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ORIGINAL PAPER

miR-196a-2 rs11614913 polymorphism is associated with vitiligo by affecting heterodimeric molecular complexes of Tyr and Tyrp1

T.-T. Cui[1] · X.-L. Yi[1] · W.-G. Zhang[1] · C. Wei[1] · F.-B. Zhou[1] · Z. Jian[1] · G. Wang[1] · T.-W. Gao[1] · C.-Y. Li[1] · K. Li[1]

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Abstract Tyrosinase and tyrosinase-related protein 1 (Tyr-Tyrp1) complex plays a critical role in the synthesis of melanin intermediates, which involves the production of reactive oxygen species (ROS) and contributes to the development of vitiligo. Based on our previous observation that rs11614913 single nucleotide polymorphism (SNP) in miR-196a-2 could affect the risk of vitiligo by influencing *Tyrp1*, we hypothesized that the same SNP could also regulate the level of *Tyr* in vitiligo. The aim of this study was to evaluate the potential association between rs11614913 SNP in miR-196a-2 and serum Tyr level in vitiligo and the regulatory role of miR-196a-2 in the expression of *Tyr* in melanocytes. The serum Tyr level was detected in 116 patients with vitiligo and 116 controls by ELISA plate assay. The expression level of Tyrp1 and Tyr in PIG1(normal melanocyte cell lines) cells was analyzed by western blotting. The ROS level and apoptosis rate in PIG1 cells transfected with si-*Tyr* or control siRNA were tested by flow cytometry. The results show that the individuals with TT+TC genotypes in miR-196a-2 and higher Tyr level in serum had an increased risk of vitiligo compared with those who had the CC genotype and lower Tyr level ($P < 0.001$). Furthermore, the rs11614913 C allele in miR-196a-2 enhanced its inhibitory regulation on the expression of *Tyr,* the down-regulation of which in melanocytes successfully reduced the intracellular ROS levels and the apoptosis rate. In conclusion, our findings suggest that miR-196a-2 polymorphisms can regulate the Tyr levels, which influences the susceptibility of vitiligo.

Keywords miR-196a-2 · Single nucleotide polymorphism · ROS · Tyrosinase · Tyrosinase-related protein 1 · Vitiligo

Introduction

Vitiligo is a common depigmentation skin disease that results from the destruction of cutaneous melanocytes. With a global incidence rate of approximately 0.1–2.0 % [1], the disease exerts a substantially negative impact on both the physiological and psychological health of patients worldwide. The pathogenesis of vitiligo is still far from completely clear, with various genetic and environmental factors considered to be involved in the development of the disease [11]. Many studies have implicated that oxidative stress is closely related to the loss of melanocytes in vitiligo epidermis [8, 16]. Moreover, recent evidence indicates that genetic variations of some genes that influence the level of reactive oxygen species (ROS) contribute to the susceptibility of vitiligo [3, 15].

Tyr and Tyrp1 act within the context of a series of reactions in the melanogenic pathway to control melanin production. Tyr and Tyrp1 are co-expressed to form multiple complex on the internal surface of melanosomes, and increase pigmentation. [22]. And the formation of Tyr-

T. T. Cui, X. L. Yi, W. G. Zhang have contributed equally to this work.

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✉ C.-Y. Li
lichying@fmmu.edu.cn

✉ K. Li
kaifmmu@fmmu.edu.cn

[1] Department of Dermatology, Xijing Hospital, Fourth Military Medical University, 127 Changlexi Road, Xi'an 710032, Shaanxi, China

Tyrp1 complex is critical for melanin production. Mutation in Tyrp1 could promote the degradation of Tyr-Tyrp1 complex and result in a decrease in melanin synthesis. Unfortunately, the synthesis of melanin intermediated by Tyr and Tyrp1 can bring a lot of cytotoxic-free radicals, which are major sources of endogenous ROS.

MicroRNAs (miRNAs) are a class of endogenous non-coding small RNAs that can regulate gene expression at the post-transcriptional level by binding to complementary sites in the 3′ untranslated region (UTR) of targeted messenger RNA (mRNA), thus impairing the translation of mRNAs or directly leading to the degradation of targeted mRNAs [20]. As common gene regulators, miRNAs have been demonstrated to participate in various physiological functions, and their abnormal expression is involved in the development of many diseases [2, 5, 18]. Moreover, some studies have shown that single nucleotide polymorphisms (SNPs) in miRNAs could alter their expression and target selection, thus playing a role in the pathogenesis of vitiligo [10, 12, 13, 19].

In our previous study, our in silico analysis found that the miR-196a-2 could potentially target genes such as platelet-derived growth factor receptor, alpha-polypeptide (PDGFRA), interleukin 2 (IL2), and mannose-binding lectin (protein C) 2 (MBL2) that are likely candidates of several oxidative stress-mediated ocular diseases. In addition, miR-196a-2 was also demonstrated to target Tyrp1, which plays an important role in melanin synthesis in melanocytes. And the rs11614913 T>C change in miR-196a-2 could down-regulate the cellular level of ROS and protect human melanocytes from apoptosis by suppressing the expression of tyrosinase-related protein 1 (Tyrp1) [6].

In melanocytes, Tyrp1 and Tyr interact with and stabilize each other by forming multi-enzyme complexes on the internal surface of the melanosomal membrane [9, 22]. As the initial and rate-limiting step of melanin production, Tyr has been reported to be more stable in the presence of Tyrp1 and degrades more quickly in mutant *Tyrp1* mouse melanocytes than in wild-type melanocytes [7, 9]. Since the miR-196a-2 T>C mutation can down-regulate the expression of Tyrp1, we hypothesized that the SNP may also lower the Tyr level by decreasing the formation of stable Tyrp1-Tyr complex, which can further protect the melanocytes from Tyr-induced ROS.

To test our hypothesis, we first performed a correlation analysis on the serum level of Tyr and the susceptibility of vitiligo. Furthermore, we evaluated the risk of vitiligo associated with the rs11614913 SNP in miR-196a-2 by Tyr levels. In addition, several in vitro experiments were also conducted to see whether the miR-196a-2 T>C mutation can down-regulate the cellular Tyr level and in turn prevent the melanocytes from ROS-induced apoptosis.

Materials and methods

Study subjects

The patients and healthy controls were selected as described in our previous research [6]. A total of 116 vitiligo patients and 116 gender- and age-matched healthy controls were recruited from Xijing Hospital, Fourth Military Medical University between 2011 and 2012. After signing informed consent forms, each subject donated 4 ml of blood, which was used for genomic DNA extraction and serum collection. The research protocol was designed and performed according to the principles of the Helsinki Declaration and was approved by the ethics review board of the Fourth Military Medical University.

Genotyping and ELISA assay

As shown in our previous study, the genotypes of the rs11614913 in miR-196a-2 were determined using PCR-restriction fragment length polymorphism (RFLP) analysis, after PCR genotyping, sequence analysis of the PCR products for the miR196a-2 gene was determined using an ABI PRISM 3700 (Carlsbad, CA, USA) automatic sequencer [6]. Serum was collected and stored at −80 °C within half a year before analysis. After thawing, the supernatants were applied to the ELISA plate assay according to protocol (Human Tyrosinase, TYR ELISA kit; Xmbio Corp., Shanghai, China). Quantitation was performed by external calibration using Tyr standards. Each sample was tested repeatedly in triplicate.

Cell culture

The immortalized normal human epidermal melanocyte cell line PIG1(a gift from Dr. Caroline Le Poole, Loyola University Chicago, Maywood, IL, USA) was cultured in Medium 254 (Cascade Biologics/Invitrogen, Portland, OR, USA) that included human melanocyte growth supplements (Cascade Biologics/Invitrogen), 5 % fetal bovine serum (Invitrogen, San Diego, CA, USA), and penicillin–streptomycin antibiotic mix (Invitrogen), in the presence of 5 % CO_2 at 37 °C.

Transient transfection

Transfections were performed with PIG1 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. All the transfections were carried out in triplicate.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The transfection efficiency was analyzed by qRT-PCR. The RNA was extracted using the RNeasy kit (Qiagen). Following quantitation, 2 ng of RNA was used to generate cDNA using random primers and Superscript III reverse transcriptase (Invitrogen). The cDNA was diluted 10-fold, and 1 μl used for the real-time reaction. qRT-PCR was performed using SYBR qRT-PCR Kit(TaKaRa) in a BIO-RAD Multicolor Real-time PCR Detection System (iQTM5). The primers were designed and synthesized by Tiangen company (Beijing, China). DNA was amplified for 32 cycles of denaturation for 30 s at 95 °C and annealing for 45 s at 56 °C for miR-196a-2, 30 s at 60 °C for Tyr and Tyrp1. The relative expression level of each mRNA was normalized by the amount of β-actin mRNA. The primers we used are listed here: miR-196a-2 (forward 5′-CCCC TTCCCTTCTCCTCCAGATA-3′ and reverse 5′-CGAAA ACCGACTGATGTAACTCCG-3′), Tyr (forward 5′-TTG GCAGATTGTCTGTAGCC-3′ and reverse 5′-GGCATTG TGCATGCTGCTT-3′), Tyrp1(forward 5′-CACAAAACC ACCTGGTTGAA-3′ and reverse 5′-CCAGCTTTGAAAA GTATGCC-3′), β-actin (forward 5′-AGAAAATCTGGC ACCACACC-3′ and reverse 5′-AGAGGCGTACAGGGA TAGCA-3′). All reactions were run in triplicates for at least three independent experiments. Relative quantification was performed according to the ΔΔCT method, and results were expressed in the linear form using the formula $2^{-\Delta\Delta CT}$. See details in Supplementary materials (Online Resource 1) for results of transfection efficiency.

Western blotting

After confirming the transfection efficiency, the protein expression of Tyr and Tyrp1 was detected by Western blotting. After transfection with miR-196a-2 expression plasmids (pcDNA3.1-miR-196a-2-T or C allele) or control plasmids (pcDNA3.1) for 48 h, the cells were first lysed with RIPA lysis buffer (Beyotime, Shanghai, China) containing a cocktail of protease inhibitors and phosphatase inhibitors for 30 min on ice and were then centrifuged at 12 000 g for 20 min. The total protein concentration was measured using BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of the protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Millipore, Bedford, MA, USA). The blot was blocked for 1 h and then incubated overnight with a primary antibody against Tyrp1, Tyr (Abcam, Cambridge, UK) or β-actin (Santa Cruz, CA, USA). After extensive rinsing, the blot was incubated with HRP-conjugated secondary antibodies (Zhongshan Biotechnology, Beijing, China) for 2 h at room temperature (RT). The immunoreactive bands were detected with an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

Melanin content assay

To determine the melanin content, PIG1 cells were plated in 6-well plates at a density of 4×10^5 cells per well overnight. After transfection for 48 h, cells were collected and washed with PBS twice, and then solubilized in 100 μl of the mixture consisting of 1 N NaOH and 10 % dimethylsulfoxide for 2 h at 37 °C. After cooling to the room temperature, the absorbance of the mixture was measured at 405 nm by spectrophotometer (Beckman Coulter) and compared with a standard curve using synthetic melanin (Sigma Chemical Co., St. Louis, MO). Each sample was analyzed in triplicates.

Tyrosinase activity assay

To determine the tyrosinase activity, PIG1 cells were plated in 96-well plates at a density of 8×10^3 cells per well overnight. After transfection for 48 h, cells were washed with PBS twice, then solubilized in 100 μl of 1 % Triton X-100 and frozen at −80 °C for 30 min immediately, followed by addition of 100 μl of 0.1 % L-DOPA solution and incubate at 37 °C for 2 h, The tyrosinase activity was quantified by spectrophotometer (Beckman Coulter) at 490 nm. The test was performed in triplicate.

Annexin V-FITC/propidium iodide apoptosis assay

To quantify apoptotic and necrotic death, PIG1 cells were plated in 6-well plates at a density of 4×10^5 cells per well overnight and were then transfected with si-Tyr or a normal control siRNA and incubated in 37 °C in 5 % CO_2 for 48 h or 72 h. The cells were then collected and re-suspended in 200 μl binding buffer, followed by the addition of 5 μl of FITC-labeled annexin V and 10 μl propidium iodide. After incubation in the dark (15 min at RT), the samples were diluted with 200 μl binding buffer. The cells were quantified by flow cytometry (Beckman Coulter, Miami, FL, USA) and analyzed by the Expo32 software (Beckman Coulter). Each sample was analyzed in triplicate.

Measurement of intracellular ROS

To determine the levels of ROS, PIG1 cells were plated in 6-well plates at a density of 4×10^5 cells per well overnight. At 48 h after siRNA transfection, the culture

medium was replaced by serum-free antibiotic-free 254, and incubated with 10 mM DCF-DA (Beyotime, Jiangsu, China) diluted in Medium 254 at 37 °C for 30 min. Subsequently, the cells were harvested and washed with PBS 3 times and analyzed with flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistical analysis

Unconditional univariate and multivariate logistic regression analyses were performed to obtain the crude and adjusted odds ratios (ORs) for the risk of vitiligo and their 95 % confidence intervals (CIs). Multivariate adjustments were made for age and gender. P values of <0.05 were considered statistically significant. All tests were two-sided for statistical significance and carried out using SAS software (version 9.1; SAS Institute, Cary, NC, USA).

Results

Characteristics of study subjects

The analysis included 116 vitiligo patients and 116 age- and sex-matched controls, all the subjects were examined and diagnosed by dermatologists. Table 1 shows the selected characteristics of the cases and controls, and the distribution frequency is in concordance with that in our previous study [6]. The mean age is 23.48 ± 1.19 years old for case and 22.23 ± 0.41 for controls ($P = 0.324$), while the proportion of men is 55.2 % for case and 56.0 % for controls ($P = 0.976$) (Table 1). The patients were considered to have a family history if they had one or more first- to third-degree relatives with this condition; the duration of more than 12 months is regarded as a long course. In total, 15 patients were with at least one other autoimmune disease (Grave's disease, autoimmune thyroiditis, chronic autoimmune hepatitis, connective tissue disease, diabetes mellitus, alopecia areata, and rheumatoid arthritis), the proportion is 12.9 %.

Association between serum Tyr levels and the susceptibility of vitiligo

We tested the serum Tyr levels of 116 vitiligo patients and 116 age- and gender-matched controls whose demographic and clinical characteristics as well as the genotype frequencies of rs11614913 were in concordance with those of the overall samples [6] (763 cases and 817 controls; data not shown). As shown in Fig. 1, we found significantly higher Tyr levels in the serum samples of 116 vitiligo patients than that of 116 healthy controls (95.48 ± 1.39 vs. 80.45 ± 1.43 pg/ml; $t = 7.52$, $P < 0.001$). For further analysis on the correlation between serum Tyr levels and the susceptibility of vitiligo, we divided serum Tyr levels into two or three categories according to the median or tertile serum Tyr level of the healthy controls. We found that the individuals with the upper median or tertile Tyr level (>80.69 ng/l or ≥89.16 ng/l) had a significantly increased risk of vitiligo (adjusted OR 4.76; 95 % CI 2.60–8.72 and OR, 6.14; 95 % CI 2.93–12.86, respectively, $P_{trend} < 0.001$) (Table 2).

Stratification analysis of the tyrosinase level and risk of vitiligo by selected variables

To explore the possible effects of the tyrosinase level in serum on different vitiligo features, we performed a stratified analysis of that and the selected variables of vitiligo, the Tyr level was divided into two categories(by median), and the concentration more than 80.69 ng/L is regarded as high level (Table 3). The high level of tyrosinase was significantly less common in subgroup of Age <20, ($P < 0.001$; adjusted OR 0.043; 95 % CI 0.016–0.113), without any remarkable difference in subgroup of Sex, Stage and Course ($P = 0.134$, $P = 0.720$, $P = 0.069$, respectively).

Risk of vitiligo associated with the rs11614913 SNP in miR-196a-2 by Tyr levels

To explore the possible effects of the rs11614913 SNP in miR-196a-2 on serum Tyr levels in vitiligo patients, we further estimated the risk of vitiligo associated with the rs11614913 SNP by Tyr levels. We divided the rs11614913 genotype in miR-196a-2 into two categories: the protective

Table 1 Clinical characteristics of the patients with vitiligo (cases) and controls

	Case, n (%) $n = 116$	Control, n (%) $n = 116$
Age (≥20/<20)	60 (51.7)/56 (48.3)	57 (49.1)/59 (50.9)
Sex (female/male)	52 (44.8)/64 (55.2)	51 (44.0)/65 (56.0)
Stage (active/stable)	96 (82.8)/20 (17.2)	–
Course (months) (≥12/<12)	72 (62.1)/44 (37.9)	–
Family history (yes/no)	22 (19.0)/94 (81.0)	–
Autoimmune diseases (yes/no)	15 (12.9)/101 (87.1)	–

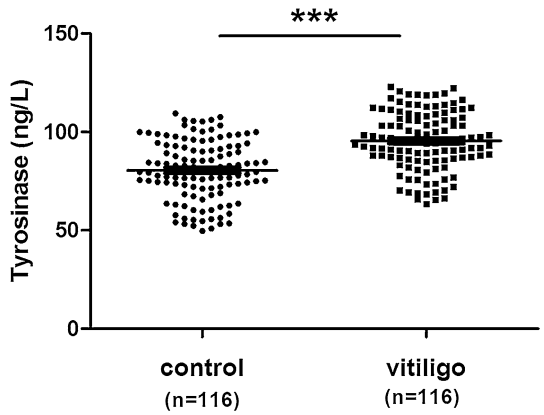

Fig. 1 Serum Tyr levels in vitiligo patients and normal controls. The serum Tyr levels in the group of patients with vitiligo ($n = 116$) were significantly higher compared with those in control group ($n = 116$). Values are mean ± SD. ***$P < 0.001$

CC genotype and the risk (TT+TC) genotype. When the individuals with CC genotype and a lower serum Tyr level (≤80.69 ng/l) were used as the reference, those with the TT or TC genotype and a higher level of serum Tyr (>80.69 ng/l) had a significantly increased risk of vitiligo (adjusted OR 4.15; 95 % CI 1.49–11.58, $P_b < 0.001$). Consistent with the preceding results, the individuals with the risk genotype and the upper tertile Tyr level (≥89.16 ng/l) showed an increase in the risk of vitiligo (adjusted OR 4.71; 95 % CI 1.34–16.58, $P_b < 0.001$) compared with those who had the protective genotype and the lower tertile Tyr level (≤74.61 ng/l) (Table 4).

miR-196a-2 C allele reduced the protein level of Tyr in PIG1 cells through inhibiting the expression of *Tyrp1*

To understand the molecular mechanisms underlying the observed risk association, we further investigated the potential association between miR-196a-2 and Tyr in vitro. The miR-196a-2 expression plasmids (rs11614913 T or C allele) were transfected into PIG1 cells, and the protein expression of Tyr and Tyrp1 was detected by western blot analysis. As shown in Fig. 2a, transfection with the miR-196a-2 expression plasmids led to a decreased expression of both Tyrp1 and Tyr compared with the control. Moreover, PIG1 cells transfected by the miR-196a-2 expression plasmids carrying the T allele (the pCDNA3.1-miR-196a-2-T) showed higher protein level of Tyr and Tyrp1 than that in the cells transfected with the plasmids carrying the C allele (the pCDNA3.1-miR-196a-2-C). Additionally, the cells transfected with si-*Tyrp1* also showed lower protein level of Tyr than normal control (Fig. 2b), which demonstrates that the previous observation of the decreased Tyr induced by miR-196a-2 is due to the down-regulation on its target gene *Tyrp1*.

miR-196a-2 C allele attenuate the melanin synthesis but did not influence the activity of tyrosinase

To clarify whether the miR-196a-2 SNP could influence the other function of melanin synthesis, the melanin content and the tyrosinase activity were tested. The miR-196a-2 expression plasmids (rs11614913 T or C allele) were transfected into PIG1 cells, after culturing for 48 h, cells were collected for analysis, as shown in Fig. 3a: the melanin content in cells transfected with plasmid carrying C allele (pcDNA-3.1-miR-196a-2-C) has an remarkable decrease compared with cells transfected with pcDNA-3.1-miR-196a-2-T or pcDNA3.1 ($P < 0.01$). These data indicate that miR-196a-2 T>C change could attenuate the melanin synthesis.

Next, to evaluate the effect of miR-196a-2 T>C change on the activity of tyrosinase, the tyrosinase activity of PIG1 cells transfected with different plasmids was also tested, as

Table 2 Logistic regression analysis of Tyr levels in vitiligo patients and controls

Tyr (ng/L)	Cases ($n = 116$)		Controls ($n = 116$)		OR[a] (95 % CI)
	N	%	*N*	%	
By median					
≤80.69	20	17.2	58	50.0	1.00 (reference)
>80.69	96	82.8	58	50.0	4.76 (2.60–8.72)
By tertile					
≤74.61	13	11.2	39	33.6	1.00 (reference)
74.61–89.16	25	21.6	39	33.6	1.94 (0.87–4.34)
≥89.16	78	67.2	38	32.8	6.14 (2.93–12.86)
Trend test	–	–	–	–	$P^b < 0.001$

95 % CI 95 % confidence interval

[a] Odds ratios (ORs) were obtained from a logistic regression model with adjustment for age and sex

[b] Two-sided Chi square test for distributions of genotype or allele frequencies between the cases and controls

Table 3 Stratified analysis of the tyrosinase level and types of vitiligo using select variables

	Tyr level (ng/L)					
	≤80.69	%	>80.69	%	*P*	Adjusted OR (95 % CI)[a]
Age					<0.001	
≥20	11	9.5	49	42.2		1.0
<20	47	40.5	9	7.8		0.043 (0.016–0.113)
Sex					0.134	1
Female	12	10.3	40	34.5		2.100 (0.786–5.610)
Male	8	6.9	56	48.3		
Stage					0.720	1
Active	16	13.8	80	69.0		0.800 (0.236–2.711)
Stable	4	3.4	16	13.8		
Course (months)					0.069	1
≥12	16	13.8	56	48.3		2.857 (0.888–9.193)
<12	4	3.4	40	34.5		

95 % CI 95 % confidence interval

[a] Odds ratios (ORs) were obtained from a logistic regression model with adjustment for age and sex

Table 4 Risk of vitiligo interacted with the rs11614913 SNP in miR-196a-2 by Tyr levels

Tyr (ng/L)	miR-196a-2 rs11614913 (case/control)			
	CC	OR (95 % CI)[a]	TT+TC	OR (95 % CI)[a]
By median				
≤80.69	6/14	1.00 (reference)	14/44	0.74 (0.24–2.30)
>80.69	17/14	2.82 (0.86–9.28)	79/44	4.15 (1.49–11.58)
By tertile				
≤74.61	4/9	1.00 (reference)	9/30	0.68 (0.17–2.73)
74.61–89.16	4/11	0.84 (0.16–4.36)	21/28	1.69 (0.46–6.27)
≥89.16	15/8	4.21 (0.98–18.11)	63/30	4.71 (1.34–16.58)
Trend test	–	–	–	$P^b < 0.001$

95 % CI 95 % confidence interval

[a] Odds ratios (ORs) were obtained from a logistic regression model with adjustment for age and sex

[b] Two-sided Chi square test for distributions of genotype or allele frequencies between the cases and controls

shown in Fig. 3b: there is no significant difference in tyrosinase activity among the three groups ($P > 0.05$). These results indicate that miR-196a-2 T>C change can decrease the synthesis of melanin through influencing the formation of TYR-TYRP1 complexes.

The down-regulation of Tyr could decrease the intracellular levels of reactive oxygen species and inhibit the apoptosis of PIG1 cells

Our previous study has demonstrated that the rs11614913 T>C in miR-196a-2 change decreases the levels of intracellular reactive oxygen species and inhibits apoptosis. To determine whether this regulatory effect depended on the expression of *Tyr*, PIG1 cells were transfected with si-*Tyr* or si-NC as negative control (transfection efficiency shown in Fig. S1b), and 2′, 7′-dichlorofluorescein diacetate (DCF-DA) and flow cytometry were used to examine the intracellular peroxide levels. As shown in Fig. 4a, b, the ROS levels in cells transfected by si-*Tyr* were significantly lower than control ($P < 0.05$), which indicates that the rs11614913 T>C change in miR-196a-2 could attenuate oxidative stress in cells possibly through its down-regulation on Tyr level.

Next, to testify whether the effect of rs11614913 T>C in miR-196a-2 change on cell apoptosis was associated with the intracellular Tyr level, PIG1 cells were transfected with the two kinds of siRNA as described above, and apoptosis assay was performed using flow cytometry. The results showed that at 48 h after transfection, the inhibition of Tyr expression significantly decreased the apoptosis rate ($P < 0.05$), and at 72 h, it is more remarkable for the

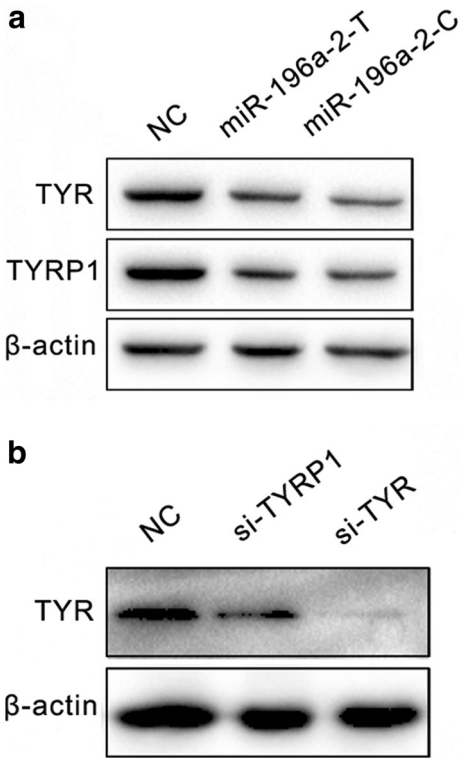

Fig. 2 miR-196a-2 reduced the protein level of Tyr in PIG1 cells by suppressing its target gene Tyrp1 **a** PIG1 cells were transfected with the plasmids of miR-196a-2-C or miR-196a-2-T. Western blot analysis showed that the rs11614913 miR-196a-2-C obviously decreased the Tyr protein expression in PIG1 cells, whereas the inhibitory effect of miR-196a-2-T is comparatively weaker. **b** PIG1 cells were transfected by si-*Tyr* or si-*Tyrp1*. Western blot analysis showed that not only si-*Tyr* but also si-*Tyrp1* can reduce the protein level of Tyr in PIG1 cells. β-actin was used as the internal control

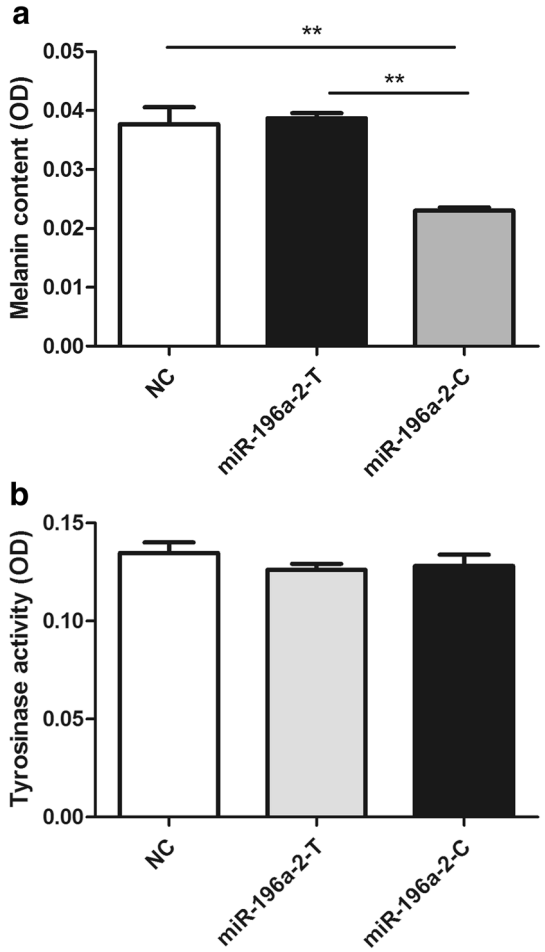

Fig. 3 miR-196a-2 C allele attenuate the melanin synthesis but did not influence the activity of tyrosinase. **a** Melanin contents were tested by spectrophotometer at 405 nm, the *bar graphs* represent the mean values for the OD data (*$P < 0.05$, **$P < 0.01$). **b** Tyrosinase activity was tested by spectrophotometer at 490 nm, the *bar graphs* represent the mean values for the OD data (*$P < 0.05$, **$P < 0.01$)

inhibitory effect of si-Tyr on apoptosis rate ($P < 0.01$) (Fig. 4c, d).

Discussion

In the present study, we discovered that the patients with vitiligo had significantly higher serum level of Tyr, and a dose–response relationship between increased vitiligo risk and elevated Tyr levels was proven. Furthermore, the genetic variation of rs11614913 T allele in miR-196a-2 was found to be closely related with the elevated serum levels of Tyr, leading to the increased risk of vitiligo. We then performed additional experiments to further understand the molecular mechanisms underlying the observed association. We found that miR-196a-2 cannot only inhibit the expression of *Tyrp1* as our previous study demonstrated, but also reduce the protein level of Tyr, and the low Tyr is associated with decreased ROS level and apoptosis rate. Furthermore, we also found that the miR-196a-2 C allele can attenuate the synthesis of melanin in melanocytes, and this result is consistent with the findings in a literature studying on the Tyr-Tyrp1 complex, and indirectly supporting our hypothesis.

In our previous report, we indicated that overexpression of miR-196a-2 with C genotype could remarkably decrease the ROS level and apoptosis rate, combined with our current results that decreased ROS and apoptosis rate were associated with low Tyr level which can be down-regulated by miR-196a-2, we may partly explain why the rs11614913 T>C in miR-196a-2 change decreases the levels of intracellular reactive oxygen species and inhibits apoptosis. To the best of our knowledge, this is the first report to demonstrate that the rs11614913SNP in miR-196a-2 may influence the expression of Tyr in human melanocytes and affect the risk of vitiligo.

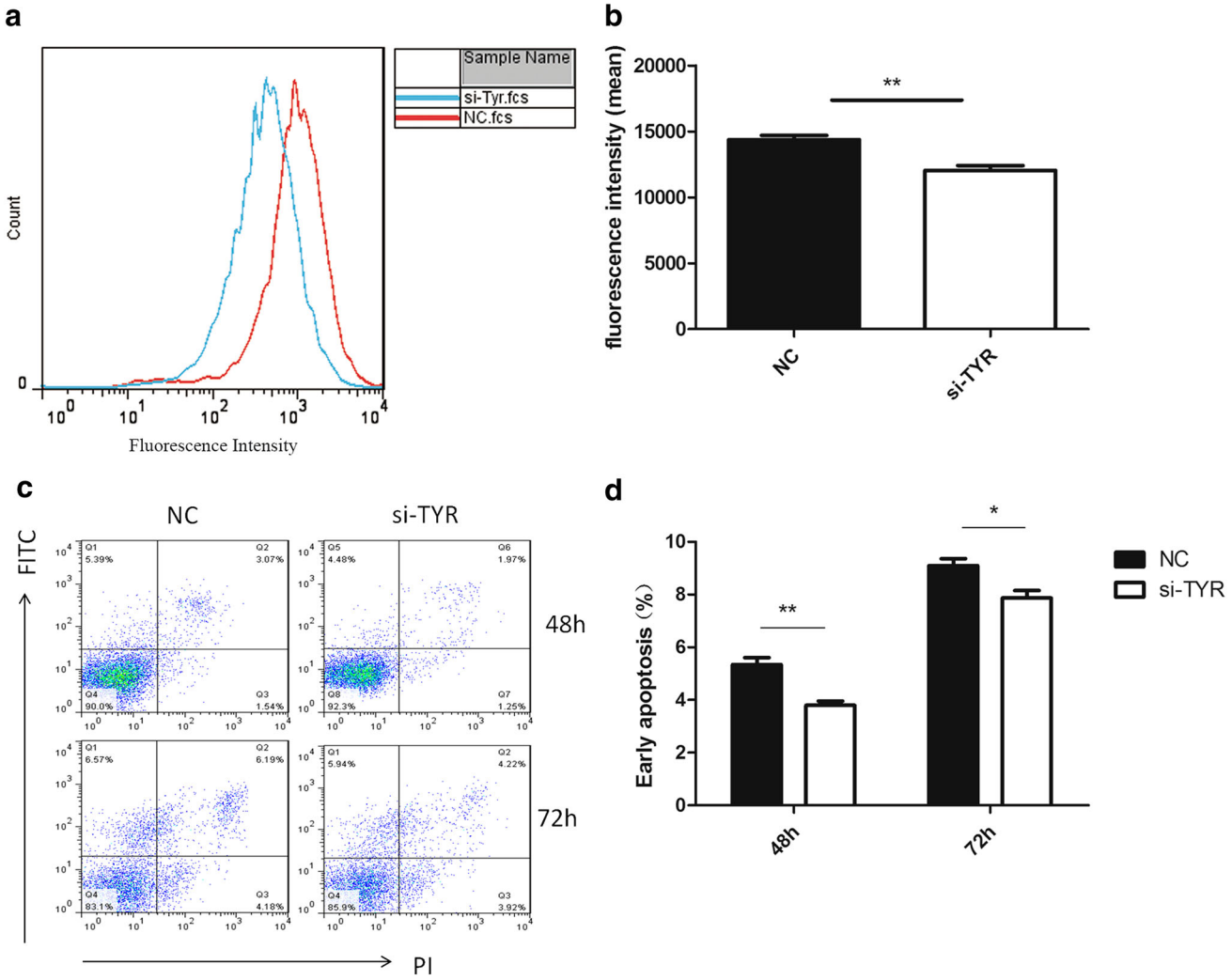

Fig. 4 The down-regulation of Tyr protein level could decrease the levels of intracellular reactive oxygen species and inhibits the apoptosis of melanocytes **a** ROS levels were monitored with flow cytometry (FCM) using DCF-DA. Representative results for ROS production after transfection with different plasmids. **b** The fluorescence intensity of the cells was calculated relative to that of normal control. **c** Cells were stained with annexin V and propidium iodide (PI) for 15 min and analyzed by FCM after cell transfection with different plasmids for 48 and 72 h respectively. Representative FCM graphs are shown. **d** The *bar graphs* represent the mean values for the FCM data ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$

Tyr and Tyrp1 are critical proteins that are involved in melanin biosynthesis [4]. Tyr catalyzes the conversion of tyrosine to dopa and subsequently to DOPA quinone, which is the initial and rate-limiting step of melanin production [9]. As for Tyrp1, it plays an intrinsic role in the formation and stabilization of the melanin-producing multi-enzyme complexes, thus preventing complexes from degradation [7]. Unfortunately, the synthesis of melanin intermediated by Tyr and Tyrp1 can bring about cytotoxic-free radicals [14, 17], which have been proved to promote the development of vitiligo. In the present study, we tested the serum level of Tyr, and found that the serum Tyr level in vitiligo patients was higher than that in healthy controls. Further stratification analysis showed that a significantly increased risk of vitiligo was associated with higher serum Tyr level. Therefore, it is reasonable to regard the serum Tyr level as a potential marker that may assist in the diagnosis of vitiligo.

Our previous study demonstrated that the rs11614913 T>C change in miR-196a-2 could inhibit the expression of *Tyrp1* at the post-transcriptional level. Since the SNP-induced down-regulation of *Tyrp1* could in turn affect the Tyr level, we wondered whether the abnormal serum level of Tyr in vitiligo was related to the rs11614913 SNP in miR-196a-2. Our association analysis revealed that the individuals with the risk TT or TC genotype and a higher level of serum Tyr had a significantly increased risk of vitiligo compared with those with the protective CC genotype and a lower serum Tyr level, indicating a potential relationship between Tyr and the rs11614913 T>C

change. Further western blot analysis proved that miR-196a-2-C could suppress the protein level of Tyr in PIG1 cells, which is an indirect regulation through the inhibition of the target gene *Tyrp1*.

The production of melanin mediated by *Tyr* could result in the accumulation of intracellular ROS in melanocytes [21]. We have previously found that the rs11614913 T>C change in miR-196a-2 decreased the levels of ROS and protected human melanocytes from apoptosis through the suppression of *Tyrp1* [6]. Combined with the observation that the expression of Tyr could be indirectly suppressed by miR-196a-2, we consider that the inhibitory effect of miR-196a-2 on the levels of ROS and the apoptosis partly attributes to the inhibition of Tyr level in PIG1 cells. In the present study, the flow cytometry analysis showed that knockdown of Tyr could independently down-regulate both the level of intracellular ROS and the apoptosis rate in PIG1 cells, which demonstrated that miR-196a-2 inhibited the levels of ROS and melanocyte apoptosis through not only the direct inhibition of Tyrp1 but also the indirect suppression of Tyr.

In summary, we provide evidences that the rs11614913 SNP in miR-196a-2 combined with the serum levels of Tyr influences the susceptibility of vitiligo in Han Chinese populations. Moreover, we found that miR-196a-2 T>C change indirectly inhibits the expression of Tyr, which contributed to its inhibitory effect on the levels of ROS and apoptosis rate in melanocytes. These findings indicate that miR-196a-2 rs11614913 polymorphism is associated with vitiligo by affecting heterodimeric molecular complexes of Tyr and Tyrp1 and may be a potential promising therapeutic target in the treatment of vitiligo.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of Fourth Military Medical University and with the 1964 Helsinki declaration and its later amendments.

Informed consent Informed consent was obtained from all individual participants included in the study.

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