

Association Between Serum miR-221-3p and Intravenous Immunoglobulin Resistance in Children With Kawasaki Disease

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Abstract

Objectives

Intravenous immunoglobulin (IVIG) resistance was a major cause of coronary artery lesions in children with Kawasaki disease (KD). However, the cause of IVIG resistance in KD remains unknown. miR-221-3p has been confirmed involved in cardiovascular diseases and rheumatoid arthritis. The purpose of this study was to investigate the association between miR-221-3p and IVIG resistance in children with KD.

Methods

55 KD patients and 29 healthy controls (HCs) were enrolled in this study. KD patients were divided into group of sensitive to IVIG (IVIG-response, n=42) and group of resistant to IVIG (IVIG-resistance, n=13). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to detect the levels of miR-221-3p.

Results

Compared with the HCs group, miR-221-3p were significantly increased in the KD group ($p < 0.05$), and the IVIG-resistance group had higher levels of miR-221-3p than those in the IVIG-response group ($p < 0.05$). CRP (C-reactive protein), PCT (procalcitonin), NLR (neutrophil-lymphocyte ratio) were positively correlated with miR-221-3p in KD patients. In addition, the group of IVIG resistance had a higher level of Kobayashi Score ($p < 0.001$). The receiver operating characteristic (ROC) curve showed that miR-221-3p had a better value for diagnosis IVIG resistance in children with KD than Kobayashi Score and the combination of both with the AUC of 0.811 (95% CI, 0.672-0.951), 0.793 (95% CI, 0.618-0.968) and 0.797 (95% CI, 0.619-0.974), respectively.

Conclusions

miR-221-3p might be involved in the pathogenesis of KD and IVIG resistance and miR-221-3p can be used as a new potential biomarker to predict of IVIG resistance in children with KD.

What Is Known

- IVIG resistance in KD was mainly caused by immune imbalance and inflammation, and miR-221-3p is involved in cardiovascular diseases and immune disease.

What Is New

- In this study, we have found the relationship between miR-221-3p and IVIG resistance in KD, and serum miR-221-3p can be used as a potential diagnostic biomarker to predict IVIG resistance in KD.

1. Introduction

Kawasaki disease (KD) is an acute self-limiting febrile vasculitis that mainly affects children under five years old [1]. Since its first discovery in 1960s, KD has been identified worldwide and now become the leading cause of acquired heart disease in children in developed countries [2]. The most severe complication of KD is coronary artery lesions, which could ultimately lead to coronary heart disease (CHD) in young adults, resulting in myocardial ischemia, infarction, and even sudden death [3-5]. Although a single dose of 2g/kg intravenous immunoglobulin (IVIG) combined with aspirin regimen considered to be the standard treatment for KD, there is still approximately 20% of the cases are insensitive to IVIG, and around 10% of them develop into serious coronary artery abnormalities [6]. As a result, further research regarding the pathogenesis of IVIG resistance in children with KD is necessary.

miR-221, a member of antiangiogenic gene-regulating miRNAs family, is encoded by a co-transcribed cluster on the X chromosome and abundant distribution in endothelial and smooth muscle cells of human vessels [11]. Lots of studies have shown that microRNAs (miRNAs) are involved in pathogenesis of some diseases, such as cardiovascular diseases, inflammatory diseases, and immunologic diseases [7-10]. Pandis et al. found that the expression levels of miR-221 were significantly higher in synovial fibroblast (SF) in patients with rheumatoid arthritis (RA) [12]. Yang et al. demonstrated that downregulation of miR-221 can significantly inhibit the levels of TNF- α , IL-6, IL-1 β and CXCL16 in fibroblast-like synoviocytes (FLS) cells in patients with RA [13]. Also, studies have found that miR-221 expression increases significantly in acute KD patients [14]. However, the relationship between miR-221 and IVIG resistance remains unclear. Thus, the purpose of this study is to investigate the role of miR-221-3p on IVIG resistance in children with KD.

2. Method

2.1 Study subjects

Fifty-five KD patients (KD group) consisting of 37 males and 18 females (average age 2.648 ± 1.832 years) and twenty-nine healthy controls (HCs) children consisting of 19 males and 10 females (average age 3.191 ± 1.075 years) were enrolled in this study from April to August of 2019 consecutively. All subjects met the criteria of American Heart Association (AHA) Scientific Statement. An IVIG non-responder was defined by recrudescence or persistent fever at least 36 hours after the initial infusion of IVIG [2] and Kobayashi system [15] was used to predict IVIG resistance. This study was approved by the institutional ethics board of Children's Hospital of Chongqing Medical University; and written informed consents were obtained from all subjects or guardians.

2.2 Sample Collection

The blood samples were collected from the KD patients on the day of admission before given IVIG and aspirin and immediately centrifuged at 4°C for 10 min at 3,000 revolutions/min to isolate the serum. The same procedures were conducted to the HCs, and all serum samples were stored at -80°C.

2.3 RNA extraction and Real-Time qPCR assay

RNA was extracted using a miRNeasy serum/plasma kit (Qiagen, Hilden, Germany, Cat. No: 217184) according to the manufacturer's instructions. The primer of miR-221-3p was quantitated by reverse transcription and Real-Time qPCR analysis using Qiagen-based RT-qPCR assay (Qiagen, Hilden, Germany, Cat. No: 339320). The cycling conditions were 95°C for 2 min followed by 40 cycles at 95°C for 10 sec and 56°C for 1 min. All reactions were performed in triplicate. The expression was quantified using the $2^{-\Delta\Delta Cq}$ values and hsa-miR-103a-3p served as a control for calibrate the expression levels of miR-221-3p.

2.4 Statistical analysis

Demographic and clinical characteristic of subjects were expressed as mean values and as total numbers according to the applied statistical methods. The distribution of these variables was evaluated with Shapiro-Wilk normality test. Normally distributed variables were compared using t-test. The differences levels of miR-221-3p in serum between KD patients and HCs were analyzed by $2^{-\Delta\Delta Cq}$ method. The correlation of miR-221-3p with other clinical characteristics was assessed with Pearson correlation. The diagnostic accuracy of miR-221-3p were measured by the receiver-operating characteristic (ROC) curves and the area under the ROC curve (AUC). Reported *p*-values are two-sided and a *p* value less 0.05 was considered the difference significantly. All analyses were performed by Statistical Package for Social Sciences for Windows 24.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 The Expression of miR-221-3p in KD Patients and Healthy Controls

Demographic features between KD group and HCs group were examined and there was no significant difference in age and gender between these two groups (*p* > 0.05). The qRT-PCR method was used to detect the expression of miR-221-3p in 55 KD patients and 19 healthy controls. The results showed that, the level of serum miR-221-3p were significantly higher in KD patients than that of in healthy controls (*p* = 0.002, Figures 1).

3.2 The Correlations between miR-221-3p and Clinical Characteristics in KD Patients

Pearson correlation analysis showed that serum miR-221-3p was positively correlated with clinical inflammatory factors in all KD patients, including percentage of neutrophils (N%), C-reactive protein (CRP), procalcitonin (PCT), and Neutrophil-Lymphocyte ratio (NLR) ($r = 0.494, p < 0.001; r = 0.545, p < 0.001; r = 0.399, p = 0.568; r = 0.436, p = 0.001$, respectively), and negative with percentage of lymphocyte (L%) ($r = -0.450, p = 0.001$) (Table 1).

Interestingly, serum miR-221-3p was significantly association with total dosage of IVIG and administration times of IVIG in KD group ($r = 0.501, p < 0.001; r = 0.558, p < 0.001$, respectively), while no relationship with the onset age, gender, and weight (all *p* > 0.05, Table 1).

3.4 Clinical Characteristics in IVIG resistance group and IVIG response group.

A total of 42 IVIG responders and 13 IVIG non-responders were enrolled in this study. IVIG non-responders were (n=13) more likely to have a longer duration of fever, a higher total dosage of IVIG ($p < 0.001$), higher levels of mean platelet volume (MPV), platelet distribution width (PDW), neutrophils, total bilirubin (TLB), alanine transaminase (ALT), aspartate aminotransferase (AST), CRP, PCT, NLR, prothrombin and lower levels of lymphocyte, platelet count (PLT), and albumin (ALB) than that of IVIG responders group, while age, gender, weight and time point of IVIG were not different (Table 2).

3.5 Expression of miR-221-3p and Score of Kobayashi System in IVIG resistance group and IVIG response group.

Statistical tests revealed that the level of serum miR-221-3p was significantly higher in group of resistance to IVIG than that of group of IVIG responses (1.782 ± 0.7 vs 1 ± 0.552 , $p = 0.002$) (Figure 2). There was also a higher Kobayashi scores in KD patients with IVIG resistance ($p < 0.001$) (Table 3).

3.5 The ROC Curves Analysis of serum miR-221-3p in predicting IVIG resistance in children with KD

Receiver operating characteristic (ROC) curve analysis was used to explore the usefulness of serum miR-221-3p, Kobayashi Score and combination of both to predict unresponsiveness to IVIG in children with KD. The results showed that serum miR-221-3p reflected obvious separation between group of IVIG resistance and group of IVIG response; the AUC was 0.811 (95% CI, 0.672-0.951, $p = 0.001$), 0.793 (95% CI, 0.618-0.968, $p = 0.002$) and 0.797 (95% CI, 0.619-0.974, $p = 0.001$), respectively (Figure 3).

4. Discussion

The characterization of KD is an inflammatory in all the medium-sized arteries, especially coronary artery lesions (CALs). Accumulating studies reported that 10% to 20% of KD patients' unresponsiveness to initial treatment with IVIG, whom had increased risk to develop of CALs [16, 17]. Early identification of IVIG resistance may be important to reduce CALs in children with KD. However, the pathogenesis of nonresponsiveness of IVIG in children with KD remains unknown.

Total number of 55 KD patients were enrolled in this study, out of which 13 of them (23.6%) were diagnosed with IVIG resistance according to the 2017 American Heart Association guidelines. Our observation showed that patients with IVIG nonresponsiveness have a longer duration of fever, a higher total dosage of IVIG and significantly higher levels of CRP, N%, PCT, NLR, ALT and AST than that of group of IVIG responders, and coincident with other reports [18-20]. The IVIG resistance group also showed lower levels of lymphocyte, PLT, albumin and sodium concentrations than that of IVIG responding group. Up to now, these laboratory data have been used as predictors of the IVIG non-responders [15, 21-24]. These results may collectively reflect the presence of severe vascular inflammation in children with KD and result in IVIG resistance.

Evidences have shown that the inflammatory cytokines and chemokines such as TNF- α , IL-1, IL-6, and ICAM-1, are excessively increased during the acute febrile phase, indicating immune activation may contribute to the pathogenesis of KD [25,26]. Recently, numerous studies have implicated that Toll-like receptors (TLRs) signaling pathway in inflammatory vascular pathological conditions may play an important role in development of KD [27-30]. Our previous study clarified that TLR4/NF- κ B signaling pathway was involved in the process of coronary artery lesions. These results may suggest that regulating expression of TLRs will protect children with KD from coronary artery lesions [31]. miR-221-3p, one of the miRNAs that is conserved among vertebrates, was detected in immunologic and inflammatory disease as well as cardiovascular disease. Evidence has shown that miR-221-3p could be a regulator of TLRs [13]. Regulation of miR-221-3p can affect the level of pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, in FLS cells when stimulated by LPS [32]. Up regulation of miR-221-3p in TLR4 can enhance the secretion of IL-6 and IL-8, and down regulation the level of anti-inflammatory factors of CXCL13 and IL-10 [33]. Consistent results were also observed that there was relationship between miR-221-3p and expression of IL-6, TNF- α and activation of NF- κ B and MAPKs in inflammatory lung disorder [34].

In our study, we found that serum miR-221-3p was elevated in children with acute phase of KD, which is consistent with previous study [14]. For the first time, we reported that serum miR-221-3p was extremely higher in group of IVIG non-responders than that of group of IVIG responders. The serum miR-221-3p levels have significantly correlated with many key inflammatory indexes, such as N%, CRP, PCT, NLR and even total dosage of IVIG and given times of IVIG in KD patients. These results elicited that miR-221-3p may exhibit an important role in initiating inflammatory reaction involved in pathogenesis of KD and IVIG resistance.

Interleukin-6 (IL-6) is one of the pro-inflammatory with pleiotropic activity. It is released by not only T cells and macrophages, but also vascular endothelial cells, fibroblasts, and many other cells in response to various stimulation including TLR ligands and proinflammatory cytokines such as TNF- α or IL-1 [35]. It also has correlation with the acute phase of KD and IVIG non-responsiveness [19]. SOCS3 is a member of the IL-6 signaling family, it directly interacts with Janus kinase (JAK) signal transducer and inhibits of transcription 3 (STAT3) pathway and regulates immune reaction, cell growth and differentiation. According to a recent study via bioinformatics analysis and dual luciferase reporter gene assays, SOCS3 was found to be the target gene of miR-221-3p in hepatocellular carcinoma (HCC), which demonstrated that regulating the miR-221-3p/SOCS3 axis may affect the JAK/STAT signaling pathway and ultimately alter HCC tumor growth [36, 37]. Furthermore, Chatterjee PK et al. [38] had reported that IL-6/STAT3 pathway may be an underlying mechanism of inflammation in vascular endothelial. Whether or not miR-221-3p/SOCS3 axis is involved in the development of KD and IVIG resistance need further study.

Kobayashi score (KS) has been used to predict CALs and IVIG resistance. The items of the KS scores included age in months, day of illness at initial treatment, N%, PLT, AST, sodium, and CRP to generate a scoring model, and had a specificity of 68% and a sensitivity of 86%. Here, we have shown that serum miR-221-3p had a better value than the Kobayashi system to predict IVIG non-responsiveness with the AUC of 0.811 by ROC analysis.

5. Conclusions

In conclusion, our study showed that KD patients with IVIG resistance had a higher level of miR-221-3p and a better value than Kobayashi score to predict IVIG resistance, miR-221-3p may be used as a proinflammatory factor and a potential biomarker of IVIG resistance in KD and provide a novel mechanism and a new therapeutic target for KD.

Declarations

Study limitations.

There are limitations in this study. Firstly, the sample size was relatively small and it was finished in a single center. Secondly, we did not collect serum samples from KD patients after being treated with IVIG in subacute and convalescent.

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Conflicts of interest

The authors reported no conflict of interest.

Data Availability

All datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability

Not applicable.

Authors' contributions

Fengchuan Jing has a substantial contribution to the conception and design of this study, acquisition of data, analysis of data and draft the article.

Haobo Weng contributes to acquisition of data, analysis of data and interpretation of data.

Qiongfei Pei contributes to interpretation of data.

Jing Zhang contributes to interpretation of data.

Ruixi Liu * has a substantial to design of the study and revise the article.

Qijian Yi * has a substantial contribution to revise the article critically for important intellectual content and final approval of the version to be published.

All authors approved the final manuscript as submitted.

Ethical approval

This study was approved by the institutional ethics board of Children's Hospital of Chongqing Medical University.

Consent to participate:

Informed consent was obtained from all subjects or guardians.

Consent for publication

All authors approved the final manuscript as submitted.

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Tables

Table 1. Correlations between serum miR-221-3p and clinical characteristics in all KD patients.

Variables	Serum Levels of miR-221-3p	
	r	p
Demographics		
Age	0.196	0.151
Weight	0.122	0.375
Time point of IVIG (day)	-0.224	0.122
Total dosage of IVIG (g)	0.501	<0.001***
Given times of IVIG	0.558	<0.001***
Blood test		
White blood cell ($10^9/L$)	0.133	0.334
Platelet count ($10^9/L$)	-0.242	0.075
Red blood cell count ($10^{12}/L$)	0.239	0.079
Hemoglobin (g/l)	-0.05	0.715
Percentage of neutrophil (%)	0.494	<0.001***
Percentage of lymphocyte (%)	-0.450	0.001***
Biochemical test		
Alanine transaminase (IU/L)	0.226	0.097
Aspartate aminotransferase (IU/L)	0.068	0.621
Albumin (g/L)	-0.237	0.082
Inflammatory factor		
C-reactive protein (mg/L)	0.545	<0.001***
Erythrocyte sedimentation rate (mm/L)	0.125	0.362
Procalcitonin (ng/ml)	0.568	<0.001***
Neutrophil/Lymphocyte ratio	0.47	0.001***
Platelet/Lymphocyte ratio	0.231	0.09
Coagulation Function		
Prothrombin (second)	0.193	0.174
Activated partial thromboplastin time (second)	0.111	0.436
Fibrinogen (g/L)	0.178	0.212

Thrombin time (second)	0.106	0.46
D-dimer (mg/L)	0.066	0.649

*** $p<0.005$

Table 2. Laboratory values between IVIG Response and IVIG Resistance.

Variables	IVIG Response (n = 42)	IVIG Resistance (n = 13)	p value
Demographics			
Age (y)	2.379±1.748	2.349±1.211	0.944
Gender (male/female)	27/15	10/3	0.51
Weight (kg)	12.719±5.284	12.192±2.803	0.643
Duration of fever (d)	6.55±1.468	8.46±2.847	0.002***
Usage of Intravenous Immunoglobulin			
Time point of IVIG (day)	6.3±1.493	5.69±2.213	0.929
Total dosage of IVIG (g)	25.357±7.1902	43.269±18.3821	p < 0.001***
Given times of IVIG	1±0	1.69±0.48	p < 0.001***
Blood test			
White blood cell (10 ⁹ /L)	14.054±4.894	17.707±8.732	0.06
Red blood cell count (10 ¹² /L)	4.271±1.111	4.342±1.37	0.833
Hemoglobin (g/l)	108.3±14.659	102.8±11.003	0.867
Platelet count (10 ⁹ /L)	411.02±129.758	304.77±128.486	0.017*
Mean platelet volume(fL)	10.129±1.202	11.177±1.044	0.006**
Platelet distribution width(fL)	11.567±2.248	13.792±2.282	0.006**
Neutrophil count (10 ⁹ /L)	0.626±0.141	0.799±0.136	0.001***
Lymphocyte count (10 ⁹ /L)	0.309±0.125	0.159±0.121	0.001***
Biochemical test			
Total bilirubin(umol/L)	5.117±2.574	16.138±18.873	p < 0.001***
Alanine transaminase(IU/L)	29.095±31.393	92.754±107.538	0.001***
Aspartate aminotransferase (IU/L)	29.181±10.245	69.331±107.585	0.018*
Albumin (g/L)	37.855±4.186	33.231± 4.059	0.002***
Sodium	137.393 ± 2.609	134.6 ± 2.9439	0.002***
Inflammatory factor			
C-reactive protein (mg/L)	52.44±33.023	101.46±62.89	0.001***

Erythrocyte sedimentation rate (mm/L)	75.62±24.528	81.38±32.577	0.564
Procalcitonin (ng/ml)	0.644±0.81	5.683±5.197	<i>p</i> <0.001***
Neutrophil/Lymphocyte ratio	3.7698±6.936	11.348±9.31	0.003***
Platelet/Lymphocyte ratio	179.563±338.748	270.5322±327.808	0.397
Coagulation Function			
Prothrombin (second)	12.335±0.917	13.182±0.929	0.016*
Activated partial thromboplastin time (second)	30.068±2.746	31.118±3.922	0.419
Fibrinogen (g/L)	6.01±1.35	6.584±1.167	0.179
Thrombin time (second)	15.075±0.74	14.945±0.906	0.67
D-dimer (mg/L)	2.13±3.853	4.364±3.835	0.107

p*<0.05, *p*<0.01, ****p*<0.005

Table 3. Kobayashi System in IVIG Response and IVIG Resistance.

Variables	IVIG Response	IVIG Resistance	<i>p</i> value
	(n = 42)	(n = 13)	
Serum sodium (mmol/L)	137.393±2.609	134.6±2.944	0.002***
Aspartate aminotransferase (IU/L)	29.181±10.244	69.331±107.585	0.018*
Percentage of neutrophil	0.626±0.141	0.799±0.16	0.001***
Time point of IVIG (day)	6.3±1.493	5.69±2.213	0.929
C-reactive protein (mg/L)	52.44±33.023	101.46±62.89	0.001***
Platelet count (10 ⁹ /L)	411.02±129.758	304.77±128.486	0.017*
Age (y)	2.379±1.748	2.349±1.211	0.944
Kobayashi Score	0.79±1.048	3.54±2.696	<i>p</i> < 0.001***

p*<0.05, **p*<0.005

Figures

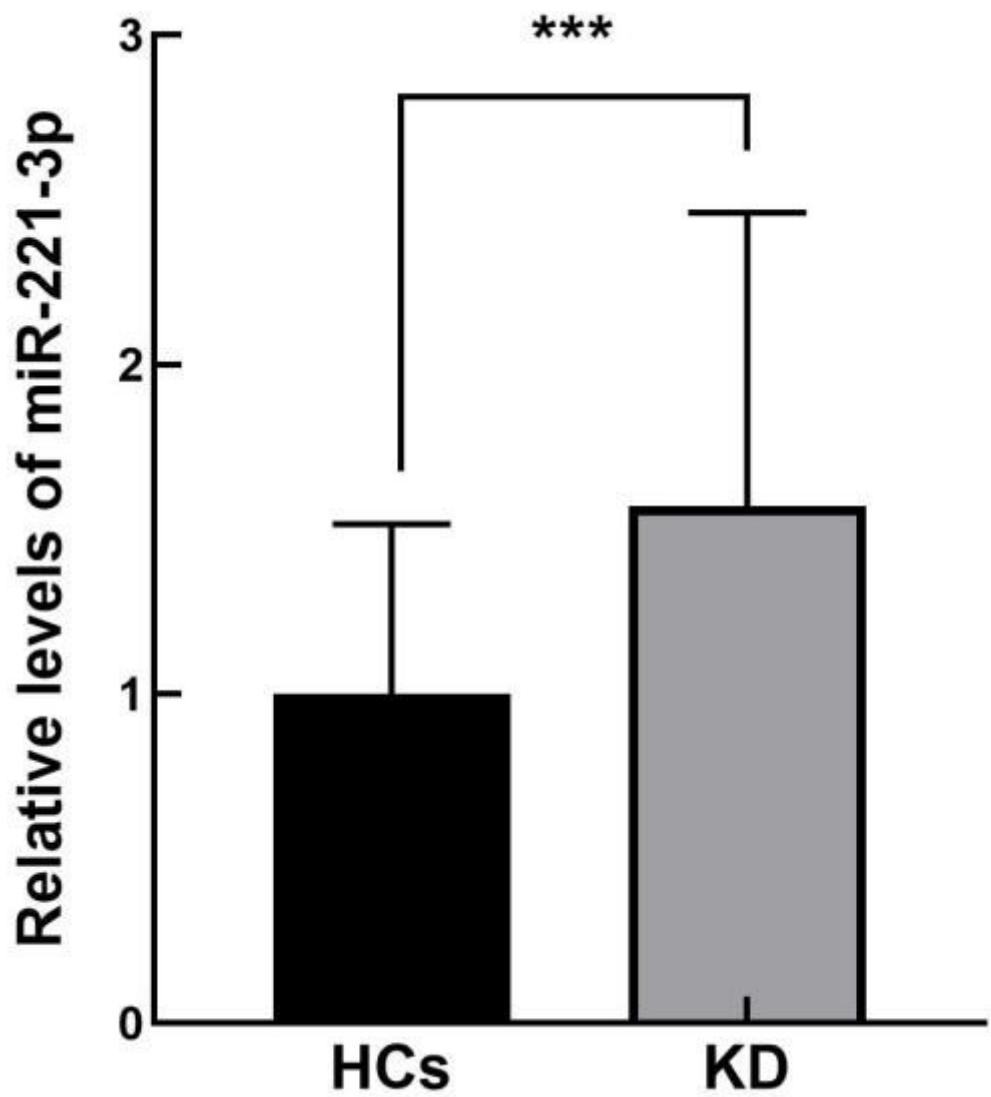


Figure 1

Serum levels of miR-221-3p in Kawasaki disease (KD) and healthy controls (HCs). ***p<0.005.

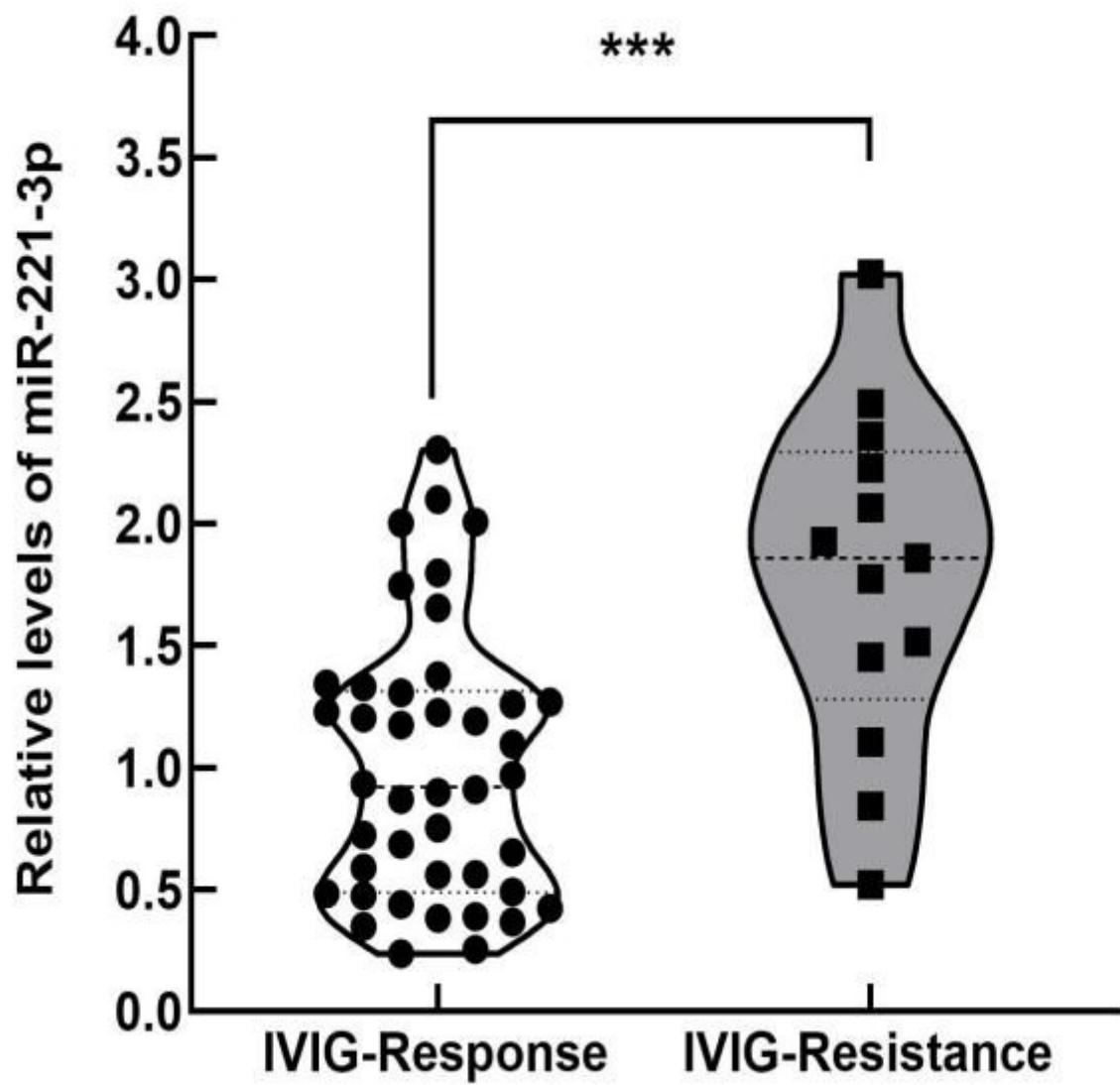


Figure 2

Serum levels of miR-221-3p in KD patients with IVIG Response and IVIG Resistance. ***p<0.005.

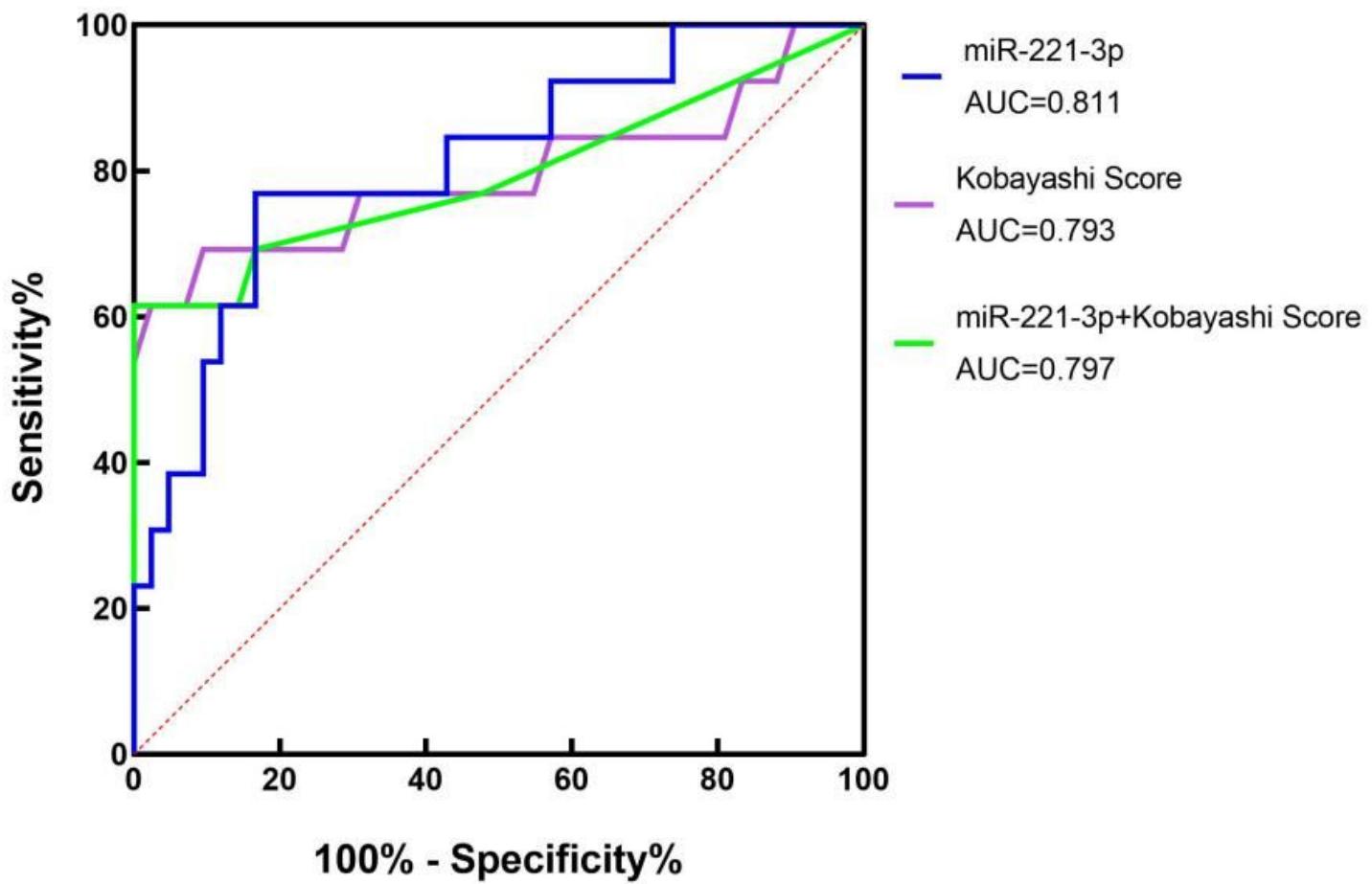


Figure 3

ROCs of various scores used to diagnosis KD patients with intravenous immunoglobulin (IVIG) resistance. Area under the curves (AUC): miR-221-3p (blue)= 0.811 (95% CI, 0.672-0.951, p =0.001), Kobayashi Score (purple) = 0.793 (95% CI, 0.618-0.968, p =0.002) and combination of these two (green) parameters = 0.797 (95% CI, 0.619-0.974, p =0.001).