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# Clinical Relevance of Differential *RARα* and *PPARβ/δ* Expression in Myelodysplastic Syndromes

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**Abstract.** *Background/Aim: Myelodysplastic syndromes (MDS) are clinically heterogeneous hematological malignancies with an increased risk of transformation to acute myeloid leukemia, emphasizing the importance of identifying new diagnostic and prognostic markers. This study sought to investigate the predictive ability of all-trans retinoic acid (ATRA)-dependent nuclear transcription factors RARα and PPARβ/δ gene expression in MDS patients. Materials and Methods: Peripheral blood specimens were collected from 49 MDS patients and 15 healthy volunteers. The specimens were further separated in Ficoll density gradient to obtain the mononuclear cells fractions. Gene expression analysis was carried out using quantitative real-time polymerase chain reaction (qRT-PCR) technique. Results: In the mononuclear cell fractions of MDS patients, RARα expression was increased ($p<0.05$) and PPARβ/δ expression was decreased ($p<0.01$) compared to healthy volunteers. When RARα and PPARβ/δ expression was compared in groups of MDS patients with different risks of disease progression, no statistically significant difference was found for RARα expression, while PPARβ/δ expression was significantly lower in the high-risk group of patients compared to the low-risk group ($p<0.05$). The expression of RARα was significantly associated with overall survival ($p<0.05$). ROC analysis showed that the expression of PPARβ/δ, rather than RARα expression, could have potential diagnostic value for MDS patients (AUC=0.75, p=0.003 and AUC=0.65, p=0.081, respectively). Conclusion: RARα and PPARβ/δ genes are putative biomarkers that may be associated with the diagnosis and prognosis of MDS.*

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Myelodysplastic syndromes (MDS) constitute clonal blood disorders that arise from hematopoietic stem cell progenitors. They are characterized by progressive cytopenia, morphologic dysplasia in one or more bone marrow cell lineages, and ineffective hematopoiesis leading to an increased risk of leukemic transformation (1). MDS is a heterogeneous group of hematological neoplasms with a wide spectrum of presentations and implications. The course and outcomes of MDS can vary significantly, ranging from minimal clinical manifestations with a near-normal life expectancy to a rapid evolution into acute myeloid leukemia (AML) (2). Currently, MDS risk assessment is based on the International Prognostic Scoring System (IPSS) that stratified MDS from low- to high-risk (3). The IPSS mainly utilized a number of hematological parameters (severity of peripheral blood cytopenia, percentage of bone marrow blast cells, and presence of cytogenetic abnormalities) for determining MDS risk category and prognosis. This classification tool has limitations and in some cases, fail to capture reliable prognostic information at the individual patient level (4).

All-trans retinoic acid (ATRA), a bioactive metabolite of vitamin A (retinol), exhibits the ability to induce differentiation of myelodysplastic cells *in vitro* and apoptosis in primary MDS cultures (5). The attempts to use ATRA in clinical studies as a treatment for MDS patients were not very encouraging: the effect was only minimal and transient (6). However, it was shown that co-administration of ATRA and decitabine benefits patients with MDS and AML (7-9).

The biologic effects of ATRA are mediated through specific ligand-activated nuclear transcription factors known as retinoid acid receptors (RARs) and members of the superfamily of

nuclear hormone receptors. RARs mediate their biologic effects by forming heterodimers with retinoid X receptors (RXR) (RAR-RXR), binding to regulatory retinoic acid response elements (RAREs) present in the promoters of their specific target genes. There are several isoforms of RARs (NR1B) – RARα (NR1B1), RARβ (NR1B2), RARγ (NR1B3) and RXRs (NR2B) – RXRα (NR2B1), RXRβ (NR2B2), RXRγ (NR2B3). The activation of all RAR isoforms *in vivo* is specifically mediated by ATRA, whereas RXRs are activated by their own ligand, 9-cis RA. This molecule has demonstrated high-affinity RXRs binding *in vitro*, but was not detected in cells unless ATRA was present first or added (10-12).

RARs play a critical role in regulating adult hematopoiesis, particularly in myeloid differentiation. Knockout mice deficient in both RARα and RARγ display an *in vitro* block to granulocyte differentiation (13). The 15;17 chromosome translocation leading to the fusion between the *RARα* and promyelocytic leukemia (*PML*) genes is associated with human acute promyelocytic leukemia (APL). The resulting dominant negative PML-RARα fusion protein inhibits the function of normal RARs and blocks the terminal granulocytic differentiation that characterizes this type of leukemia (14). ATRA is effectively used in the treatment of APL. The pharmacological concentrations of ATRA target the PML/RARα fusion protein, inducing its proteolytic degradation within the silencing complex. This activation initiates the cell differentiation program, leading to growth arrest and apoptosis of APL cells (15).

The RXRs belong to the orphan family of the nuclear receptor superfamily as their natural ligands were initially unknown (12). RXRs can regulate transcription as homodimers or they can be recruited as obligatory partners for heterodimer formation by RARs, as well as by other nuclear receptors, particularly peroxisome proliferator-activated receptors (PPARs) (16, 17). PPARs form another ligand-activated transcription factor family within the nuclear receptor superfamily, playing a crucial role in regulating the metabolic homeostasis of the cell (18). The PPAR family includes PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). The transcriptional activity of PPAR is mediated by PPAR/RXR heterodimers. These transcription factors control genes responsible for the oxidation of lipids, with unsaturated fatty acids identified as PPAR ligands (19, 20). It has been shown that ATRA can also function as a specific ligand for the PPARβ/δ/RXR heterodimer (21).

This study evaluated the significance of the expression of two ATRA-dependent receptors, *RARα* and *PPARβ/δ*, for the development and survival of patients with MDS.

## Materials and Methods

*Study population*. This study included a cohort of 49 patients with primary MDS (29 females and 20 males) with a median age of 69.8 years (range=59-77 years). Patients were diagnosed between January 2011 and August 2018. The myelodysplastic syndrome was verified on the basis of cytological examination of peripheral blood cells. All patients were followed-up from the time of MDS diagnosis until May 2019, with consideration given to the transformation to AML and/or patient deaths during the follow-up period. Patient risk assessment was conducted according to the 2017 World Health Organization (WHO) classification (22) and IPSS (3). Additional patients' characteristics are presented in Table I. The patients' karyotype was classified using the International System for Human Cytogenetic Nomenclature (ISCN) (23). The control group consisted of 15 volunteers free of neoplasms or any other abnormalities, with a median age of 65.3 years (range=61-68 years). The clinical and hematologic variables in the control group were within the normal ranges.

Table I. *Clinical variables of 49 patients with myelodysplastic syndrome*.

| Variable | Value |
|---|---|
| Median age (range), years | 69.8 (59-77) |
| Sex, n | |
| Female | 29 |
| Male | 20 |
| WHO classification, n | |
| MDS-SLD | 7 |
| MDS-RS | 2 |
| MDS-MLD | 11 |
| MDS-EB (EB1+EB2) | 22 |
| MDS-del(5q) | 7 |
| IPSS classification, n | |
| Low | 13 |
| Intermediate-1 | 12 |
| Intermediate-2 | 9 |
| High | 15 |
| Karyotype*, n | |
| Good | 29 |
| Intermediate | 2 |
| Poor | 6 |
| n/d | 12 |
| Mean hemoglobin count±SEM, g/dl | 6.58±1.62 |
| Mean platelets count±SEM, $\times10^9$/l | 118.57±62.08 |
| Mean leukocyte count±SEM, $\times10^9$/l | 4.68±3.58 |
| Bone marrow blasts, n | |
| >5% | 24 |
| <5% | 25 |
| AML progression, n | 14 |
| Death, n | 25 |
| Survival time±SD, months | 24.65±23.03 |

WHO: World health organization; MDS-SLD: myelodysplastic syndrome with single-lineage dysplasia; MDS-RS myelodysplastic syndrome with ring sideroblasts; MDS-MLD: myelodysplastic syndrome with multilineage dysplasia; MDS-EB (EB1+EB2): myelodysplastic syndrome with excess blasts; MDS-del(5q): myelodysplastic syndrome with isolated 5q- deletion; IPSS: International Prognosis Scoring System; AML: acute myeloid leukemia. *Cytogenetic subgroups; Good: normal karyotype, isolated-Y, del(5q) or del(20q); Poor: ≥3 abnormalities or chromosome 7 anomalies; Intermediate: other abnormalities; n/d: not determined.

*Ethics approval*. The present study was approved (approval no. 6/2016) by the ethics commission of the A.S. Loginov Moscow Clinical Scientific Center (Moscow, Russia). All patients and volunteers provided written informed consent to participate in the present study.

*Inclusion and exclusion criteria*. The patients included were required to have one or more of the following criteria: *de novo* MDS female and male patients aged ≥18 years old or MDS patients that had previously received only supportive care (red blood cell and/or platelet transfusions for severe anemia and severe thrombocytopenia improvement, correspondingly). Patients treated with erythropoiesis-stimulating agents (in cases of chromosome 5q deletion) were also included in the study. Patients previously treated with hypomethylating agents and/or received immunosuppressive therapy were excluded from the study. The patients with the hypoplastic variant of MDS as well as patients that refused to participate or to provide informed consent in the study were not included.

*Mononuclear cells preparation and cDNA synthesis*. The peripheral blood specimens were separated in Ficoll density gradient (Paneko, Moscow, Russia) and the obtained mononuclear cells fraction was used for further analysis. Total RNA was isolated using TRI REAGENT (MRC, Cincinnati, OH, USA) according to the manufacturer's instructions. Reverse transcription PCR reactions were performed using 2 μg of total RNA, employing random 6 oligonucleotide primers (Syntol, Moscow, Russia) and RevertedAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

*Quantitative real-time polymerase chain reaction (qRT-PCR)*. The amplification was carried out in the Bio-Rad CFX (Bio-Rad, Hercules, CA, USA) instrument using Eva Green dye (BIOTIUM, Fremont, CA, USA) and qPCR Master Mix (Syntol, Moscow, Russia). PCR conditions for all genes were the following: initial heating of reaction mixture to 95˚C for 5 min, followed by 39 repeated cycles of – denaturation (95˚C for 20 s), annealing of primers (59˚C for 25 s), and elongation (72˚C for 20 s). Each sample was measured in triplicate. For normalization of expression data, the Ribosomal Protein L27 (*RPL27*) gene was used. The relative gene expression was determined according to the $2^{\Delta Ct}$ equation [ΔCt=Ct (*RPL27*) – Ct (test gene), where Ct – threshold cycle of gene in the exponential phase of amplification curve]. The following primer sequences were used: *RARα* 5'- AAGCCCGAGTGCTCTGAGA -3' (forward), 5'- TTCGTAGTGTATTTGCCCAGC -3' (reverse); *PPARβ/δ* 5'- GCCTCTATCGTCAACAAGGAC -3' (forward), 5'- GCAATGAATAGGGCCAGGTC -3' (reverse); *RPL27* 5'- ACCGCTA CCCCCGCAAAGTG-3' (forward), 5'-CCCGTCGGGCCTTGCG TTTA-3' (reverse).

*Statistical analyses*. All qRT-PCR experiments were performed in triplicate. The experimental data were analyzed using Graph Pad Prism software version 5.02 (GraphPad Software Inc., Boston, MA, USA). Data are expressed as the mean±SEM and compared using either two-tailed Student's *t*-test to determine significant *p*-values for comparison between two groups or one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post-hoc* test for multiple comparisons. Correlation was estimated using Pearson's rank test. Overall survival was analyzed using the Kaplan-Meier method with a log-rank test. Receiver operating characteristic (ROC) analysis was conducted to assess the diagnostic value of *RARα* and *PPARβ/δ* gene expression as candidate biomarkers. Area under ROC curve (AUC) was used to compare the discriminatory performance of putative markers to determine their utility as a novel diagnostic test. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. The difference between data was statistically significant at $p<0.05$ (*), $p<0.01$ (**).

## Results

*RARα* expression was studied by using qRT-PCR in the peripheral blood of 49 patients with MDS and in a control group of 15 healthy donors. The individual values of *RARα* expression in MDS patients varied considerably as compared to the individual values of *RARα* expression in healthy donors. The medium level of *RARα* expression was elevated in MDS patients and the difference between the mean levels of *RARα* expression between MDS patients and healthy donors was statistically significant ($p<0.05$) (Figure 1A).

The individual levels of *PPARβ/δ* expression in the control group of healthy donors showed more variability than *RARα* expression, and the individual levels of *PPARβ/δ* expression in MDS patients varied even more. The mean level of *PPARβ/δ* expression in MDS patients was lower than that in the control group, and this difference was statistically significant ($p<0.01$) (Figure 1B).

To analyze whether there were differences in *RARα* and *PPARβ/δ* expression among groups of MDS patients with different risk levels of disease development, the patients were stratified according to the IPSS system in low (13 patients), intermediate-1 (12 patients), intermediate-2 (9 patients) and high (15 patients) risk groups. The medium levels of *RARα* and *PPARβ/δ* expression in these groups were compared with those in the control group of healthy donors. Unfortunately, the *RARα* expression data variations in each group were too large and no statistical difference between the groups was found (Figure 1C). It is worth noting the elevation in *RARα* expression mean levels in the low-risk, intermediate-1, and intermediate-2 groups of MDS patients as compared to the control group, whereas *RARα* expression in the high-risk group of MDS patients showed a tendency to decrease.

Unlike *RARα* expression, a difference in *PPARβ/δ* expression was observed between the groups. The mean level of *PPARβ/δ* expression in the low group of MDS patients did not differ from that in the control group; however, *PPARβ/δ* expression was lower in the intermediate-1, intermediate-2, and high-risk groups of MDS patients compared to the control and low-risk groups of MDS patients. The difference was statistically significant between the control and high-risk groups, as well as between the low and high-risk groups ($p<0.01$ and $p<0.05$, respectively) (Figure 1D).

Surprisingly, despite the fact that *RARα* expression was significantly elevated and the *PPARβ/δ* expression, on the

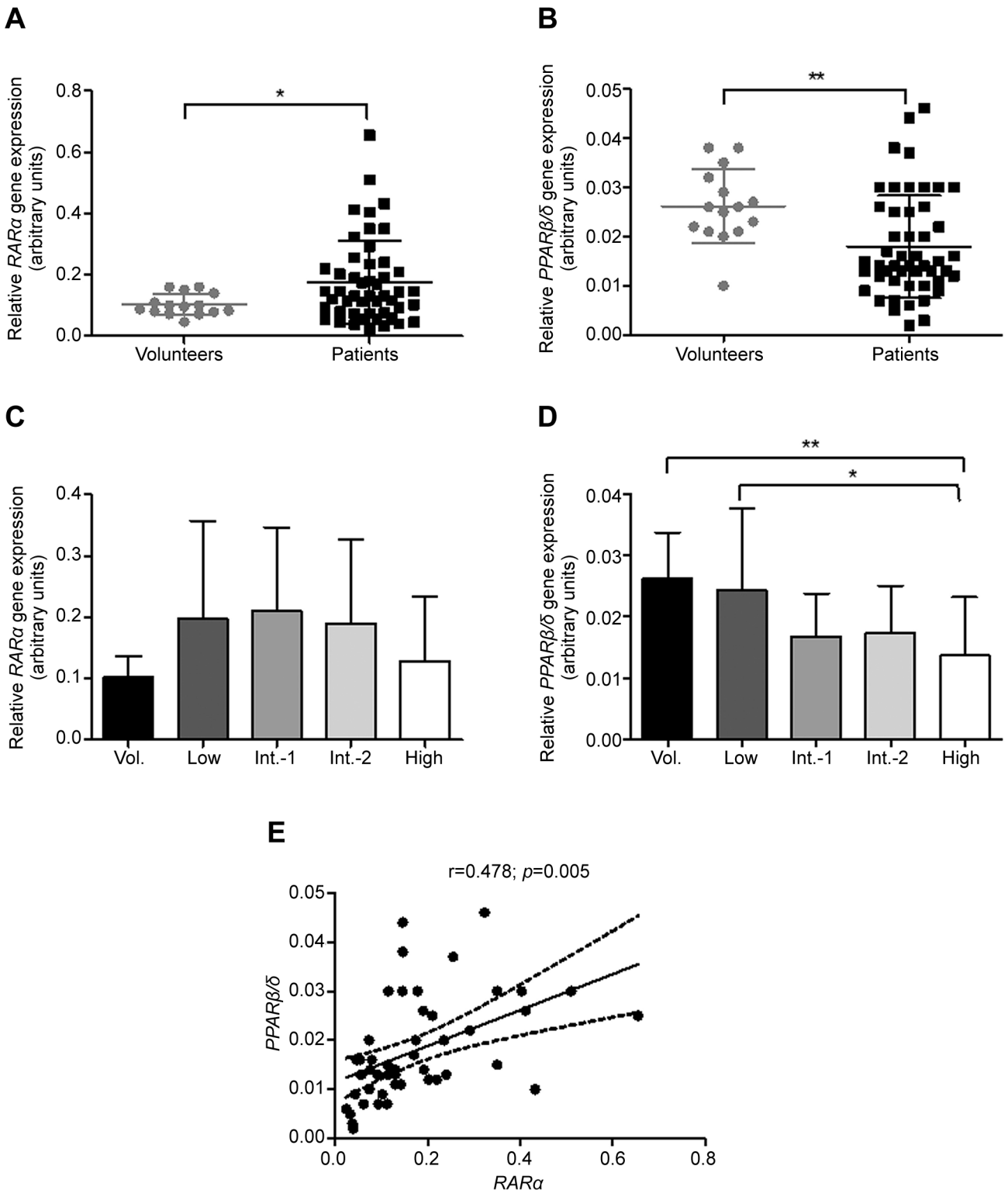


Figure 1. *Comparison of baseline RARα (A) and PPARβ/δ (B) expression levels in myelodysplastic syndrome (MDS) patients (n=49) and healthy volunteers (n=15) tested through qRT-PCR. Relative RARα (C) and PPARβ/δ (D) gene expression in groups of MDS patients with low, intermediate-1 (Int.1), intermediate-2 (Int.2) and high-risk of disease development according to IPSS. E) Pearson's correlation analysis of the association between RARα and PPARβ/δ gene expression in MDS patients. Data are presented as mean values±SD and were analyzed using an unpaired two-tailed Student's t-test; p<0.05 (*), p<0.01 (**).*

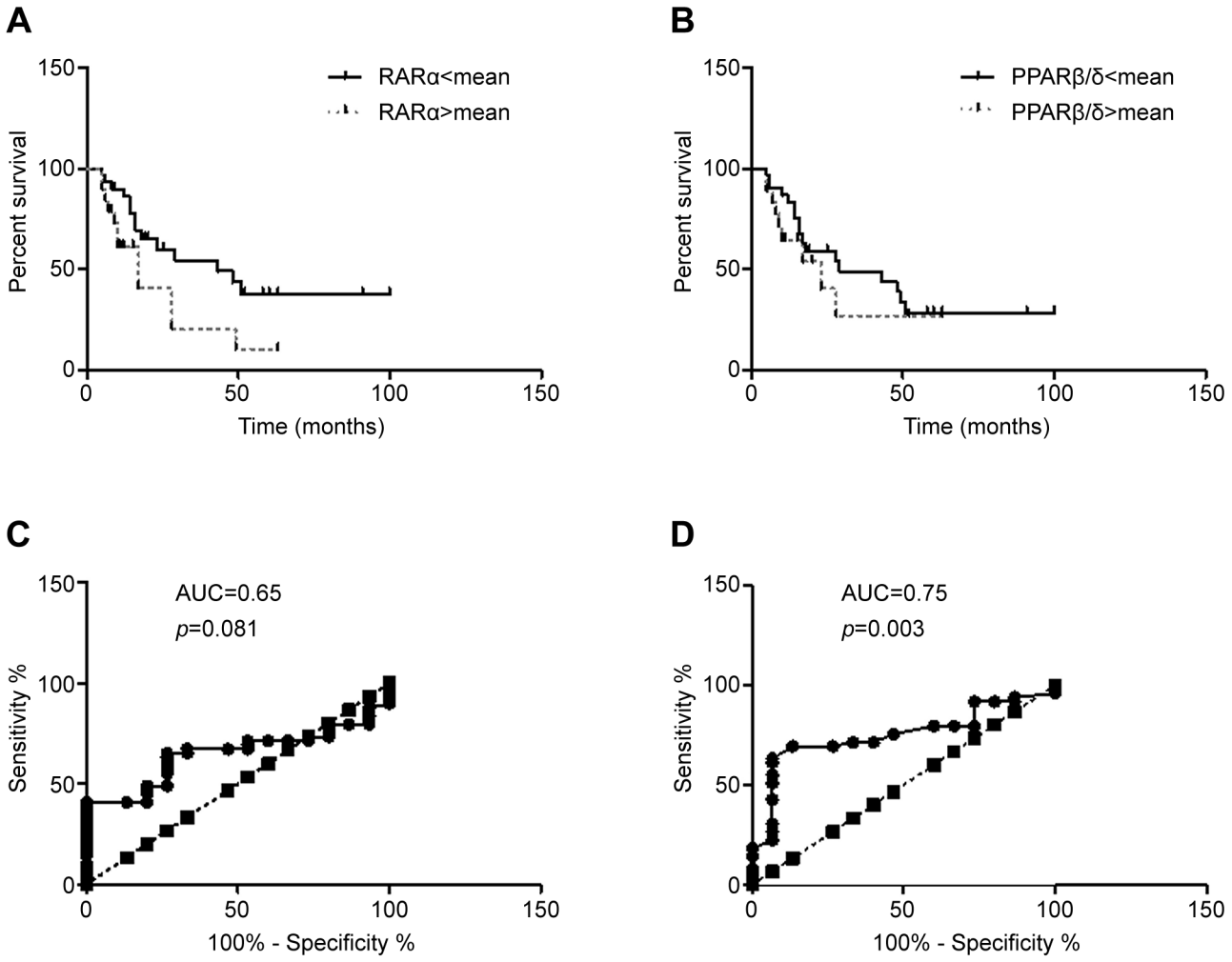


Figure 2. *Kaplan-Meier plots showing overall survival (OS) when the mean level of RARα (A) and PPARβ/δ (B) gene expression was used to dichotomize myelodysplastic syndrome (MDS) patients. Receiver operating characteristic (ROC) curves analysis of RARα (C) and PPARβ/δ (D) gene expression for validation as potential biomarkers of MDS. AUC: Area under the ROC curve.*

contrary, was significantly reduced in the group of MDS patients compared to the control group, we revealed a positive association between *RARα* and *PPARβ/δ* expression in MDS patients ($p$=0.005) (Figure 1E).

We also conducted a comparison of the survival rates between MDS patients with high and low levels of *RARα* and *PPARβ/δ* expression. The groups of MDS patients with expression levels higher and lower than the mean levels were compared.

Survival in the group of MDS patients with *RARα* expression lower than the mean level was significantly better than that in the group of patients with higher levels of *RARα* expression ($p$<0.05) (Figure 2A). The median survival was 43 months in the group with the low *RARα* expression levels and 17 months in the group with high levels of *RARα* expression.

The same tendency was found in the groups of patients differed according to *PPARβ/δ* expression (Figure 2B). The patients with *PPARβ/δ* expression lower than the mean level had better survival as compared to the group of MDS patients with high levels of *PPARβ/δ* expression, but the difference between these two groups was not statistically significant. The median survival in groups of MDS patients with low and high levels of *PPARβ/δ* expression was 29 months and 23 months, respectively.

The diagnostic value of putative biomarkers could be evaluated through ROC curve analysis. We applied ROC analysis to the same cohort of 49 MDS patients and 15 healthy donors. The AUC for *RARα* was 0.65 ($p$=0.081), indicating that *RARα* expression has no potential diagnostic value (Figure 2C). On the contrary, the data on *PPARβ/δ* expression suggest a potential diagnostic value (AUC=0.75,

$p$=0.003) in discriminating between MDS patients and healthy volunteers (Figure 2D).

## Discussion

The expression of RAR in cancer has gained attention due to its role in mediating the growth inhibitory activities of ATRA, a chemotherapeutic agent used in several cancers. Aberrant expression of RARα has been observed in various types of cancer. RARα over-expression has been identified in patients with hepatocellular carcinoma and laryngeal squamous cell carcinoma (24, 25). RARα was expressed and correlated with tumor grade in patients with the prostate carcinoma (26). The RARα isoform RARα2 expression was associated with disease progression of myeloma (27). Conversely, RARα expression in certain cancers can be considered a positive predictive marker. In pancreatic ductal adenocarcinoma (PDAC), the expression of RARα and RARβ as well as RXRα and RXRβ was down-regulated and the expression of RARα and RXRβ was associated with better overall survival of PDAC patients, whereas reduction of retinoids and their receptors was associated with worse patient survival outcomes (28). RARα expression also correlated with the expression of estrogen receptor α (ER) and predicted positive clinical outcomes in ER-positive breast cancer patients undergoing endocrine therapy (29). Additionally, ER-negative human breast carcinoma cell lines, RARα over-expression restored growth inhibition by ATRA (30).

In our study, *RARα* expression was elevated in the blood samples of patients with MDS as compared to healthy volunteers. Earlier, Bar *et al.* using oligonucleotide microarrays, found a statistically significant increase in the expression of genes involved in the RAR-RXR pathway in mononuclear cells from patients with advanced MDS disease as compared to patients with refractory anemia (31). When *RARα* expression in different risk groups of MDS patients was compared, no statistically significant difference in *RARα* expression between these groups was found. However, there was a tendency towards decreased *RARα* expression in the high-risk group of MDS patients. Reduced *RARα* expression seemed to be associated with better outcomes for MDS patients: those with lower *RARα* expression than the mean level had a more favorable survival compared to those with expression levels higher than the mean (43 months *vs.* 17 months, $p$<0.05). However, ROC analysis did not identify *RARα* expression as a predictive marker.

The potential role of PPAR signaling in MDS development has been suggested. Sankaran *et al.* showed that ineffective erythropoiesis, resembling human MDS, could be induced in mice by the deletion of retinoblastoma protein (pRb), a central regulator of the cell cycle (32). The study showed a significant decrease in PPARγ co-activator 1β (PGC1β) expression in pRbΔ/Δ mice. The authors suggested that the defect in the PGC pathway may be related to defective mitochondrial biogenesis and a block in differentiation.

Further investigation into the mechanisms downstream of pRb deficiency in pRbΔ/Δ mice was performed by Sen *et al.* (33). They showed that a phenotype strongly resembling refractory anemia associated with MDS, induced in mice by deleting pRb in hematopoietic stem cells (HSCs), could be rescued through enhanced PPAR signaling. *In vivo* over-expression of PPARγ co-activator 1β (PGC1β), or treatment with bezafibrate, a pan-PPAR agonist, normalized the blood parameters, suggesting that an enhanced PPAR signaling pathway rescued MDS-like anemia.

We found that in MDS patients, *PPARβ/δ* expression was lower than that in healthy volunteers, and a decrease in *PPARβ/δ* expression was observed in groups of patients with MDS progression. mRNA expression of this gene in the low-risk group was significantly higher than that in the high-risk group of patients ($p$<0.05). Our data regarding the decreased expression of one of the PPAR family genes, *PPARβ/δ*, in MDS patients compared to healthy volunteers align with the suggestion of the importance of PPAR signaling in MDS development.

Both RARα and PPARβ/δ are activated by ATRA. Shaw *et al.* have demonstrated that ATRA was a specific ligand for PPARβ/δ, as well as for RARs, whereas PPARα and PPARγ were not activated by retinoic acid (21). The authors concluded that while ATRA exerts growth inhibition through activating RAR, it may promote anti-apoptotic activities and cell growth *via* activation of PPARβ/δ.

The role of PPARβ/δ in tumorigenesis is controversial (34). It has been demonstrated that ligand activation of PPARβ/δ inhibits human breast cancer cell line tumorigenicity and cell proliferation in human keratinocytes (35, 36). In contrast, PPARβ/δ activation stimulated the proliferation of human breast and prostate cancer cell lines, increased the resistance of keratinocytes to apoptosis, and this activity was shown to be mediated by activation of the anti-apoptotic Akt1 signaling pathway (37, 38).

We analyzed the survival of MDS patients in groups differing in *PPARβ/δ* expression, but no statistical significant difference between the groups with high and low levels of expression was found. At the same time, according to the ROC analyses, *PPARβ/δ* expression may be considered as a predictive marker for MDS patients.

As mentioned earlier, both RARα and PPARβ/δ bind to and are activated by ATRA. Therefore, the resulting effect of ATRA may depend on the partitioning of exogenous retinoids between these different transcriptional signaling pathways. Studies have shown that the delivery of exogenous retinoids from the cytosol to either nuclear RARs or PPARβ/δ is highly dependent on the relative abundance of their critical intracellular ligand–binding proteins cellular retinoic acid–binding protein 2 (CRABP2) and fatty acid–binding protein

5 (FABP5) (39). Thus, the effects of ATRA could vary based on the cell content, producing opposite outcomes.

## Conclusion

Our results, demonstrating a decrease in *PPARβ/δ* expression in patients with MDS, in accordance with the existing data on the impact of PPAR-signaling on MDS development, as well as the ROC analysis of *PPARβ/δ* expression in patients with MDS and healthy volunteers performed in this study indicate that *PPARβ/δ* expression could serve as diagnostic and prognostic indicator for MDS diagnosis and development. Moreover, the statistically significant relationship between *RARα* gene expression and overall survival of MDS patients revealed in this study also suggests that the expression of *RARα* could be considered a putative prognostic biomarker in MDS.

## Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

## Authors' Contributions

Conceptualization: NK and AK. Methodology: NK, AK and GD. Investigation: NK, NKo, AS and AV. Formal analysis: NK and AK. Supervision: NK, AK and GD. Validation: NK and AK. Writing - original draft: NK and AK. Writing - review & editing: NK, AK and GD.

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