Biochemical and Biophysical Research Communications 374 (2008) 101–105

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Allele-specific targeting of hsa-miR-657 to human IGF2R creates a potential mechanism underlying the association of ACAA-insertion/deletion polymorphism with type 2 diabetes

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ARTICLE INFO

Article history:
Received 25 June 2008
Available online 9 July 2008

Keywords:
microRNAs
3′ untranslated region
DNA sequence polymorphism
Insulin-like growth factor 2 receptor
Translational regulation
Type 2 diabetes

ABSTRACT

The biological mechanism of a recent discovered association of type 2 diabetes with the ACAA-insertion/deletion polymorphism at the 3′UTR of the *IGF2R* gene has remained unclear. A very recently emerging novel polymorphic control layer by microRNAs (miRNAs) makes it possible to elucidate this issue. In this study, a prediction by web tools MicroInspector and miRanda demonstrated that DNA sequence polymorphism (DSPs) ACAA-insertion/deletion in *IGF2R* 3′UTR is located within the hsa-miR-657 and hsa-miR-453 binding sites. And luciferase reporter assay revealed that hsa-miR-657 acts directly at the 3′UTR of the *IGF2R*. Furthermore, ACAA-deletion exerted a further repression compared with ACAA-insertion, indicating that hsa-miR-657 regulates *IGF2R* gene expression in a polymorphic control manner. Importantly, we also demonstrated that hsa-miR-657 can translationally regulate the IGF2R expression levels in Hep G2 cells. Thus, our findings testify the possibility that the ACAA-insertion/deletion polymorphism may result in the change of IGF2R expression levels at least in part by hsa-miR-657-mediated regulation, contributing to the elucidation for the pathogenesis of type 2 diabetes and raise the possibility that miRNAs or in combination with functional DNA sequence polymorphism may be valuable in the treatment of human type 2 diabetes.

MicroRNA (miRNA) are novel single-stranded RNAs of ~22 nucleotides in length that are transcribed from either independent noncoding RNAs or the introns of protein-coding genes [1]. There are accumulating evidence showing that miRNAs are involved in a variety of significant biological processes, and with changes of expression levels, some specific miRNA may act as regulator in pathogenesis of a number of human disorders such as chronic lymphocytic leukemia [2], cardiac hypertrophy [3], and cardiac arrhythmogenic potential [4].

At least 20–30% of all human genes are estimated to be regulated by miRNAs through targeting sequences in their 3′ untranslated region (3′UTR) [5,6]. To date, a number of studies have suggested that DNA sequence polymorphisms (DSPs) occurring within or in close proximity to miRNA binding sites may contribute to phenotypic variation, include disease susceptibility and important traits [7]. The first direct study showed that a 3′UTR variation in candidate gene SLITRK1 strengthens the binding of hsa-miR-189 with target site, consequently leading to enhanced down-regulation of SLITRK1 and contributes to the association of SLITRK1 variant with Tourette's syndrome (TS) [8]. Then other groups demonstrated more striking novel findings, for example, a 3′UTR variant in the sheep Gdf8 gene creates a novel target site for miR-1 and miR-206, which contributes to translational inhibition of the myostatin gene and the muscular hypertrophy of Texel sheep [9], and a naturally occurring SNP near the hsa-miR-24 binding site in the 3′UTR of human dihydrofolate reductase (DHFR) affects DHFR expression by interfering with hsa-miR-24 function, resulting in DHFR overexpression and methotrexate resistance [10].

Insulin-like growth factors (IGFs) have been shown to participate in normal growth, and especially fetal pancreas β-cell development [11]. IGF-II receptor is a multifunctional glycoprotein involved in the process of tumor suppression, immunity and invasion of *Listeria* species [12–14]. IGF-II receptor gene (*IGF2R*) is located at chromosome 6q26, a region that has shown to be related to insulin resistance and obesity-related metabolic phenotypes [15]. Very recently, Villuendas et al. applied a case-control association study in Spaniards to test a common ACAA-insertion/deletion (144/140 bp) polymorphism in *IGF2R* for association with type 2 diabetes and insulin-resistant traits, which led to the identification that diabetes patients were more frequently homozygous for the wild type 144 bp allele of *IGF2R* compared with controls, indicating

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doi:10.1016/j.bbrc.2008.06.102

that *IGF2R* 140 bp variant had a potential protective role against type 2 diabetes [16]. In addition, a previous study by Costello et al. had developed a highly sensitive quantitative human IGF-II/M6-P receptor immunoassay and had shown that the soluble receptor levels in noninsulin-dependent diabetes mellitus (NIDDM) and insulin-dependent diabetes (IDDM) patients were mildly elevated above those in control healthy subjects [17]. Although it has been hypothesized that the ACAA-insertion/deletion polymorphism at the 3′UTR of the *IGF2R* might participate in the pathogenesis of type 2 diabetes [16], its molecular mechanism has not been clearly elucidated.

Here, we identify that DNA sequence polymorphism ACAA-insertion → ACAA-deletion is located within the hsa-miR-657 binding site in *IGF2R* 3′UTR. Furthermore, the presence of ACAA-deletion was tested to strengthen the inhibitory interaction between *IGF2R* 3′UTR and hsa-miR-657. Importantly, we demonstrate that hsa-miR-657 can translationally regulate the IGF2R expression levels in Hep G2 cells. Thus, our findings result in the possibility that the ACAA-insertion/deletion polymorphism may contribute to the IGF2R expression levels at least in part by hsa-miR-657-mediated regulation, which is possibly involved in the pathogenesis of type 2 diabetes.

Materials and methods

Hybridization prediction. The *IGF2R* 3′UTR sequence was obtained from http://www.ncbi.nlm.nih.gov/Genbank/index.html, and the putative binding miRNAs with the ACAA-insertion/deletion polymorphism region (NCBI SNPs Data Base: rs8191962) were identified by means of MicroInspector (http://mirna.imbb.forth.gr/microinspector/) [18] and MiRanda (http://microrna.org) [19]. The candidate mature miRNA sequences were obtained from miRBase (http://microrna.sanger.ac.uk/sequences/) [20], and the minimum free energy hybridization of the two candidate miRNAs (hsa-miR-453 and hsa-miR-657) and the human *IGF2R* 3′UTR harboring either the ACAA-insertion or ACAA-deletion was predicted by miRanda software v1.0b.

Cell culture. HEK 293 cells and Hep G2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose) (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA). All cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

Generation of recombinant constructs. A 1038 bp fragment (33nt–1070nt downstream from the *IGF2R* stop codon) containing almost 70% length of the entire IGF2R 3′UTR was PCR-amplified utilizing a sense XbaI-adapter primer (5′-GCTCTAGACGGG ACAGCCAAGCACCTC-3′) and an antisense XbaI-adapter primer (5′-GCTCTAGAATGCTAGGGCCGAGGCTCT-3′) using Pfu 2× MasterMix (Tiangen Biotech, Co. Ltd., Beijing, China). The total cDNA derived from human monocyte cell line THP-1, prepared previously by us, was used as template. The XbaI restriction enzyme digested PCR product was inserted at the XbaI site which is located downstream of the luciferase gene in the pGL3-promoter vector (Promega, Madison, WI, USA). The authenticity and orientation of the fragment were confirmed by sequencing, and the resulting plasmid was designated IGF2R-INS which contains ACAA-insertion/deletion polymorphism region. In addition, a plasmid with a deletion of ACAA-insertion/deletion polymorphism region, designated IGF2R-DEL, was generated using the TaKaRa MutanBEST Kit (TaKaRa Biotech, Co. Ltd., Dalian, China). Briefly, a mutagenic deletion primer (5′-TGAATTCAACCTGCCCAA AGAT-3′), where the sequence (ACAA) to be deleted was missing from the middle of the primer, and a reverse primer (5′-CCAGCCGGCAAAAATACTGA-3′) were synthesized and utilized in a PCR reaction according to the manufacturer's instructions. Then following blunting kination and ligation, the remaining reaction was used for transformation. The deletion of ACAA sequence in IGF2R-DEL was confirmed by dideoxy chain termination sequencing.

Cotransfection of miRNA with plasmids and assay of luciferase activity. Hsa-miR-453 (sense 5′-AGGUUGUCCGUGGUGAGUUCGCA-3′, antisense 5′-CGAACUCACCACGGACAACCGUU-3′), hsa-miR-657 (sense 5′-GGCAGGUUCUCACCCUCUCUAGG-3′, antisense 5′-UAGA GAGGGUGAGAACCUGUCUU-3′), and nonspecific (sense 5′-UUC UCCGAACGUGUCACGUTT-3′, antisense 5′-ACGUGACACGUUCGG AGAATT-3′) duplex miRNAs were obtained from GenePharma (Shanghai, China). HEK 293 cells were seeded in 96-well plates at a density of 0.2×10^5 cells/well with 150 μL/well fresh 10% fetal bovine serum-containing DMEM 24 h before transfection. Each well containing cells were cotransfected with 100 ng each construct (IGF2R-INS or IGF2R-DEL) coupled with 50 ng of the pRL-tk vector as an internal control, and 20 pmol each miRNA (hsa-miR-453 or hsa-miR-657 or nonspecific duplex miRNA) with final concentration of 33 nM, and 0.8 uL Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The nonspecific duplex miRNA was transfected as negative control.

Twenty-four hour incubation after transient cotransfection assay, the HEK 293 cells were then harvested for measurement of their firefly luciferase and *Renilla* luciferase activities with a Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (BERTHOLD, Wildbad, Germany). The experiments were performed three times.

Transfection, RNA extraction, and RT-qPCR. Hep G2 cells were seeded in 6-well plates at a density of 0.2×10^5 cells/well with 2 mL/well fresh 10% fetal bovine serum-containing DMEM 24 h before transfection. Each well containing cells were transfected with 100 pmol each miRNA (hsa-miR-453 or hsa-miR-657 or nonspecific duplex miRNA) with final concentration of 33 nM, and 16 μL Lipofectamine2000. The nonspecific duplex miRNA was transfected as negative control.

RNA extraction was performed by using TRIZOL® Reagent (Molecular Research Center, Inc.) following the manufacturer's instructions. Briefly, 48 h after transfection assay, the supernatants from each well were harvested for subsequent soluble IGF2R ELISA before RNA extraction. Hep G2 cells were washed with PBS 2 times and lysed directly by adding 1 mL of TRIZOL® Reagent. Chloroform (0.2 mL) and 0.5 mL of isopropyl alcohol were used in aqueous phase separation and in precipitation of RNA. After the wash with 75% ethanol, the total RNA was dissolved. Then following a treatment with DNase I (RNase Free) (TaKaRa, Dalian, PR China), the total RNA were re-dissolved for RT-qPCR detection.

The total RNA was reverse transcribed to cDNA with ExScript™ RT Reagent Kit (Perfect Real Time) (TaKaRa). The reverse transcription mixture comprised 6.5 μL RNA (500 ng), 2 μL 5× ExScript™ Rtase Buffer, 0.5 μL dNTP Mixture, 0.5 μL Oligo dT, 0.25 μL ExScript™ RTase, 0.25 μL RNase inhibitor. Real-time PCR mixture consisted of 0.5 μL sense primer (10 μM), 0.5 μL antisense primer (10 μM), 12.5 μL SYBR® Premix ExTaq™ and 9 μL dH2O (SYBR® Premix Ex Taq™ (Perfect Real Time), TaKaRa Biotechnology Co., Ltd., Dalian, PR China). The primer sequences were as follows: IGF2R sense primer, 5′-GCACGACTTGAAGACACGCA-3′, IGF2R antisense primer, 5′-TCCCAGGGTTTTCCCACAC-3′; β-actin sense primer, 5′-GT CCACCTTCCAGCAGATGT-3′, β-actin antisense primer, 5′-TGC CAATCTCATCTTGTTTTCT-3′. Real-time PCR was carried out using an instrument RG-3000A (Gene Company Ltd.) with the annealing temperature at 55 °C. The data were analyzed using the Two Standard Curve method.

Soluble IGF2R ELISA. The concentration of the harvested supernatants from Hep G2 was determined by a highly sensitive quantitative human IGF-II/M6-P receptor immunoassay as described previously [17].

Statistical analysis. Statistical significance was determined by Student's *t* test, with a value of *p* below 0.05 considered to be statistically significant. Data are presented as means ±SD.

Results

ACAA-insertion/deletion is located within the hsa-miR-453 or hsa-miR-657 potential target sequence in IGF2R 3′UTR

To interpret the association of the ACAA-insertion/deletion polymorphism at the 3′UTR of the *IGF2R* gene with type 2 diabetes, we predicted that there might be an interaction between some specific miRNAs and the 3′UTR region encompassing ACAA-insertion/deletion sequence, which probably results in differences in human IGF2R expression. Using MicroInspector web server, we determined that this polymorphism is located in the target region of hsa-miR-453, hsa-miR-657, and hsa-miR-9. Then, a further analysis by miRanda, which is the most specialized for the calculation of ΔGs [21], led to the focus on the former two (hsa-miR-453 and hsa-miR-657) as candidates. As shown in Fig. 1, the minimal free energy of hybridization was predicted. The difference between the IGF2R 3′UTR (ACAA-insertion) and the IGF2R 3′UTR (ACAA-deletion), in the energies of hybridization with hsa-miR-453, is −2.76 kCal/mol, indicating that the former hybridization is relatively more stable than the latter (Fig. 1A.). In contrast, the hybridization of the IGF2R 3′UTR (ACAA-insertion) with hsa-miR-657 is weaker than that of IGF2R 3′UTR (ACAA-deletion), and the difference of 3.91 kCal/mol means that variation of ACAA-insertion → ACAA-deletion would strengthen the predicted existing hybridization of hsa-miR-657 with IGF2R 3′UTR.

hsa-miR-657 acts directly at the 3′UTR of the IGF2R in a polymorphic control pattern

To begin to verify the above predictions, the human *IGF2R* 3′UTR (33–1070 bp downstream from the *IGF2R* stop codon) was subcloned immediately downstream from the firefly luciferase gene, and the resulting constructs were designated IGF2R-INS and IGF2R-DEL, which harbor ACAA-insertion and ACAA-deletion, respectively (Fig. 2A). To test the efficacy of miRNAs candidates in regulating luciferase expression, either IGF2R-INS or IGF2R-DEL was cotransfected into HEK 293 cells with hsa-miR-453 or hsa-miR-657 or nonspecific miRNA (N.C.), and relative luciferase activities were determined. The reporter gene assay showed that, in HEK 293 cells cotransfected with hsa-miR-657, relative luciferase activities of both constructs were significantly decreased below that of N.C., indicating the functional potential of hsa-miR-657 to repress the expression of IGF2R. Importantly, transfection of IGF2R-DEL resulted in a modest but statistically significant further repression of luciferase expression compared with that of IGF2R-INS, confirming that hsa-miR-657 mediated interaction with IGF2R 3′UTR was enhanced by the polymorphic change of ACAA-insertion → ACAA-deletion. In contrast, the overexpression of hsa-miR-453 mimics had no statistically significant effect on the relative luciferase activities of both IGF2R-INS and IGF2R-DEL compared with N.C. (Fig. 2B). Taken together, these results indicate that of the two miRNA candidates for the human *IGF2R* identified by *in silico* analysis, only hsa-miR-657 can interfere with luciferase activity via direct action on the human *IGF2R* 3′UTR.

hsa-miR-657 exerts translational repression of IGF2R expression in Hep G2 cell line

To determine the effect of hsa-miR-657 on IGF2R mRNA levels, we chose Hep G2 cell line as a model, which contains abundant of IGF2R mRNA and possesses ACAA-insertion sequence in IGF2R

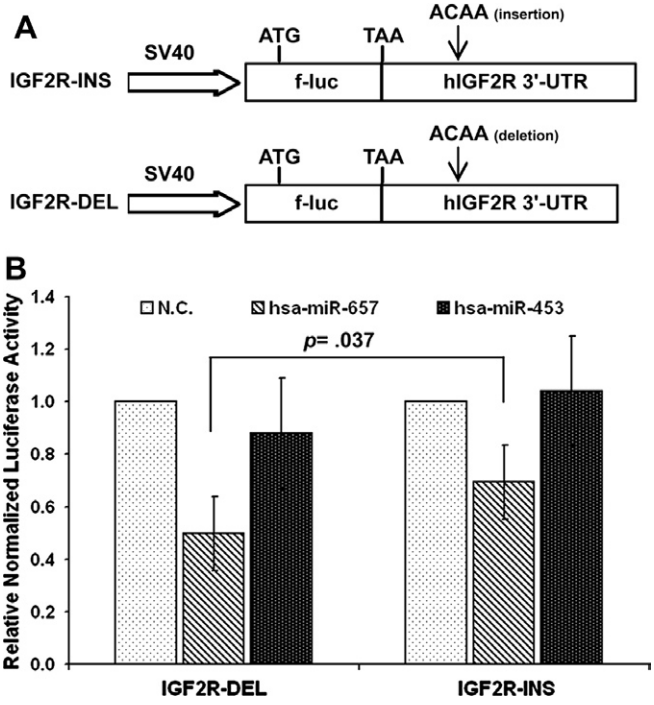

Fig. 2. Effects of hsa-miR-453 and hsa-miR-657 on the luciferase reporter genes bearing 3′UTR segments from human *IGF2R*. (A) The schematic representation of the luciferase reporter constructs. The *IGF2R* 3′UTR fragments harboring ACAA-insertion or ACAA-deletion was inserted downstream of the luciferase gene. (B) HEK 293 cells were cotransfected with IGF2R-INS or IGF2R-DEL, pRL-tk, and either negative control miRNA (N.C.) or hsa-miR-657 or hsa-miR-453 at the concentrations of 33 nM. Firefly luciferase activity was normalized to *Renilla* luciferase expression. The values relative to the mean of N.C. group data are shown.

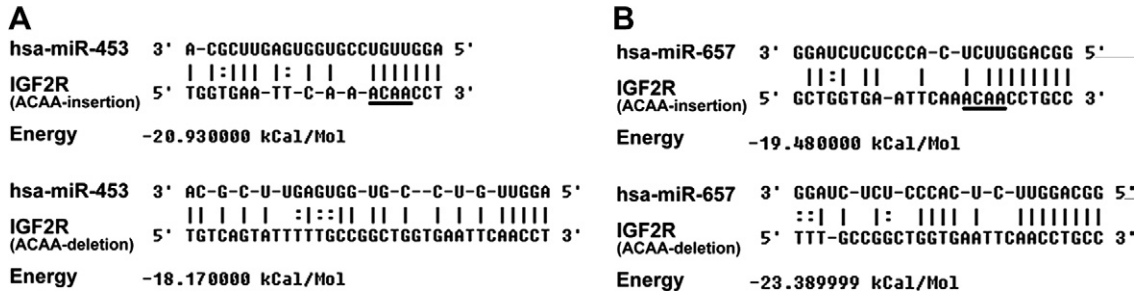

Fig. 1. The IGF2R ACAA-insertion/deletion polymorphism occurs within the hsa-miR-453 and hsa-miR-657 binding sites. Binding of hsa-miR-453 (A) and hsa-miR-657 (B) to IGF2R 3′UTR target sites containing the ACAA sequence (ACAA-insertion) and no ACAA sequence (ACAA-deletion) was predicted. ACAA is indicated by an underline, and the minimum free energy of the RNA duplex was analyzed by miRanda.

3′UTR [22]. Hep G2 cells were transfected with miRNAs, and the human IGF2R steady state mRNA levels were quantitated by RT-qPCR experiments. The data demonstrate that hsa-miR-657 does not significantly reduce human IGF2R mRNA levels compared with N.C. and mock (Fig. 3A), indicating that hsa-miR-657 does not target human IGF2R mRNA for degradation. Furthermore, to investigate the effect of hsa-miR-657 on IGF2R protein levels, the soluble IGF2R in the supernatants, obtained from Hep G2 cells transfected with miRNAs, were quantitated by ELISA. The data exhibit that Hep G2 cells transfected with hsa-miR-657 show a significant reduction in the expression of human soluble IGF2R compared with N.C. (Fig. 3B). Taken together, these data reveal that hsa-miR-657 significantly reduces human IGF2R expression by inhibition of translation but not degradation of mRNA.

Discussion

Besides the influences by environmental factors such as the increased sedentary lifestyles and more plentiful food, an increasing number of novel genetic components identified by genome-wide association studies (GWAS) have been found to predispose to type 2 diabetes. Furthermore, performing studies focusing on functional variants in these findings are indispensable.

To date the vast majority of functional variants identified are those that alter the primary sequence and consequently change the structure of proteins. Notably, the regulatory variations increasingly emerged to play an important role in genetic variation a few years ago. Different from a variety of advances elucidating the functional variants which are mainly located in promoter or enhancer region, about 2–3 years ago, a new layer of allele specific miRNA-mediated gene regulation was discovered to participate in those specific associations that variants in 3′UTR in susceptible genes are associated with human disorders [7,23,24].

Thus, the recent two findings by Villuendas et al. and Costello et al. naturally led us to hypothesize that ACAA-insertion/deletion polymorphism located at the 3′UTR of *IGF2R* gene may affect the expression of IGF2R via a specific miRNA-mediated gene regulation, which consequently at least in part contributes to the association of IGF2R with type 2 diabetes. In this study, we present evidence that the ACAA-insertion/deletion polymorphism within hsa-miR-657 target region in *IGF2R* 3′UTR alters the interaction between IGF2R and hsa-miR-657. Furthermore, by overexpressing hsa-miR-657 mimics, we testify that the expression of soluble IGF2R protein in ACAA-insertion expressing cells, rather than mRNA, is regulated by hsa-miR-657. Our findings suggest that the binding between *IGF2R* 3′UTR harboring ACAA-insertion and hsa-miR-657 is weaker than that of ACAA-deletion, which leads to relative higher levels of soluble IGF2R protein (Fig. 4).

In general, the genetic basis of type 2 diabetes mellitus has been considered disturbances either in insulin action or in β-cell function by most research. Peripheral insulin resistance is believed to lead to the increased insulin secretion, which is an indicator of adaptive mechanisms of the pancreas β-cells and therefore is considered the cause of "β-cells exhaustion" with subsequent development of hyperglycemia [11]. Our findings reveal the possibility of an allele specific miRNA regulation mechanism may participate in the pathogenesis of type 2 diabetes. Since it is assumed that decreased IGF2R in the mouse may stimulate growth via the reduced clearance of plasma circulating IGF2 [25], and IGF2R can be developmentally regulated, especially during fetal development and organogenesis [26], we propose that relative higher soluble IGF2R protein levels may result in increased clearance of plasma circulating IGF2. Given that insulin-like growth factors (IGFs) have been shown to participate in normal growth, and especially fetal pancreas β-cell development [11], we suggest the possibility that hsa-miR-657, or other miRNAs, is involved in this process. Namely, relative higher levels of IGF2R, caused by the type 2 diabetes related variant in allele specific miRNA-mediated regulation manner, could lead to decreased IGF2, which might result in relatively insufficient development of fetal pancreas β-cell and the subsequent development of type 2 diabetes. While, we do not yet know if this allele specific miRNA-mediated regulation mechanism really occurs in the development of pancreatic islet in type 2 diabetes *in vivo*, which may elucidate the association of DNA sequence polymorphism with this complex disease. Future studies concerning this issue remain to be done to test this model.

In conclusion, one mechanism identified by us underlying the association of ACAA-insertion/deletion polymorphism with type 2 diabetes [16] is that hsa-miR-657 regulates *IGF2R* gene expression by binding to 3′UTR in an allele specific manner, which may prob-

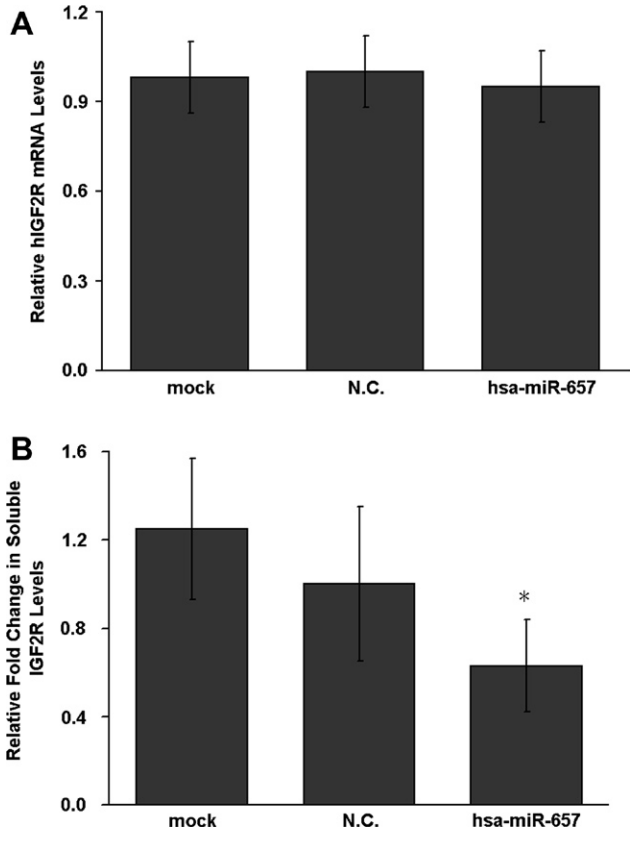

Fig. 3. hsa-miR-657 inhibits human IGF2R expression. Hep G2 cells were either mock-transfected or transfected with negative control miRNA (N.C.) or hsa-miR-657. (A) RT-qPCR was performed to utilize total RNA isolated from transfected cells, and the relative gene expression of *IGF2R* mRNA was normalized to *β-actin* expression. (B) Soluble IGF2R protein levels were determined as described under Materials and methods. The values relative to the mean of N.C. group data are shown.

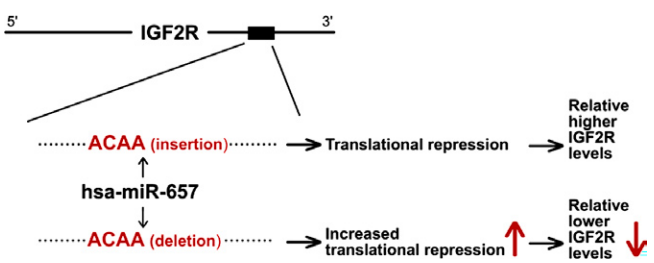

Fig. 4. A model for molecular mechanism of ACAA-insertion/deletion polymorphism mediated hsa-miR-657 regulation of human IGF2R expression change. The ACAA-insertion allele in the 3′UTR of *IGF2R* possesses relatively weak hsa-miR-657 binding, which leads to relative higher IGF2R levels.

ably bring at least in part a novel mechanisms for the pathogenesis of type 2 diabetes. Moreover, in the future it will possibly rise that hsa-miR-657 or in combination with functional DNA sequence polymorphism may be valuable in the treatment of human type 2 diabetes.

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