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Original Article

The SNP rs915014 in MTHFR regulated by miR-661 associates with atherosclerosis

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Abstract: Genetic polymorphisms of methylene tetrahydrofolate reductase (*MTHFR*) were associated with atherosclerosis. This study analyzed *MTHFR* polymorphisms at the 3'-untranslated region for association with risk and outcome of atherosclerosis in a Chinese Han population. 500 patients and 600 healthy volunteers were enrolled for *MTHFR* rs915014 genotyping identified based on bioinformatics approach. The binding of miR-661 to *MTHFR* rs915014 was determined by luciferase assay, *MTHFR* expression was assessed using qRT-PCR, and plasma homocysteine levels were assayed by ELISA. Cigarette smoking, alcohol consumption, diabetes, hypertension and low levels of serum high-density lipoprotein-C were associated with an increased risk of developing ischemic stroke. MTHFR rs915014 AG and GG genotypes were significantly associated with increased risk of rs915014 compared with the GG genotype. The qRT-PCR confirmed that *MTHFR* rs915014 AG or GG genotypes could facilitate miR-661 binding leading to low *MTHFR* levels in cells. In addition, patients carrying the *MTHFR* rs915014 AG or GG genotypes were associated with accumulation of serum tHcy and a poor atherosclerosis outcome. In conclusion, this study demonstrates that the *MTHFR* rs915014 SNP is associated with increased risk in developing atherosclerosis, miR-661 binding, low *MTHFR* levels in cells.

Keywords: Atherosclerosis, MTHFR, polymorphism, homocysteine, miR-661

Introduction

Atherosclerosis is a devastating and life-threatening disease that is characterized by an accumulation of fibrous elements and lipids in large arteries and is the leading cause of mortality and morbidity worldwide [1-3]. Unstable atherosclerotic plaques are characterized by a relative preponderance of inflammatory cells and heightened proteolytic activity. It is believed that endothelial cells apoptosis results in the denudation or dysfunction of the intact endothelial monolayer, which causes lipid accumulation, monocyte adhesion, and inflammatory reactions leading to atherosclerotic lesion [4, 5].

Methylene tetrahydrofolate reductase (*MTHFR*) is the key enzyme involved in plasma homocysteine (tHcy) metabolism by catalyzing the conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate, a methyl donor during tHcy remethylation [6]. The tHcy is a crucial intermediate in methionine metabolism and causes excessive production of reactive oxygen species (ROS) [7]. This plays an important role in the regulation of cell signaling and homeostasis. *MTHFR* is localized at chromosome 1 p36.3 of human genome, and to date there are over 40 point mutations or SNPs in the *MTHFR* gene identified [8-10]. The polymorphism 1801133 involves substitution of C to T at position 677 (C677T), causing the conversion of alanine to valine. This missense mutation will result in approximately 70% and 35% reductions of normal *MTHFR* enzymatic activity in TT and CT genotype carriers [11].

To date, non-coding RNAs (ncRNAs) including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), have gained increasing attention in tumor malignant processes [12, 13]. MicroRNAs (miRNA) are small, non-coding RNA molecules. MicroRNAs (miRNAs) are a family of small noncoding RNAs with 20-25 nucleotides, which function through binding to their comple-

mentary sites on the 3'-UTR of target mRNAs to mediate mRNA degradation and translation repression [14-16]. Through binding to the 3' untranslated region (3'-UTR) of messenger RNA (mRNA), miRNA can cause degradation of mRNA, thus regulating the expression of mRNA at post-transcription level [17, 18]. Genetic variations in the 3'-UTR of certain genes are able to change the expression of the genes and alter the risk of a variety of human cancers [19, 20]. Thus, in this study, we assessed genetic variants in the *MTHFR* 3'-UTR in atherosclerosis patients and healthy control individuals, and then associated these genetic variants with miRNA levels using a case-control study design in a Chinese Han population.

Materials and methods

Study subjects

In this study, we enrolled 500 patients diagnosed with atherosclerosis from Zhengzhou Central Hospital Affiliated to Zhengzhou University between July 2009 and April 2016. Diagnosis of patients with atherosclerosis was established clinically. We also recruited 600 healthy volunteers from this hospital during the same period of time and they were matched to the cases by age and gender. All control individuals visited our hospital for annual health check-ups and were free of atherosclerosis. This study was approved by the Ethics Committee of Zhengzhou University and each participant signed a written informed consent.

Genomic DNA extraction and genotyping

Blood samples were collected after a 12 h overnight fasting period and then separated into serum, red blood cells, and buffy coat. Genomic DNA from peripheral whole blood of every validation subject was extracted by using QIAamp DNA blood mini kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After quantification, these DNA samples were subjected to DNA genotyping using the TaqMan SNP Genotyping Assay. Briefly, genomic DNA samples of 10 ng each were amplified using PCR in a total volume of 5 μL containing TaqMan Universal Master Mix, 80x SNP Genotyping AssayMix, DNase-free water and 10-ng DNA. The PCR conditions were 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in a 384-well ABI 7900HT Real Time PCR System as described previously.

Bioinformatic analysis of miRNAs binding to MTHFR SNP and linkage disequilibrium analysis among MTHFR polymorphisms

Bioinformatics software (http://www.bioguo.org/miRNASNP/) was used to detect the candidate SNPs which could affect *MTHFR* gene regulation via miRNAs.

Cell line, culture, and mIR-661 transfection

Embryonic Kidney 293T cells were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37°C. The miR-661 mimics and control were obtained from Genepharma (Shanghai, China) and transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Construction of luciferase-based reporter plasmids and luciferase reporter assay

MTHFR 3'-UTR fragments containing either A or G alleles were amplified using PCR from genomic DNA and cloned into pGL3-promoterless luciferase-based plasmids (Promega, Madison, WI, USA) multiple cloning sites. *MTHFR* 3'-UTR fragments potentially binding to miR-661, predicted by bioinformatics analysis or a mutated sequence with the predicted target sites, were cloned using PCR from genomic DNA and inserted into the pGL3 promoter vector (Genscript, Nanjing, China). The cloned plasmids were amplified and verified by DNA sequencing. Next, 293T cells were plated onto 24-well plates and transfected with 100 ng of pGL3-*MTHFR* wild, pGL3-*MTHFR* mutant, and miR-661 mimics, respectively. A Renilla luciferase vector pRL-SV40 (5 ng) was also co-transfected as a normalization and transfection efficiency control. Each experiment was performed in triplicate and luciferase activity was assessed 48 h after transfection using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity.

ELISA of plasma homocysteine levels

Plasma levels of tHcy in patients was assessed using a tHcy ELISA kit (Green Stone, Bern, Switzerland), according to the manufacturer's

Table 1. Frequency distributions of selected variables in patients and healthy controls

Characteristics	Cases (n=500)	Control (n=600)	*p* value
Age (year)	64 (49-79)	65 (51-78)	0.51
Sex (Male/Female)	329/171	399/201	0.81
Smoking (Yes/No)	367/133	344/256	<0.001
Drinking (Yes/No)	307/193	301/299	<0.001
Diabetes (Yes/No)	299/201	109/491	<0.001
Hypertension (Yes/No)	402/98	111/489	<0.001
BMI (kg/m^2)	24.3 (19.1-28.2)	24.4 (20.5-28.3)	0.33
Total cholesterol (mmol/L)	5.01 (4.04-5.39)	4.59 (4.05-5.34)	0.21
HDL-C (mmol/L)	1.38 (1.22-1.49)	1.23 (1.11-1.52)	0.02
LDL-C (mmol/L)	2.58 (2.54-3.12)	2.54 (2.22-3.11)	0.49

Table 2. SNPs located in the MTHFR gene 3'-UTR and the predicted miRNAs

SNP	Chromosome	HGVS Names	miRNA
rs55763075	1:11790377	XM_005263460.1:c.*303G>A	miR-34b
rs915014	1:11789412	XM_005263458.1:c.*1268A>G	miR-661
rs868014	1:11789390	XM_005263459.1:c.*1150T>C	miR-1203
rs114290429	1:11788822	XM_005263460.1:c.*303G>A	miR-521
rs4846048	1:11786195	XM_005263459.1:c.*4345C>T	miR-522

Table 3. Genotype frequencies of the MTHFR rs915014 polymorphism among atherosclerosis patients and control group

Genotype	Cases (n=500)		Controls (n=600)		OR (95% CI)[a]	*P* Value[a]
	N	%	N	%		
rs915014						
AA	301	60.2	415	69.2	1.00	
AG	127	25.4	122	20.3	1.44 (1.01-2.19)	0.014
GG	72	14.4	63	10.5	1.51 (1.09-1.98)	0.015
G carrier	199	39.8	185	30.8	1.68 (1.29-2.65)	0.002

[a]The ORs, 95% CIs and *P* value were calculated after adjusting for age, gender, parental smoking, drinking and other characteristics listed in **Table 1**.

protocol. The level of plasma tHcy was normalized to the kit standard and the data were summarized as Mean ± SD.

Statistical analysis

The association between different genetic variants and ischemic stroke risk was evaluated by calculation of the odds ratios (ORs) and 95% confidence intervals (CIs) using univariate and multivariate logistic regression analysis. The difference in association of *MTHFR* mRNA levels with three *MTHFR* genotypes and of the relative luciferase activity between the wild and mutant genotype were evaluated by using an independent-sample *t* test. All statistical analyses were two-sided and P<0.05 was considered statistically significant using SPSS 13.0 (SPSS, Chicago, IL, USA) or SAS software (version 9.1.3; SAS Institute, Cary, NC, USA).

Results

Characteristics of participants

Characteristics of patients with atherosclerosis and healthy controls are shown in **Table 1**. Specifically, the controls were matched with cases for age and gender. Cigarette smoking (P< 0.001), alcohol consumption (P<0.001), diabetes and hypertension (P<0.001), a low level of serum high-density lipoprotein-C (HDL-C; P=0.02) were significantly associated with ischemic stroke. However, BMI and total serum cholesterol concentration were not associated with ischemic stroke (**Table 1**).

Identification of MTHFR polymorphisms in 3'-UTR

In this study, we mainly focused on the relationship of the SNPs in the *MTHFR* 3'-UTR to ischemic stroke risk and outcome. We first searched the GenBank of Single Nucleotide Polymorphism (SNP) database (https://www.ncbi.nlm.nih.gov/snp) to identify potential *MTHFR* genetic variants in the 3'-UTR using the following parameters: Organism (Homo sapiens); Function Class (3'-UTR); Global MAF (0.05-0.1); Validation Status (by-1000 Genomes). We identified five *MTHFR* polymorphisms (**Table 2**).

We then assessed genotype frequencies of these five *MTHFR* SNPs in 500 atherosclerosis

Table 4. Stratified analysis of rs915014 genotype with clinicopathological parameters of atherosclerosis patients

Feature	Genotype			AG vs AA* *P* Value	GG vs AA* *P* Value
	AA	AG	GG		
Gensini score (Mean/SD)	32.00/2.00	45.00/4.00	58.00/4.00	0.01	0.005
tHcy (mmol/L) (Mean/SD)	15.2/2.2	16.2/2.1	18.8/2.5	0.03	0.02
MTHFR (Mean/SD)	0.08/0.18	0.03/0.12	0.01/0.22	0.02	0.01

*Student t test for either genotype distributions or allele frequencies between cases and controls. MTHFR: Relative expression of MTHGR mRNA in atherosclerosis patients.

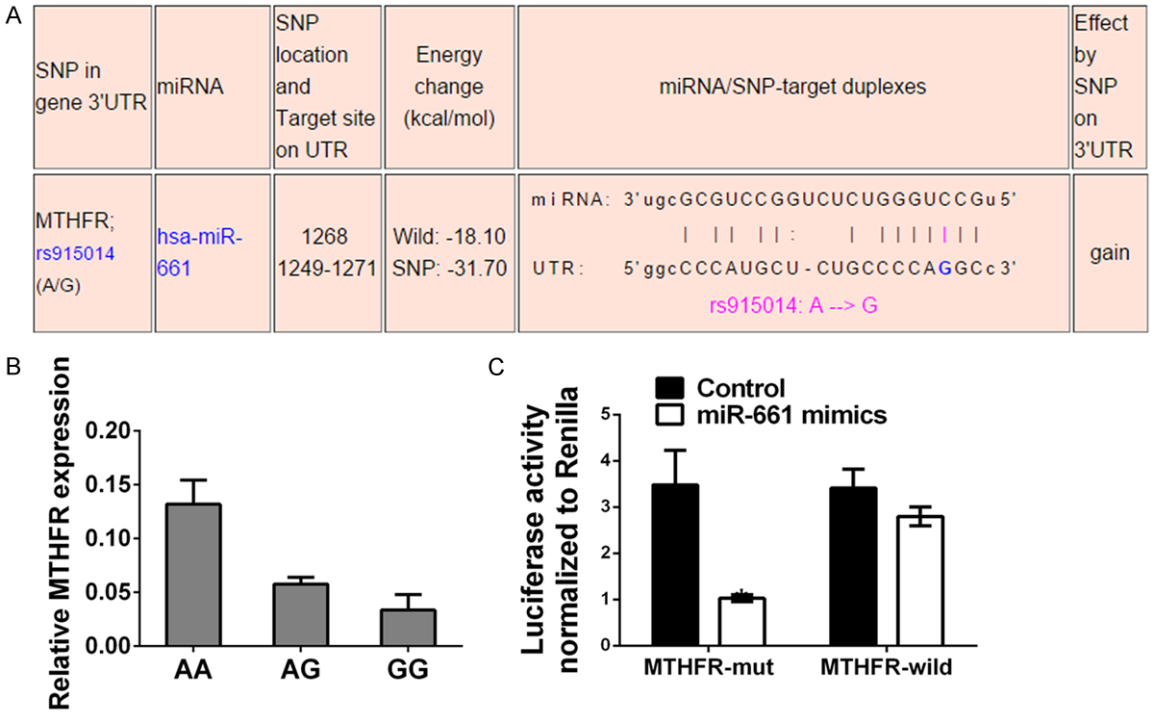

Figure 1. Binding of miR-661 to *MTHFR* rs915014 minor alleles. A: Bioinformatics analysis of potential miR-661 binding to *MTHFR* rs915014 polymorphisms. B: The mRNA expression of *MTHFR* was determined by qRT-PCR in patients with different genotypes. C: Luciferase assay. 293T cells were co-transfected with miR-661 mimics or control renilla luciferase vector pRL-SV40 for 48 h. Both firefly and renilla luciferase activities were measured in the same sample using a luciferase assay kit. Firefly luciferase signals were normalized with renilla luciferase signals. Data were presented as mean ± SE. *P<0.05.

patients and 600 healthy controls. We found that the *MTHFR* SNP rs915014 had a statistically different distribution between atherosclerosis patients and healthy controls. The *Chi-square* test confirmed that the genotype of rs915014 was in Hardy-Weinberg equilibrium distribution pattern in the healthy control group (P=0.32). The logistic regression analysis revealed that *MTHFR* rs915014 AG and GG genotypes significantly associated with increased risk of developing of atherosclerosis compared with the AA genotype (OR: 1.44; 95% CI: 1.01-2.19 for AG genotype, while OR: 1.51; 95% CI: 1.09-1.98 for GG genotype; **Table 3**). A higher number of G-alleles were also associated with an increased risk of ischemic stroke (OR: 1.68; 95% CI: 1.29-2.65).

Association of the MTHFR rs915014 SNP with a poor atherosclerosis outcome

Next, we assessed the association of the *MTHFR* rs915014 SNP with atherosclerosis outcome. The Gensini score system was applied to evaluate the outcome of atherosclerosis. We found that patients carrying either the *MTHFR*

rs915014 AG or GG genotype had an increased Gensini score. Moreover, we assessed levels of *MTHFR* mRNA in patients carrying the *MTHFR* rs915014 AA, AG and GG genotypes. Our data showed that patients carrying the *MTHFR* rs915014 AG or GG genotypes had a higher level of *MTHFR* compared with patients with the AA genotype. We then assayed levels of serum tHcy in patients and found that patients carrying the *MTHFR* rs915014 AG or GG genotype were associated with the accumulation of serum tHcy (**Table 4**).

MTHFR rs915014 SNP alteration of miR-661 binding

Thus far, we revealed that the *MTHFR* rs915014 SNP was associated with risk and poor ischemic stroke outcome. We then performed bioinformatics analysis to determine whether this *MTHFR* rs915014 SNP could have miRNA binding sites. We found that miR-661 could bind to this particular SNP of the minor allele (**Figure 1A**). We therefore performed a luciferase assay to confirm such binding and found that luciferase activity of G-allele-specific pGL3 construct was significantly suppressed by miR-661. Luciferase activity of the A-allele-specific pGL3 construct was not altered by miR-661 transfection in 293T cells (**Figure 1B**, **1C**).

Discussion

MTHFR is an important enzyme in the generation of ROS and subsequent cell damage. Indeed, in ischemic conditions, ROS generation is enhanced and can lead to damage to various types of cells, including brain endothelial cells, leading to formation of thrombosis and loss of brain function. Our current study provides direct evidence showing that this novel *MTHFR* rs915014 polymorphism increases the risk of atherosclerosis compared with healthy controls. Mechanistically, the *MTHFR* rs915014 polymorphism alters the binding of miR-661 to negatively regulate *MTHFR* expression, providing an epigenetic mechanism of gene regulation and expression. In this study, it was the first time to reveal the functional SNP located in the 3'UTR of *MTHFR* acting as a risk factor for the atherosclerosis. Besides, miR-661 was frequently documented in human malignant tumors especially the lung cancer, here we first demonstrate the involvement of miR-661 in atherosclerosis. This might providing demystify for the detailed mechanism study in future.

There is accumulating evidence demonstrating that SNPs localized at miRNA binding sites (miRSNPs) could affect the binding of miRNAs to the target genes and in turn result in reduction or increase in translation of the target mRNA and altered susceptibility to cancer. For example, previous studies showed that the rs2910164 polymorphism harboring the sequence for miR-146a could influence susceptibility to gastric cancer in a Chinese population, while rs4143815 and rs4819388 SNPs in the 3'-UTR of B7-H1 and B7-H2 genes, respectively, associated with development of gastric cancer.

In summary, our current study demonstrated that the function SNP rs915014 in the 3'UTR of *MTHFR* AG and GG genotypes were associated with increased risk of atherosclerosis compared with the GG genotype. The miR-661 is able to bind to *MTHFR* with G allele and down-regulate *MTHFR* expression resulting in an increased level of serum tHcy and a poor atherosclerosis outcome. The detection of rs915014 in atherosclerosis combining with tHcy might be a possible prognosis biomarker in the future.

Disclosure of conflict of interest

None.

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References

[1] Kim SH, Kim GJ, Umemura T, Lee SG and Cho KJ. Aberrant expression of plasma microRNA-33a in an atherosclerosis-risk group. Mol Biol Rep 2017; 44: 79-88.

[2] Vikram A, Kim YR, Kumar S, Li Q, Kassan M, Jacobs JS and Irani K. Vascular microRNA-204 is remotely governed by the microbiome and impairs endothelium-dependent vasorelaxation by downregulating Sirtuin1. Nat Commun 2016; 7: 12565.

[3] Chen C, Cheng G, Yang X, Li C, Shi R and Zhao N. Tanshinol suppresses endothelial cells

apoptosis in mice with atherosclerosis via lncRNA TUG1 up-regulating the expression of miR-26a. Am J Transl Res 2016; 8: 2981-2991.

[4] Liu G, Li Y and Gao XG. microRNA-181a is upregulated in human atherosclerosis plaques and involves in the oxidative stress-induced endothelial cell dysfunction through direct targeting Bcl-2. Eur Rev Med Pharmacol Sci 2016; 20: 3092-3100.

[5] Xie W, Li L, Zhang M, Cheng HP, Gong D, Lv YC, Yao F, He PP, Ouyang XP, Lan G, Liu D, Zhao ZW, Tan YL, Zheng XL, Yin WD and Tang CK. MicroRNA-27 prevents atherosclerosis by suppressing lipoprotein lipase-induced lipid accumulation and inflammatory response in apolipoprotein E knockout mice. PLoS One 2016; 11: e0157085.

[6] Yildiz SH, Ozdemir Erdogan M, Solak M, Eser O, Arikan Terzi ES, Eser B, Kocabas V and Aslan A. Lack of association between the methylenetetrahydropholate reductase gene A1298C polymorphism and neural tube defects in a Turkish study group. Genet Mol Res 2016; 15.

[7] van der Put NM, Gabreels F, Stevens EM, Smeitink JA, Trijbels FJ, Eskes TK, van den Heuvel LP and Blom HJ. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998; 62: 1044-1051.

[8] Zhou BS, Bu GY, Li M, Chang BG and Zhou YP. Tagging SNPs in the MTHFR gene and risk of ischemic stroke in a Chinese population. Int J Mol Sci 2014; 15: 8931-8940.

[9] Cui X, Navneet S, Wang J, Roon P, Chen W, Xian M and Smith SB. Analysis of MTHFR, CBS, glutathione, taurine, and hydrogen sulfide levels in retinas of hyperhomocysteinemic mice. Invest Ophthalmol Vis Sci 2017; 58: 1954-1963.

[10] Dinc N, Yucel SB, Taneli F and Sayin MV. The effect of the MTHFR C677T mutation on athletic performance and the homocysteine level of soccer players and sedentary individuals. J Hum Kinet 2016; 51: 61-69.

[11] Misselbeck K, Marchetti L, Field MS, Scotti M, Priami C and Stover PJ. A hybrid stochastic model of folate-mediated one-carbon metabolism: effect of the common C677T MTHFR variant on de novo thymidylate biosynthesis. Sci Rep 2017; 7: 797.

[12] Berindan-Neagoe I, Monroig Pdel C, Pasculli B and Calin GA. MicroRNAome genome: a treasure for cancer diagnosis and therapy. CA Cancer J Clin 2014; 64: 311-336.

[13] Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C and Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007; 297: 1901-1908.

[14] Kozomara A and Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 2014; 42: D68-73.

[15] Liu N, Jiang F, He TL, Zhang JK, Zhao J, Wang C, Jiang GX, Cao LP, Kang PC, Zhong XY, Lin TY and Cui YF. The Roles of MicroRNA-122 overexpression in inhibiting proliferation and invasion and stimulating apoptosis of human cholangiocarcinoma cells. Sci Rep 2015; 5: 16566.

[16] Rothschild SI, Gautschi O, Batliner J, Gugger M, Fey MF and Tschan MP. MicroRNA-106a targets autophagy and enhances sensitivity of lung cancer cells to Src inhibitors. Lung Cancer 2017; 107: 73-83.

[17] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T and Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and nontumorous tissues. Oncogene 2006; 25: 2537-2545.

[18] Tang J, Zhuo H, Zhang X, Jiang R, Ji J, Deng L, Qian X, Zhang F and Sun B. A novel biomarker Linc00974 interacting with KRT19 promotes proliferation and metastasis in hepatocellular carcinoma. Cell Death Dis 2014; 5: e1549.

[19] Liu Y, Chai Y, Zhang J and Tang J. A function variant at miR-501 alters susceptibility to hepatocellular carcinoma in a Chinese Han population. Cell Physiol Biochem 2016; 38: 2500-2508.

[20] Cipolla GA, Park JK, de Oliveira LA, Lobo-Alves SC, de Almeida RC, Farias TD, de SLD, Malheiros D, Lavker RM and Petzl-Erler ML. A 3'UTR polymorphism marks differential KLRG1 mRNA levels through disruption of a miR-584-5p binding site and associates with pemphigus foliaceus susceptibility. Biochim Biophys Acta 2016; 1859: 1306-13.