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ORIGINAL ARTICLE

MIR4532 gene variant rs60432575 influences the expression of *KCNJ11* and the sulfonylureas-stimulated insulin secretion

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Abstract

Purpose Diabetes mellitus is a major chronic disease and causes over one million deaths. *KCNJ11* genetic polymorphisms influence the response of first-line oral antidiabetic agent sulfonylureas. *Hsa-miR-4532* correlates with diabetic nephropathy and has a high abundance in urine. *MIR4532* rs60452575 G>A variant changes the mature sequence of *hsa-miR-4532*. We studied whether the genetic polymorphisms of *MIR4532* rs60452575 would influence *KCNJ11* expression and sulfonylurea-stimulated insulin secretion or not.

Methods To estimate the influence that rs60452575 G>A variant has on the interaction of *hsa-miR-4532* and *KCNJ11*, we constructed a pmirGLO vector containing 3′ UTR of *KCNJ11* and co-transfected it with wild-type and mutant *hsa-miR-4532* mimics into HEK293 cells; and we overexpressed wild-type and mutant *hsa-miR-4532* mimics into HEK293 cells and MIN6 cells to access its effects on *KCNJ11* expression and response of sulfonylureas.

Results *MIR4532* rs60452575 G>A variant appeared to disrupt the repression of *KCNJ11* expression in both cell lines, and reduce the sulfonylurea-stimulated insulin secretion by breaking the binding of the *hsa-miR-4532* to 3′ UTR of *KCNJ11* in MIN6 cells.

Conclusions Our study indicates that *MIR4532* rs60452575 variant influences *KCNJ11* expression and sulfonylurea response. It might be a potential predictive factor of sulfonylureas therapy.

Keywords MicroRNA · Polymorphism · KCNJ11 · Diabetes · Sulfonylureas · Insulin

Introduction

Diabetes mellitus is a chronic disease and a major public health problem that causes over 1.4 million deaths per year [1] and affects more than 425 million people (8.8% in prevalence) [2, 3]. β-cell dysfunction and insulin resistance are the primary pathological causes of type 2 diabetes, which accounts for more than 90% of all cases of diabetes [4]. Sulfonylureas are the first-line drugs of antidiabetic pharmaco-therapy agents besides insulin and metformin [5]. They stimulate the release of insulin in β-cells by binding to sulfonylurea receptor 1 (SUR1) which forms the ATP-sensitive potassium (K_{ATP}) channel with inwardly rectifying potassium channel ($K_{ir}6.2$) [6], but secondary failure is always the sword of Damocles for sulfonylureas therapy [7]. *KCNJ11* (the gene which encodes $K_{ir}6.2$ protein) is a very important pharmacogenomic biomarker of sulfonylurea. Oral sulfonylureas are more effective than insulin in patients carrying *KCNJ11* mutations [8]. Previous researches showed that the E23K variant of *KCNJ11* is involved

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in the secondary sulfonylurea failure [9, 10]. However, whether the genetic polymorphisms in the regulators of *KCNJ11* influence its expression and function remains unknown.

MicroRNA (miRNA) is a class of non-coding RNA molecules containing about 22 nucleotides (nt) [11] and plays an inhibitory regulatory role by binding to *cis*-elements in the 3′ untranslated region (UTR) of mRNAs [12]. *Hsa-miR-4532*, an miRNA highly expressed in human urine, was significantly downregulated in the urine of patients with diabetic nephropathy and was associated with diabetic nephropathy [13]. Polymorphisms in miRNA coding gene or the binding area of target miRNAs might influence the expression and the biological function of target genes [14]. A common variant rs60432575 G>A (minor allele frequency is 8.7% in Beijing Chinese, https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) on its encoding gene, *MIR4532*, causes the sequence alteration of mature *hsa-miR-4532*. We postulated that rs60432575 of *MIR4532* could influence *hsa-miR-4532* binding to *KCNJ11* and sulfonylurea response.

In this study, we found that the rs60432575 G>A variant on *MIR4532* influences the abundance of $K_{ir}6.2$ and sulfonylurea response. Our study might provide a new insight that single nucleotide polymorphisms (SNPs) on miRNA encoding genes might regulate some important drug biomarker genes and influence the response of corresponding drugs.

Methods

Cell culture and transfection

Human embryonic kidney HEK293 cells and pancreatic β-cell line MIN6 cells [15] were both purchased from cell resource center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's high glucose (4.5 g/L) and low glucose (1.0 g/L) medium (Gibco Company, USA), respectively, supplemented with 10% fetal bovine serum (Gibco Company, Changsha, China) at 37 °C with an atmosphere of 95% O_2/5% CO_2 in a humidified incubator. All transfections in our study were performed via Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, USA) at 70%–80% confluency according to the manufacturer's instruction.

Dual luciferase reporter vector construction and luciferase reporter assay

The 3′ UTR of *KCNJ11* was synthetized and constructed into the pmirGLO [16, 17] plasmid (GenePharma, Shanghai, China) in the multiple cloning site (MCS) as shown in Fig. 1a. HEK293 cells [18] cultured in 48-well plates were co-transfected with 7.5 pmol for one type of the three types of miRNA mimics (GenePharma, Shanghai, China) and 500 ng for one type of the four types of different constructed pmirGLO plasmids, respectively. PmirGLO plasmids contain both 3′ UTR of *KCNJ11* (including wild type and three mutant types as showed in Fig. 1b) and firefly luciferase gene and *Renilla* luciferase gene. Each type of pmirGLO plasmid was co-transfected with four groups of miRNA mimics [blank, negative control (NC), wild type, and mutant type]. MiRNA mimics (GenePharma, Shanghai, China) are synthetized according to miRBase 21 (http://www.mirbase.org/), and negative control (NC) is a 21-nt scrambled sequence which does not interact with other sequences. Stable NC: 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′ (anti-sense), wild type (WT): 5′-CCCCGGGGAGCCCGGCG-3′ (sense) and 5′-CCGGGCUCCCCGGGGUU-3′ (anti-sense), and mutant type (MT): 5′-CCCCAGGGAGCCCGGCG-3′ (sense) and 5′-CCGGGCUCCCUGGGGUU-3′ (anti-sense) [19, 20]. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) within 24 h after transfection [21].

RNA isolation and quantitative real-time PCR (qRT-PCR)

After the transfection of the three types of miRNA mimics and 24 h culture, total RNA of HEK293 cells was extracted by trizol (Takara, Dalian, China). cDNA was obtained using the PrimeScript RT master mix reagent Kit (Takara, Japan) according to the manufacturer's instructions. The primers (synthesized by Biosune, Wuhan, China) of RT-PCR were used as follows, *KCNJ11*: 5′-CCCCACAGGATCTCATCGG-3′ (forward) and 5′-GCCAACAGCCCACTCTACG-3′ (reverse); *GAPDH*: 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse). qRT-PCR was conducted on LightCycler 480 (Roche, Switzerland) by two-step PCR method with SYBR Green (Takara, Japan). Reactions were run for 40 cycles. The relative expression of *KCNJ11* mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

After the total proteins of HEK293 and MIN6 cells were lysed by Radio Immunoprecipitation Assay (RIPA, Beyotime Biotechnology, Shanghai, China), they were supplemented with 0.1% (v/v) 100 mM phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, Shanghai, China) isopropanol solution. Primary antibodies against

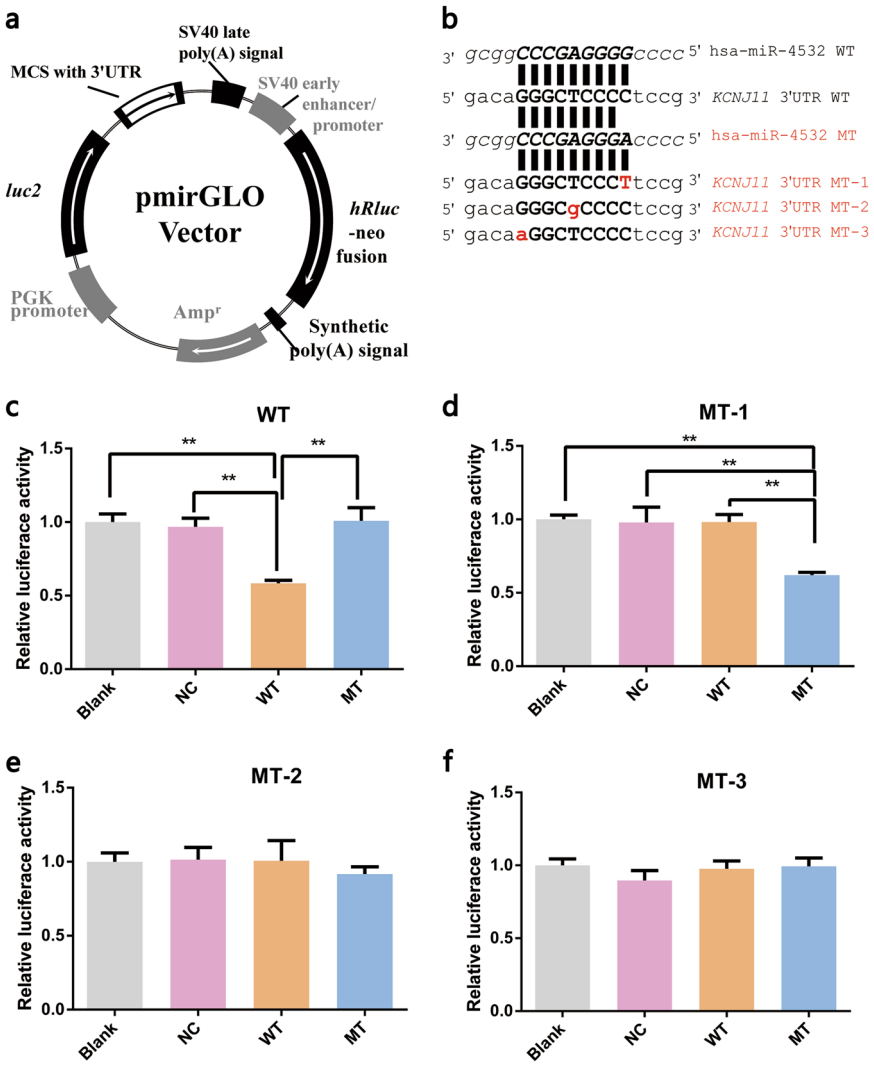

Fig. 1 Rs60432575 G>A variant disrupts hsa-miR-4532 binding to the *KCNJ11* 3′ UTR. **a** In the skeleton of the plasmid we constructed, the 3′ UTR of *KCNJ11* was inserted into the multiple clone site (MCS) and it controls the expression of *luc2*. The pmirGLO vector contains both *luc2* and *hRluc* (internal control). **b** The target binding of wild type (WT) *hsa-miR-4532* and *KCNJ11* 3′ UTR. Rs60432575 G>A variant causes one pair of base mismatch. We synthesized three artificial mutant types (MT) of *KCNJ11* 3′ UTR to validate the specificity of *hsa-miR-4532* and *KCNJ11* 3′ UTR (MT-1, MT-2, MT-3), and MT-1 rescues the mismatch of mutant type *hsa-miR-4532* and *KCNJ11* 3′ UTR. In HEK293 cells, the co-transfection of each plasmid and each miRNA mimic was performed and the luciferase activity was measured. **c** In the wild-type *KCNJ11* 3′ UTR group, wild-type *hsa-miR-4532* significantly repressed the expression of *KCNJ11* but mutant-type *hsa-miR-4532* did not. **d** In the MT-1 *KCNJ11* 3′ UTR group, mutant-type *hsa-miR-4532* significantly repressed the expression of *KCNJ11* but wild-type *hsa-miR-4532* did not. In MT-2 **e** and MT-3 **f** *KCNJ11* 3′ UTR group, no significant difference was observed; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $n = 3$ for each group. Error bars represent SEM

$K_{ir}6.2$ (Abcam, USA) and β-tubulin (Abcam, USA) were diluted to 1:400 and 1:200, respectively, and second antibody (Arigo, USA) was diluted to 1:10000.

Enzyme-linked immunosorbent assay (ELISA)

MIN6 cells were cultured in 6-well plates and transfected by three type of miRNA mimics. Then the cells were washed with Krebs-Ringer buffer (KRB) gently. Gliclazide, glybenclamide, glimepiride, repaglinide, and nateglinide were purchased from Sigma, and firstly dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) as saturated solution then diluted with KRB to 50% inhibitory concentrations (IC_{50}) (the IC_{50} concentrations are 50 nmol/L, 0.13 nmol/L, 3.0 nmol/L, 21 nmol/L, and 0.8 μmol/L, respectively) [22]. About 1 mL drugs solution and blank KRB were added to the plates. After 30-min incubation, 0.5 mL medium from each well was collected for the assessment of insulin secretion [23]. Mouse insulin ELISA kit (Mercodia, USA) was used to measure the level of insulin according to the manufacturer's instruction.

Statistical analysis

Data were expressed as the mean ± SEM. All the experiments were repeated three times independently. Statistical analysis was carried out by the software SPSS version 23 (IBM, Chicago, IL, USA). One-way ANOVA followed by a post-hoc Bonferroni's test was used when comparing multiple groups. Statistical significance was accepted when $p < 0.05$.

Results

MIR4532 rs60432575 variant disrupts the binding of *hsa-miR-4532* to the 3′ UTR of *KCNJ11* mRNA

To validate the binding target area, we predicted (*KCNJ11* 2966-2974) by PolymiRTS 3.0 database [24] and RNAhybrid [25], we designed and synthetized four types (one wild type and three artificial mutant types) of 3′ UTR of *KCNJ11*, and constructed each of them into pmirGLO plasmid to examine the binding specificity (Fig. 1a, b). And they were co-transfected into HEK293 cells, with miRNA mimics. In the cells treated with pmirGLO plasmid containing wild-type *KCNJ11* 3′ UTR, the relative luciferase/*Renilla* luciferase activity ratio was significant reduced in wild-type *hsa-miR-4532*-treated cells than the blank group and NC-treated cells. And mutant-type rs60432575 group had no apparent change compared with blank and NC (Fig. 1c). Whereas in the mutant-1 group, mutant-type *hsa-miR-4532* group was significant lower than the other three groups which were not markedly different (Fig. 1d). And in mutant-2 and mutant-3 groups, the inter-group difference was not significant (Fig. 1e, f). These results imply that *hsa-miR-4532* binds to the 3′ UTR of *KCNJ11* mRNA by perfect centered miRNA (2966-2974) and represses the expression of *KCNJ11*. However, the G>A variant disrupted the binding and can be restored by a corresponding base pair alteration on the 3′ UTR of *KCNJ11* mRNA.

MIR4532 rs60432575 variant influences the abundance of K_{ir}6.2 at *KCNJ11* mRNA level

To examine whether wild-type and mutant-type *hsa-miR-4532* can downregulate the abundance of *KCNJ11* mRNA and K_{ir}6.2 protein, qRT-PCR and western blotting are performed with HEK293 cells and MIN6 cells transfected with three types of miRNA mimics (NC, WT, and MT). Wild-type *hsa-miR-4532* overexpression significantly downregulates the levels of *KCNJ11* mRNA (Fig. 2a) and K_{ir}6.2 protein (Fig. 2b–e), whereas the overexpression of mutant-type *hsa-miR-4532* neither showed a marked reduction in the abundance of *KCNJ11* mRNA (Fig. 2a) nor K_{ir}6.2 protein (Fig. 2b–e). These data demonstrate that miR-4532 represses the abundance of K_{ir}6.2 protein through decreasing the level of *KCNJ11* mRNA, and the rs60432575 variant disrupted the repression of *hsa-miR-4532* on *KCNJ11* mRNA.

MIR4532 rs60432575 variant influences the sulfonylurea-stimulated insulin secretion

To explore the consequence of repression of K_{ir}6.2 by wild-type *hsa-miR-4532*, we overexpressed wild-type and mutant-type *hsa-miR-4532* in MIN6 cells, and then examined the sulfonylurea-stimulated insulin secretion by ELISA kit. Downregulation of insulin secretion of MIN6 cells was found in wild-type *hsa-miR-4532* overexpression subgroup after three kinds of sulfonylurea (gliclazide, glybenclamide, and glimepiride) stimulated, but mutant-type *hsa-miR-4532* subgroup did not show any alteration compared with NC subgroup (Fig. 3b–d). We then considered whether *hsa-miR-4532* target upstream genes of *KCNJ11* caused a downregulation of insulin secretion rather than the repression of *KCNJ11* itself. Thus, we performed several glinide-treated groups as control drug group. Glinides have a same upstream pathway but a different K_{ATP} channel sub-unit gene (also its biomarker), *KCNQ1* rather than *KCNJ11*, compared to sulfonylureas[26]. We found that the overexpression of wild-type and mutant-type miR-4532 did not influence the glinide-stimulated insulin secretion (Fig. 3e, f). It ruled out the possibility that miR-4532 influences insulin secretion by targeting upstream genes of *KCNJ11*. Compared with NC and mutant type subgroups, a small, and not significant, decrease was observed in wild-type subgroup among the blank group (Fig. 3a), repaglinide-treated group (negative control, Fig. 3e), and nateglinide group (negative control, Fig. 3f), indicating that the overexpression of miR-4532 itself does not remarkably influence insulin secretion without drug treatment. These results indicate that *MIR4532* rs60432575 variant influences the sulfonylurea-stimulated insulin secretion.

Discussion and conclusion

As a complex metabolic disease, diabetes mellitus affects almost 1/11th adults worldwide [2]. β-cell dysfunction and insulin resistance represent the major pathogenic mechanisms, and glycemic control is the key for antidiabetic therapy. Apart from insulin replacement and metformin, secretagogues (including sulfonylureas and glinides) that stimulate the pancreas to release insulin are commonly used.

In our study, rs60432575 G>A variant disrupts the repression attributed to *hsa-miR-4532* binding to the 3′ UTR of *KCNJ11* mRNA by one base-pair mismatch on

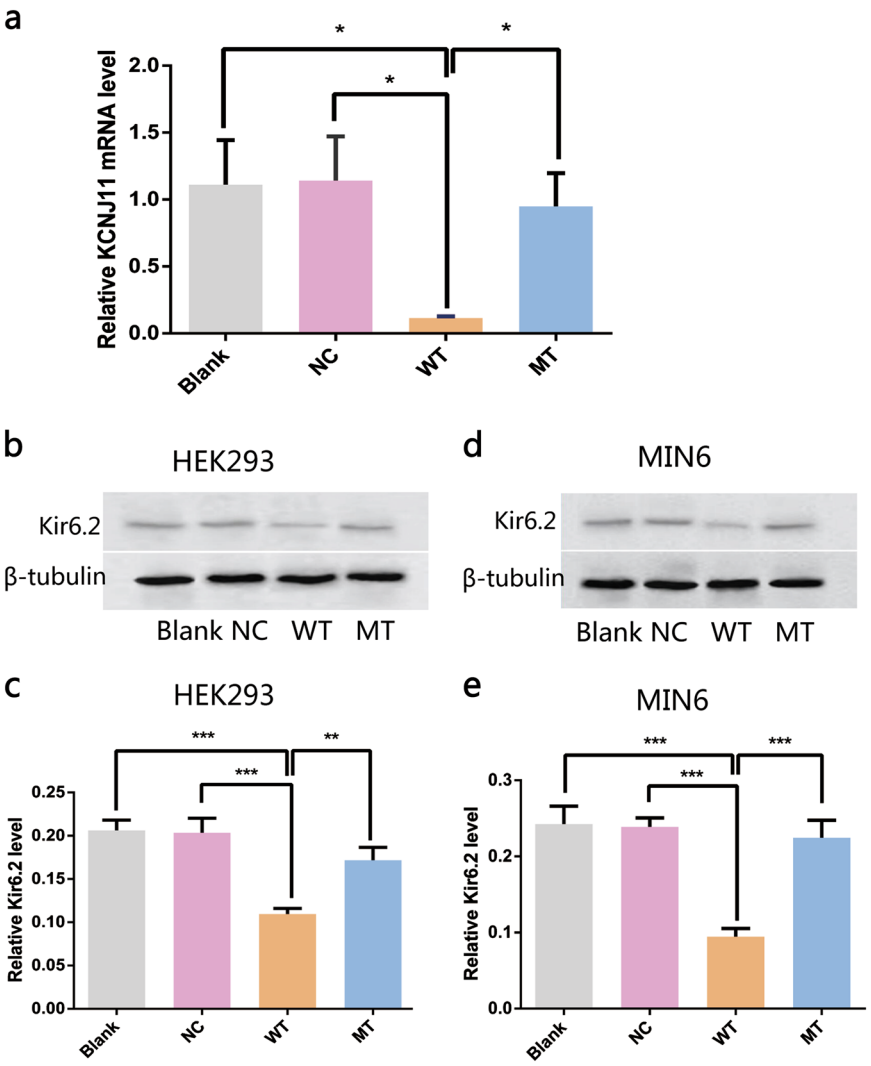

Fig. 2 Rs60432575 G>A variant influences the abundance of $K_{ir}6.2$ at KCNJ11 mRNA level. The relative expression levels of *KCNJ11* mRNA and $K_{ir}6.2$ protein after the overexpression of *hsa-miR-4532* wild type (WT), mutant type (MT), and negative control (NC). **a** The overexpression of wild-type *hsa-miR-4532* significantly reduced the level of *KCNJ11* mRNA in HEK293 cells. Whereas the overexpression of the mutant type did not reduce the level of *KCNJ11* mRNA in HEK293 cells. The western blotting image of $K_{ir}6.2$ protein and β-tubulin protein of HEK293 cells **b** and MIN6 cells **d**. The relative Kir6.2 protein level of HEK293 cells (**c**) and MIN6 cells **e**. The abundance of $K_{ir}6.2$ protein was repressed by wild-type *hsa-miR-4532* overexpression but not mutant-type *hsa-miR-4532* overexpression in HEK293 cells **b**, **c** and MIN6 cells **e**, **f**; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $n = 3$ for each group. Error bars represent SEM

original perfect centered miRNA binding sites (rather than seed region) [27]. When *KCNJ11* mRNA, which encodes $K_{ir}6.2$ protein, was decreased by the overexpression of *hsa-miR-4532* WT, $K_{ir}6.2$ (sub-unit of SUR1 complex) also decreased. This leads to the downregulation of target of sulfonylureas, SUR1 complex. And the response of sulfonylureas reduced too. Consequently, the phenomena including the reduction of the abundance of $K_{ir}6.2$ and the decrease of sulfonylurea-stimulated insulin secretion when wild-type *hsa-miR-4532* was overexpressed were absent when mutant-type *hsa-miR-4532* was overexpressed. However, we did not observe it in glinides-treated groups. Although sulfonylurea and glinides have similar pharmacological feature, the SUR1 sub-unit gene which influences the glinides response is not *KCNJ11* but *KCNQ1* [26]. Thus, despite *hsa-miR-4532* could downregulate *KCNJ11*, it would not influence the response of glinides. In summary, the *MIR4532* rs60432575 influenced the *KCNJ11* expression and sulfonylurea-stimulated insulin secretion (Fig. 4). To our knowledge, our study is the first to show that a polymorphism of an miRNA gene could contribute to the change of effect of antidiabetic agents. Given that *hsa-miR-4532* is highly expressed in urine, *MIR4532* rs60432575 might have the potential to be tested as a predictive biomarker of the response of sulfonylureas and secondary sulfonylurea failure by a non-invasive urine test.

KCNJ11, the drug target encoding gene of sulfonylureas, has been reported to be a very important biomarker to predict the glycemic response of sulfonylureas [28]. The K alleles of the E23K polymorphism show an increased glycemic response; and neonatal monogenic diabetes patients who are carriers of morbigenous K allele could be effectively treated with sulfonylureas [8]. Besides *KCNJ11*, previous studies have shown that numerous SNPs affect the incidence and progress of type 2 diabetes or the response of antidiabetes drugs, such as *TCF7L2* [29–31]. Multiple biomarkers have been discovered and developed to predict the response of antidiabetes agents [32]. Most of the

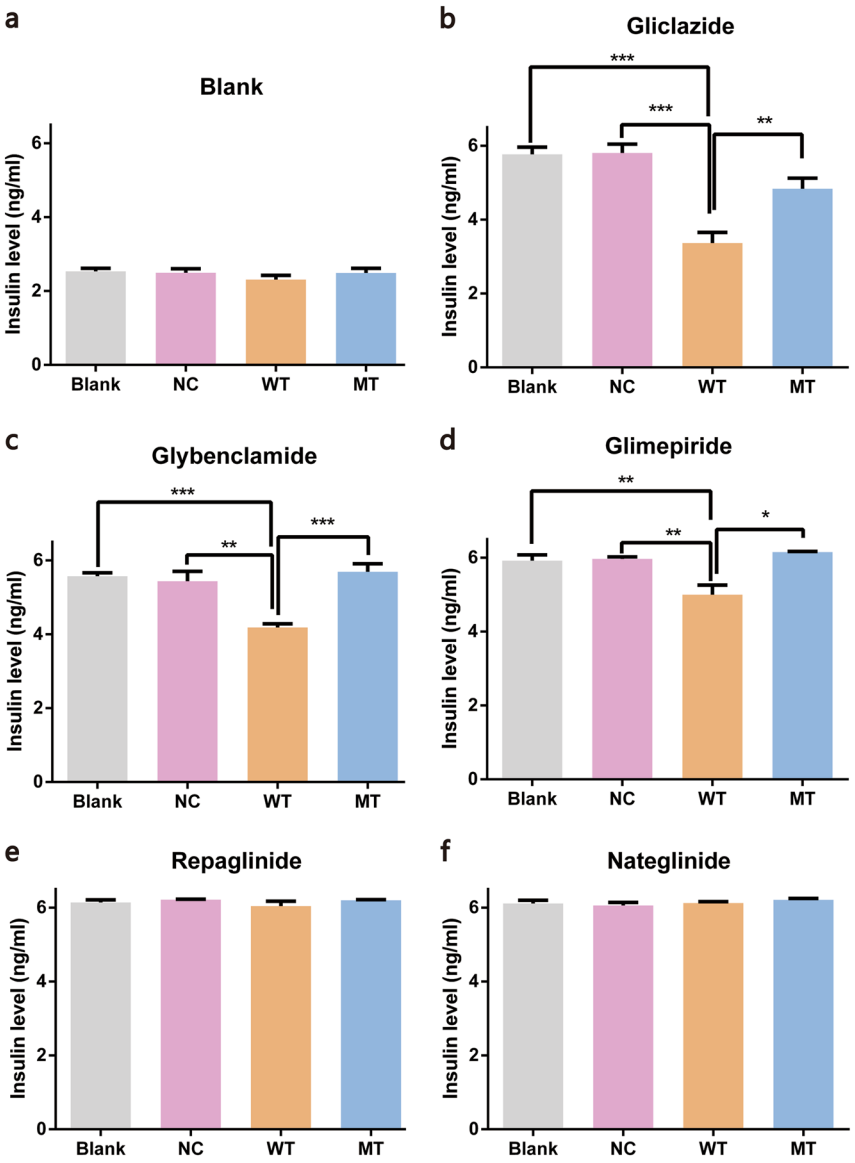

Fig. 3 Rs60432575 G>A variant influences the sulfonylurea-stimulated insulin secretion. We overexpressed wild-type (WT) and mutant-type (MT) hsa-miR-4532 in MIN6 cells and then we measured sulfonylurea-stimulated insulin secretion by insulin ELISA kit. Wild-type *hsa-miR-4532* overexpression significantly reduced the sulfonylureas (gliclazide, glybenclamide, and glimepiride)-stimulated insulin secretion (brown bars in **b**–**d**), and no marked difference was observed in MT *hsa-miR-4532* overexpression-treated groups (blue bars in **b**–**d**) and glinides (repaglinide and nateglinide)-treated groups **e**, **f**. Sulfonylureas- or glinides-treated groups secreted more insulin than the blank group **a**; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $n = 3$ for each group. Error bars represent SEM

pharmacogenomic researches are focused on polymorphisms of drug target genes (e.g., *PPARG* for thiazolidinediones), transporter genes (e.g., *SLC22A1* for metformin), or metabolism enzyme genes (e.g., *CYP2C9* for sulfonylureas) themselves [33]. And little was explored about the variants in the regulatory roles of key pharmacological proteins. Nonetheless, due to the lack of knowledge of miRNA target genes, most miRNA researches are focused on the expression of miRNA itself [34, 35]. More effort should be exerted on SNPs of miRNA-encoding genes. For example, Ciccacci et al. reported that rs6715345 C>G on *hsa-miR-375* and rs531564 C>G on *hsa-miR-124a* were associated with type 2 diabetes susceptibility [36]. Besides, the molecular variants of *hsa-miR-194* and *hsa-miR-219a-2-3p* could also potentially affect *KCNJ11* expression by disrupting miRNAs target *KCNJ11* [37].

Regarding *hsa-miR-4532*, it is a novel miRNA, and reports on its physiological and pathological functions are scarce until now. A previous study reported that it is involved in diabetic nephropathy and has a relatively high abundance in urine [13]. It is our innovativeness that we studied a sulfonylurea response-related polymorphism, which is located on an miRNA gene rather than on a gene that the drug directly acts upon. It would expand our knowledge that miRNA gene polymorphisms might influence drug response via regulating pharmacologically related genes which are also the targets of those miRNAs.

Our study has some limitations. Firstly, due to the lack of human langerhans' islet β-cell model, a mouse β-cell was used instead. Though humans and mice have a close genetic relationship, species difference could not be ignored and should be considered. Secondly, overexpressions of miRNA

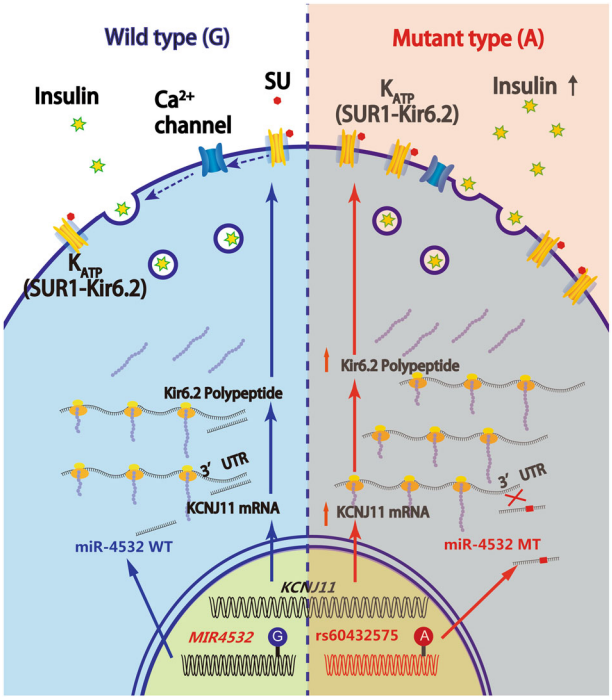

Fig. 4 *MIR4532* rs60432575 influences the abundance of $K_{ir}6.2$ and sulfonylurea-stimulated insulin secretion at the *KCNJ11* mRNA level. The wild-type *MIR4532* encodes wild-type (WT) miR-4532. WT *hsa-miR-4532* binds to the 3′ UTR of KCNJ11 and represses the abundance of $K_{ir}6.2$ protein, reduces the secretion of insulin stimulated by sulfonylurea (SU), displayed as left half. The mutant type (MT) rs60432575 G>A variant disrupts miR-4532 bound to the 3′ UTR of *KCNJ11* mRNA, and increases the level of *KCNJ11* mRNA, $K_{ir}6.2$ protein, and sulfonylurea-stimulated insulin secretion (right half). Solid arrows present the information flow of central dogma, and dotted arrows present the indirect interactions of K_{ATP} channels, Ca^{2+} channels, and insulin-storing vesicles. They are cell nucleus, cytoplasm, and tissue fluid from bottom to top and separated with nuclear membrane (blue double-line semi-ellipse) and cell membrane (blue single-line arc)

mimics were performed rather than the natural expression of endogeneous miRNA. Thirdly, the possibility that other targets of miR-4532 might regulate the expression of *KCNJ11* could not be ruled out, even though an experiment on glinide-treated control group was performed to check the possible involvement of an upstream gene of K_{ATP} channel. Fourthly, since our study is based on cellular experiment, a clinical trial should be performed on healthy volunteers and type 2 diabetes patients in the future.

In conclusion, our study demonstrated that *MIR4532* rs60432575 G>A variant disrupts *hsa-miR-4532* binding to *KCNJ11* and alters its repressive effect on $K_{ir}6.2$ expression and therefore influences sulfonylurea-stimulated insulin secretion. However, more studies should be conducted with clinical specimens to explore whether the SNP influences sulfonylurea response in vivo.

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Compliance with ethical standards

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

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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