observed. Membrane-cytoplasmic staining was allowed. Nuclear staining was considered non-specific. Staining results were compared with the number and localization of CD3+ and CD34+ cells (T-cells and progenitor cells, respectively). Detailed staining parameters and negative control image are available in Supporting information file.

Table 1
Patients' characteristics

Characteristic	Value
Patient median age, y (range)	51 (18-75)
Sex of patient, M/F	29/26
Disease type	
MDS with ring sideroblasts (MDS-RS)	1 (1.8%)
Hypoplastic MDS	1 (1.8%)
MDS with multilineage dysplasia	9 (16.4%)
MDS with isolated del(5q)	3 (5.5%)
MDS with excess blasts 1	15 (27.3%)
MDS with excess blasts 2	25 (45.4%)
Chronic myelomonocytic leukemia 2	1 (1.8%)
Risk profile according to IPSS-R score	
Very low	0 (0%)
Low	10 (18.2%)
Intermediate	18 (32.7%)
High	20 (36.4%)
Very high	7 (12.7%)
Number of transplanted patients	23 (41.8%)
Secondary MDS	3 (5.5%)
Type of received treatment	
Low dose Ara-C	11 (20%)
Hypomethylating agents	25 (45.4%)
Lenalidomide	1 (1.8%)
Cyclosporine A	7 (12.7%)
Deferasirox	8 (14.5%)
Erythropoiesis-stimulating agents	3 (5.5%)

The following clinical outcomes were analyzed: 3-year overall (the length of time from the date of diagnosis that patients diagnosed with the disease are still alive), relapse-free (the length of time after primary treatment ends that the patient survives without any signs or symptoms of the disease) survival and time-to-progression (the length of time from the date of diagnosis until the disease starts to get worse). We used IWG 2006 criteria [18] to define type of response, with progression defined either as transformation in acute leukemia for non-transplanted patients or any signs of disease recurrence for patients in posttransplant period. We performed univariate analysis using Chi-square test for categorical data and Kruskal-Wallis test to compare multiple groups. Logistic regression was performed to analyze the connection between categorical and continuous variables. Survival analysis was carried out by means of Kaplan-Meier product estimate method with log-rank test for univariate survival curves comparisons, while cumulative incidence functions with Gray test were built for time-to-progression analysis. For multivariate analysis we used Cox regression with Fine-Gray test. We looked for connection between checkpoint expression level and age, IPSS/WPSS/IPSS-R scores, blood and blast counts, transfusion dependency, and respective clinical outcomes. Statistics was computed using SAS 9.4 software (100, SAS Campus, Drive Cary, NC 27513-2414 USA), p-value of less than 0.05 was considered statistically significant.

Results

In the studied biopsy specimens expression of PD-L1 and LAG-3 was not observed in any of the cases, while in the control samples (available in Supporting information file) membrane staining was obtained on part of the cells. In a number of observations, accumulations of CD3+PD-1+ cells were determined in the bone marrow (available in Supporting information file); in individual samples, a small number of scattered PD-1+ cells was determined. There was no correlation between PD-1+ cell counts and CD34+ cell counts.

Intensive expression of TIM-3 was observed in all cases. Generally TIM-3 was determined on cells of the myeloid and erythroid lineages (Figure 1A, 1B). Also, in many cases, moderate Gal-9 expression was observed on large cells (Figure 1C, 1D). When comparing parallel sections with TIM-3 and Gal-9 staining, the coexpression of these markers on hematopoietic cells cannot be excluded. In clusters of CD3+PD-1+ cells, only single cells were stained with antibodies to TIM-3.

The general checkpoint expression pattern in relation to the whole patient population is available in Supporting information file. 75% of patients had coexpression of PD-L2, TIM-3, and CD80, and 25% of patients coexpressed additionally either PD-1 or Gal-9 or both.

Three-year overall and relapse-free survival in the whole group were 53.4% and 19.7%, respectively. In univariate analysis we observed significant connection between CD80, PD-L2, PD-L1, Gal-9

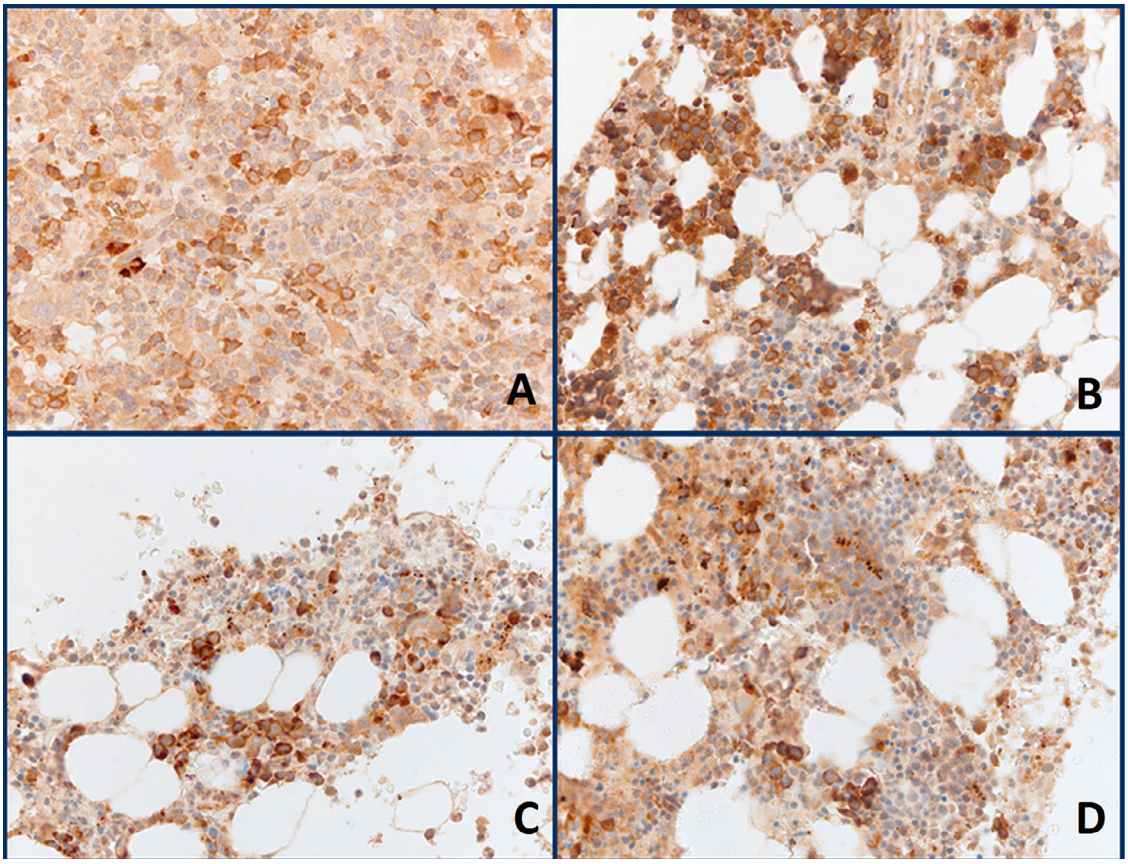

Fig. 1. A, B. Membrane expression of TIM-3 on cells of the myeloid and erythroid bone marrow. Immunohistochemical reaction, x400; C, D. Membrane expression of Gal-9 on bone marrow cells. Immunohistochemical reaction, x400

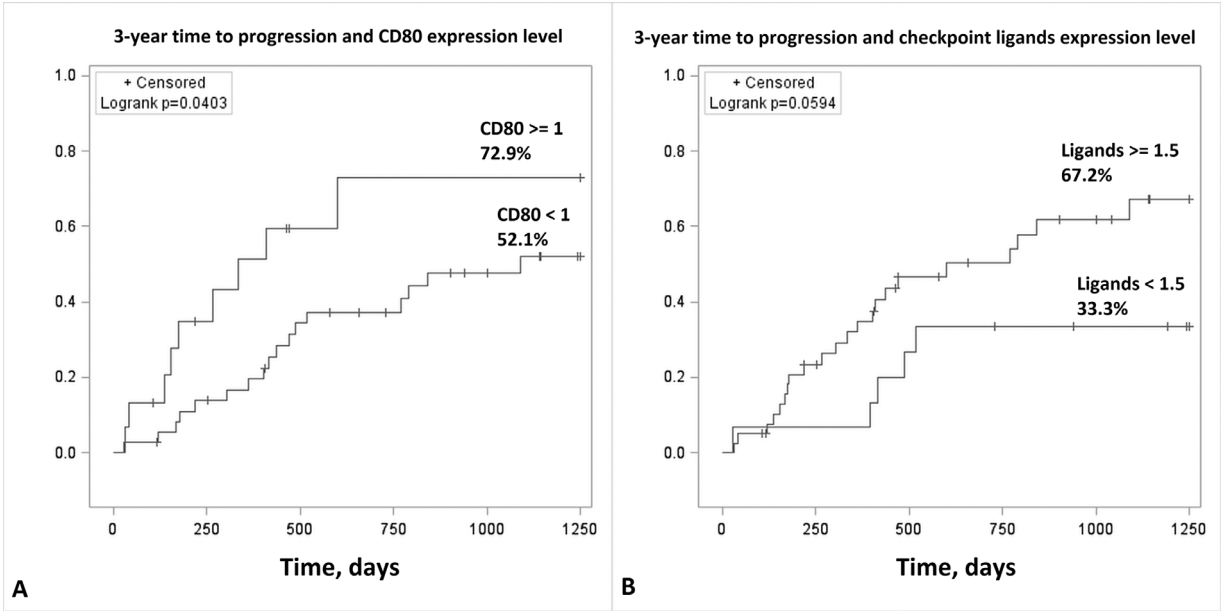

Fig. 2. A. 3-year time to progression according to CD80 expression level; B. 3-year time to progression according to checkpoint ligands expression level

expression levels and 3-year time-to-progression. At 3-year follow-up the incidence of disease progression in patients with high (more than 1 point) CD80 level was 72,9%, while patients with low (less than 1 point) CD80 level had disease progression incidence of 52,1% ($p = 0.04$, Figure 2A). Similar observation was made with overall checkpoint ligands (CD80, PD-L2, PD-L1, Gal-9) expression level – at 3-year follow-up patients with high (more than 1.5 point) checkpoint ligands expression level had disease progression incidence of 67,2%, while patients with low (less than 1.5 point) checkpoint ligands expression level had disease progression incidence of 33,3% ($p = 0.059$, Figure 2B).

In multivariate analysis we confirmed negative impact of both CD80 (HR 3.35, 95%CI 1.17-9.75, $p = 0.008$) and overall checkpoint ligands expression (HR 1.35, 95% CI 0.93-1.90, $p = 0.02$) level on 3-year time-to-progression. This negative impact was independent of IPSS-R score

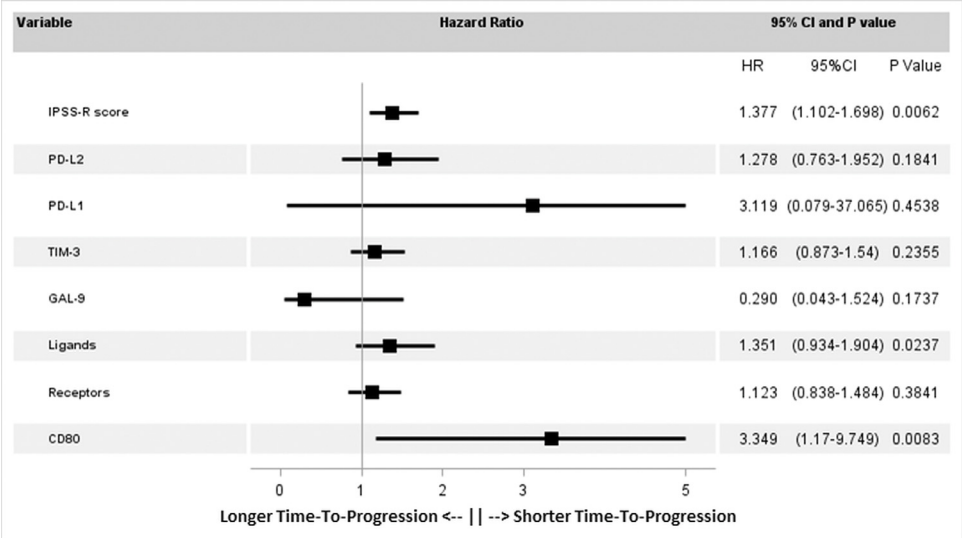

Fig. 3. Multivariate analysis of 3-year time-to-progression

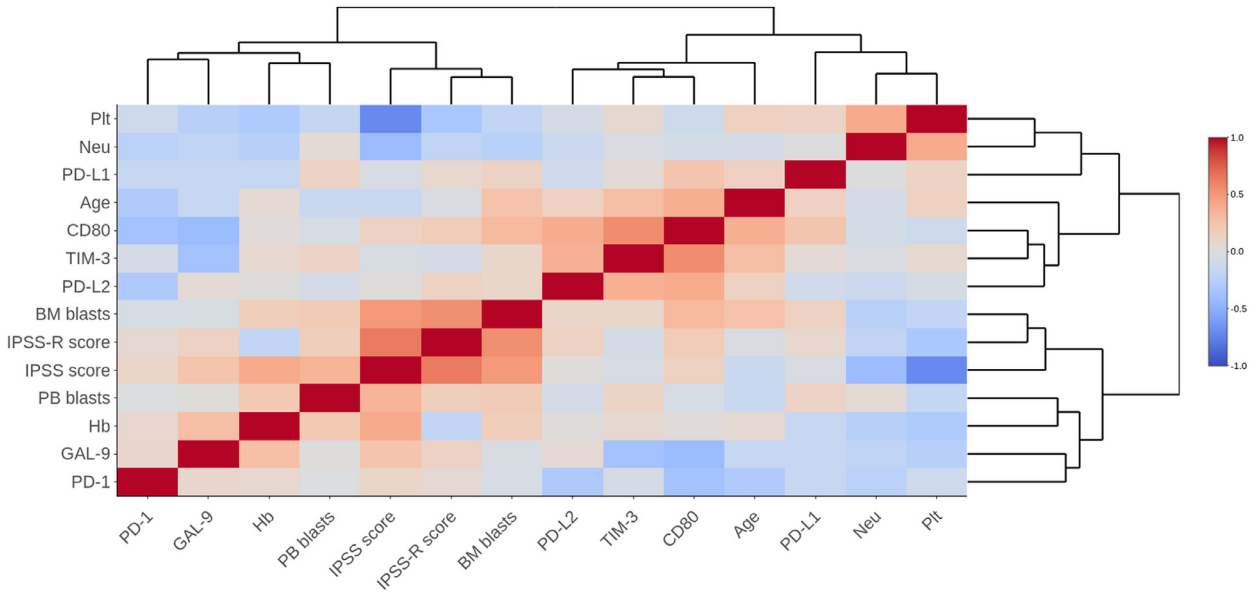

Fig. 4. Cluster analysis of associations between observed clinical parameters and checkpoint expression level (Plt – platelet count, Neu – neutrophil count, BM blasts – bone marrow blasts, PB blasts – peripheral blood blasts, Hb – hemoglobin)

(Figure 3). In cluster analysis we observed an association between the expression of checkpoint molecules CD80, PD-L2, TIM3, the number of bone marrow blasts and risk according to IPSS and IPSS-R scales (Figure 4).

Discussion

Many studies have described immune mechanisms in MDS development, especially, at early stages, and even association with autoimmune diseases is anecdotally reported [19]. In our study we once more confirmed this evidence demonstrating the expression of several checkpoint molecules in bone marrow of MDS patients. Considering the lack of PD-L1 and LAG-3 expression and absence of internal positive control in the bone marrow the study could be the subject to criticism. Messenger RNA level measurement is one of the solutions to confirm the results of IHC and should be addressed in further research.

Our study is a single-center experience so it is associated with higher risk of bias. Indeed, our clinical facility is specialized in performing allogeneic transplants and as a reference center we admit mostly patients who can be considered for this intensive treatment modality. It explains why our study group does not reflect the structure of general MDS patients' population, i.e. younger median age and higher percentage of high risk individuals. This potential pitfall can be further analyzed when more similar studies in different MDS populations will be carried out by other research groups.

Currently, there are two short reports on similar studies in MDS, one showing impact of PD-L1, PD-L2, PD-1 and CTLA-4 on response to HMAs and prognosis [20]. Compared to our results one of those studies showed different patterns of PD-L1 and PD-L2 expression [21] and neither assessed TIM-3/Gal-9 axis. In spite of observations regarding both PD-1/PD-L1 and CTLA-4 involvement in MDS pathogenesis, the clinical trial of nivolumab and ipilimumab demonstrated only modest results: overall response rate of 0% and 22% for nivolumab and ipilimumab after HMAs failure, respectively, underlining the probably more intricate biological patterns of these molecules participation in MDS pathobiology, but in this study no combinations of checkpoint inhibitors was evaluated [15]. Indeed, our results defined the wide range of molecules including CD80, TIM-3 and Gal-9 often coexpressed

simultaneously. These can lead to further attempts of combining different checkpoint inhibitors. Also further prediction of response in MDS might require the use of multiplex immunohistochemistry to define the exact cell types expressing various checkpoints.

In our study we observed an association between the expression of checkpoint molecules CD80, PD-L2, TIM3, the number of bone marrow blasts and risk score, and independent influence of CD80 and checkpoint ligands on time-to-progression. On the other hand, we didn't observe any considerable effect regarding overall and relapse-free survival. Probably, the checkpoint expression assay can leastwise identify the subgroup of patients at risk of earlier relapse and aggressive disease course with indication for closer survey and more intensive treatment modalities. We didn't perform subgroup analysis because of the small study group and we used quite restricted panel of molecules so future research has to investigate broader number of checkpoints in larger population as well as the methodology should be refined and standardized.

The valuable result of this work was the identification of high level of TIM-3 and Gal-9 expression on the bone marrow cells with the possible coexpression of these markers on granulocytic and erythroid cells. The literature describes the autocrine loop TIM-3/Gal-9, which may be important in maintenance of the MDS clone and the evolution of MDS into acute leukemia [22]. Now several clinical trials (NCT03066648, NCT03946670, NCT03940352) are in progress in attempt to introduce TIM-3 inhibitors into clinical practice. Considering our results combined therapeutic approaches may be the preferable ones.

In conclusion, our relatively small study outlined the potential clinical impact of different checkpoint molecules in MDS and managed to cast some light on the direction of further research.

Acknowledgments

This work was supported by Russian Science Foundation, grant № 17-75-20145.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.lrr.2020.100215.

References

[1] X. Ma, Epidemiology of Myelodysplastic Syndromes, Am J Med 125 (2012) S2–S5.
[2] P Greenberg, C Cox, MM LeBeau, et al., International Scoring System for Evaluating Prognosis in Myelodysplastic Syndromes, Blood 89 (1997) 2079–2088.
[3] EP Alessandrino, MG Della Porta, A Bacigalupo, et al., WHO classification and WPSS predict posttransplantation outcome in patients with myelodysplastic syndrome: a study from the Gruppo Italiano Trapianto di Midollo Osseo (GITMO), Blood 112 (2008) 895–902.
[4] PL Greenberg, H Tuechler, J Schanz, et al., Revised International Prognostic Scoring System for Myelodysplastic Syndromes, Blood 120 (2012) 2454–2465.
[5] T de Witte, D Bowen, M Robin, et al., Allogeneic hematopoietic stem cell transplantation for MDS and CMML: recommendations from an international expert panel, Blood 129 (2017) 1753–1762.
[6] G Montalban-Bravo, G Garcia-Manero, Myelodysplastic syndromes: 2018 update on diagnosis, risk-stratification and management, Am J Hematol 93 (2018) 129–147.
[7] R Bejar, KE Stevenson, B Caughey, et al., Somatic mutations predict poor outcome in patients with myelodysplastic syndrome after hematopoietic stem-cell transplantation, J Clin Oncol 32 (2014) 2691–2698.
[8] P Fenaux, GJ Mufti, E Hellstrom-Lindberg, et al., Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: a randomised, open-label, phase III study, Lancet Oncol 10 (2009) 223–232.
[9] H Kantarjian, J-PJ Issa, CS Rosenfeld, et al., Decitabine improves patient outcomes in myelodysplastic syndromes: results of a phase III randomized study, Cancer 106 (2006) 1794–1803.
[10] P Fenaux, A Giagounidis, D Selleslag, et al., A randomized phase 3 study of lenalidomide versus placebo in RBC transfusion-dependent patients with Low-/Intermediate-1-risk myelodysplastic syndromes with del5q, Blood 118 (2011) 3765–3776.
[11] VH Duong, K Lin, T Reljic, et al., Poor outcome of patients with myelodysplastic syndrome after azacitidine treatment failure, Clin Lymphoma Myeloma Leuk 13 (2013) 711–715.
[12] T Prebet, T Cluzeau, S Park, et al., Outcome of patients treated for myelodysplastic syndromes with 5q deletion after failure of lenalidomide therapy, Oncotarget 8 (2017) 81926–81935.
[13] DA Sallman, A. List, The central role of inflammatory signaling in the pathogenesis of myelodysplastic syndromes, Blood 133 (2019) 1039–1048.
[14] P Cheng, EA Eksioglu, X Chen, et al., S100A9-induced overexpression of PD-1/PD-L1 contributes to ineffective hematopoiesis in myelodysplastic syndromes, Leukemia 33 (2019) 2034–2046.
[15] G Garcia-Manero, NG Daver, G Montalban-Bravo, et al., A Phase II Study Evaluating the Combination of Nivolumab (Nivo) or Ipilimumab (Ipi) with Azacitidine in Pts with Previously Treated or Untreated Myelodysplastic Syndromes (MDS), Blood 128 (22) (2016) 344.
[16] Y Pico de Coaña, G Masucci, J Hansson, et al., Myeloid-derived suppressor cells and their role in CTLA-4 blockade therapy, Cancer Immunol Immunother 63 (2014) 977–983.
[17] T Asayama, H Tamura, M Ishibashi, et al., Functional expression of Tim-3 on blasts and clinical impact of its ligand galectin-9 in myelodysplastic syndromes, Oncotarget 8 (2017) 88904–88917.
[18] BD Cheson, PL Greenberg, JM Bennett, et al., Clinical application and proposal for modification of the International Working Group (IWG) response criteria in myelodysplasia, Blood 108 (2006) 419–425.
[19] RS Komrokji, A Kulasekararaj, NH Al Ali, et al., Autoimmune diseases and myelodysplastic syndromes, Am J Hematol 91 (2016) E280–E283.
[20] H Yang, CE Bueso-Ramos, CD DiNardo, et al., Expression Of Immune Checkpoints PD-L1, PD-L2, PD-1 and CTLA4 Predict For Prognosis and Resistance To Hypomethylating Agents (HMAs) In Myelodysplastic Syndromes (MDS), Blood 122 (2013) 2767–2767.
[21] M Dail, L Yang, C Green, et al., Distinct Patterns of PD-L1 and PD-L2 Expression By Tumor and Non-Tumor Cells in Patients with MM, MDS and AML, Blood 128 (2016) 1340–1340.
[22] Y Kikushige, T Miyamoto, J Yuda, et al., A TIM-3/Gal-9 Autocrine Stimulatory Loop Drives Self-Renewal of Human Myeloid Leukemia Stem Cells and Leukemic Progression, Cell Stem Cell 17 (2015) 341–352.