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Human angiotensinogen +11525 C/A polymorphism modulates its gene expression through microRNA binding

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Mopidevi B, Ponnala M, Kumar A. Human angiotensinogen +11525 C/A polymorphism modulates its gene expression through microRNA binding. *Physiol Genomics* 45: 901–906, 2013. First published August 6, 2013; doi:10.1152/physiolgenomics.00056.2013.—Hypertension is a serious risk factor for cardiovascular disease. Like other complex disease, hypertension is caused by a combination of genetic and environmental factors. The renin-angiotensin system plays an important role in the regulation of blood pressure. Angiotensinogen (AGT) gene is associated with essential hypertension in Caucasians, Japanese, and Asian-Indian subjects. AGT gene may also be associated with cardiac hypertrophy, coronary atherosclerosis, and microangiopathy related cerebral damage. Human AGT gene has a C/A polymorphism at nucleoside 11525 (rs7079) that is located in the 3′-untranslated region (3′-UTR) and is modestly associated with increased blood pressure. We show here that miR-31 and miR-584 bind strongly to the hAGT 3′-UTR containing 11525C allele compared with 11525A allele. We also show that transfection of miR-31 and miR-584 downregulates the hAGT mRNA and protein levels in human liver cells. These studies may provide new therapeutic approach to reduce hypertension.

human angiotensinogen gene; 3′-untranslated region; single nucleotide polymorphism; miRNA; rs7079; hypertension; blood pressure regulation

HYPERTENSION IS A SERIOUS risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure (7). It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. The incidence of hypertension and complications due to hypertension is even greater in the African American population (8). It has been shown that lowering blood pressure has a beneficial effect on the severity of other cardiovascular diseases (20). Hypertension is a polygenic disease, and it has been estimated by segregation analysis and twin studies that genetic differences account for ~45% of the interindividual differences in blood pressure. The renin-angiotensin system plays an important role in the regulation of blood pressure (10, 19). The octapeptide, angiotensin II, is one of the most active vasopressor agents and is obtained from its precursor molecule, angiotensinogen (AGT), by the combined proteolytic action of renin and angiotensin converting enzyme (ACE). Angiotensin receptor blockers and ACE inhibitors are therefore potent antihypertensive agents (4, 22). AGT is primarily synthesized in the liver and to a lesser extent in the fat, kidney, brain, heart, adrenal, and vascular walls (3). The plasma concentration of AGT is close to the Michaelis constant of the enzymatic reaction between renin and AGT (9). For this reason, a rise in plasma AGT levels can lead to a parallel increase in the formation of angiotensin II that may ultimately result in hypertension.

AGT gene is associated with essential hypertension in Caucasians (12), Japanese (11), and Asian Indian subjects (21) and may also be associated with cardiac hypertrophy (25), coronary atherosclerosis (14), and microangiopathy-related cerebral damage (26, 27). Human AGT gene has a C/A polymorphism at +11525 (rs7079) located in the 3′-untranslated region (3′-UTR). Ono et al. (23) have shown that the rs7079A allele is associated with Japanese nonalcoholic steatohepatitis, suggesting that this allele increases the expression of AGT gene in these subjects. However, the role of this polymorphism in the regulation of hAGT expression is not known. MicroRNAs (miRNAs) are endogenously synthesized short noncoding RNAs of ~20–22 nucleotides that have been shown to play an important role in modulating mammalian gene expression and regulate several key biological functions (16–18). miRNAs normally inhibit the expression of their target genes via post-transcriptional mechanisms (1, 2). It is estimated that there are >1,000 human miRNAs and that the expression of ~60% of the human protein coding genes can be downregulated by miRNAs. Single nucleotide polymorphism (SNPs) in the 3′-UTR may create, destroy, or modify the efficiency of miRNA binding to the 3′-UTR of a gene and may modulate its expression (28).

We show here that nucleotide sequence of the hAGT gene containing 7079C allele binds to two miRNAs (miR-584 and miR-31) and these miRNAs downregulate the expression of hAGT gene containing 11525C allele in human liver cells.

MATERIALS AND METHODS

Cell culture. Human liver cells (Hep3B) and human embryonic kidney cells (HEK293) were purchased from American Type Culture Collection (Manassas, VA) and were grown as a monolayer in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C.

Luciferase reporter constructs. The full-length 3′-UTR of human angiotensinogen (hAGT) gene containing the 11525C allele was PCR-amplified from human BAC (RP11-505D24) clone using forward 5′-GGA CTA GTG GCC AGG GCC CCA GAA C-3′ and reverse 5′-CGA CGC GTT TTT TGG AGG CTT ATT G-3′ primers. The forward primer contains an *Spe*1 restriction site at the 5′-end, and the reverse primer contains an *Mlu*1 restriction site at its 3′-end for cloning purpose. The amplified product was treated with restriction enzymes *Spe*1 and *Mlu*1 and was directionally cloned into the multiple cloning site of pMIR-REPORT miRNA expression reporter vector system (Life Technologies). This vector contains the firefly luciferase gene under the control of a mammalian CMV promoter/terminator system and miRNA target cloning region downstream of the luciferase coding sequence. Plasmid DNA was isolated from recombinant colonies and sequenced to ensure the authenticity and designated as hAGT-pMIR/Luc-11525C.

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The hAGT-pMIR/Luc-11525C plasmid was used as a template to mutate the 11525C allele to 11525A allele using the QuikChange Lightning Site-Directed Mutagenesis (Agilent Technologies) Kit and designated as hAGT-pMIR/Luc-11525A. For site-specific mutagenesis, we used a forward mutagenic primer (5′-TTC TGT TTG AAT GCG GAA C A A TAG CTG GTT ATT TCT CCC-3′) and a complementary reverse mutagenic primer (5′-GGG AGA AAT AAC CAG CTA T T G TTC CGC ATT CAA ACA GAA-3′) in PCR as per the manufacturer's recommendation. Following PCR, the product was treated with *Dpn*I endonuclease to digest the parental DNA and leave intact the mutated plasmid DNA. The mutation, 11525A, was confirmed by dideoxy-chain termination sequencing. Finally, transformed bacterial cultures were grown, and the reporter constructs were purified by use of a Hi speed Plasmid Medi Kit (Qiagen).

Transfection and luciferase assay. The Hep3B and HEK293 cells were seeded at 8×10^4 cells/well in 12-well plates containing 1 ml of complete medium having 10% FBS. All the transfections were optimized and performed using siPORT NeoFx transfection reagent (Ambion) as per the manufacturer's recommendations. In brief, 1.5 μl of NeoFX transfection reagent was mixed with 48.5 μl OptiMEM (Invitrogen, 11058021), and the mixture was incubated at room temperature for 10 min. miR-31, miR-584 mimics, or its inhibitors (Ambion) (50 nM) were diluted in 50 μl of OptiMEM and then added into the NeoFX transfection agent. The final mixture was incubated at room temperature for another 10 min and used for transfection. A scrambled mirVana miRNA mimic negative control, purchased from Ambion (cat. #4464058), does not have any binding site for eukaryotic 3′-UTRs and was used as a negative control. Mutated miR mimics were also purchased from Ambion. Sequences of mutated miR mimics are shown in Fig. 1. Mock transfections were performed in the absence of additional miRNA mimics. The cotransfection experiments were performed with 100 ng of pMIR/Luc reporter constructs. The pMIR-REPORT β-galactosidase reporter control vector was used to normalize the transfection efficiency. After 48 h of incubation, the cells were washed with 1× ice-cold PBS, and the luciferase and β-galactosidase activities were determined by Dual-Light Luciferase and β-galactosidase Reporter Gene Assay System (Life Technologies) as per the manufacturer recommendations.

Real-time PCR. Total RNA was isolated from miRNA transfected cells with RNeasy Plus minikit (Qiagen). We reverse-transcribed 1 μg of RNA into cDNA using first-strand cDNA synthesis kit (Fermentas). Quantitative (Q) real-time RT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and Applied Biosystems thermocycler (7500 Fast Real-Time PCR System). Primers for human AGT (PPH01807A), and human GAPDH (PPH00150E) were purchased from SuperArray Bioscience After 95°C incubation for 10 min, 40 cycles of PCR (95°C for 30 s, 60°C for 30 s) were then performed using 2 μl of cDNA, 50 nM PCR primers, and 12.5 μl of SYBR Green PCR Master Mix in 25 μl reactions. Threshold cycles for three replicate reactions were determined, and relative hAGT gene expression was calculated following normalization with human GAPDH.

Western blot. The cell lysates from miRNA transfected cells were prepared using Qproteome Mammalian Protein Prep Kit (Qiagen) as per the manufacturer's recommendations. Lysates were cleared by centrifugation, and a total of 30 μg/well protein was resolved by 10% SDS-PAGE and then was electro-blotted onto a polyvinylidene difluoride membrane. The membranes were blocked for 1 h in Odyssey blocking buffer, followed by an overnight incubation at 4°C with 1:2,000 dilution of hAGT monoclonal antibody raised in rabbit (Epitomics) and then with 1:10,000 dilution of secondary antibody conjugated with IRDye800 or IRDye700 at room temperature for 60 min, protected from light along with gentle shaking. The blots were visualized with an Odyssey Imaging System (LI-COR) and were subjected to quantitative analyses using ImageJ. The results were averaged and normalized with β-actin.

Statistical analysis. All data are expressed as means ± SD of at least three independent experiments. Statistical analyses were performed with Student's two-tailed *t*-test. The data were in normal distribution and were checked by the D'Agostino-Pearson omnibus test using GraphPad Prism V6. Values of $P < 0.05$ were considered significant.

RESULTS

hAGT +11525 C/A polymorphism (rs7079) occurs in the miR-31 and miR-584 binding site. The 3′-UTR of the hAGT gene contains a C/A polymorphism at +11525 (rs7079). Since miRNAs may bind to a nucleotide sequence located in the 3′-UTR of a gene and modulate its expression by posttranscriptional or posttranslational mechanism, we were interested in finding whether miRNA binds to this region of the hAGT gene and modulates its expression. Computer analysis by TargetScan and miRanda revealed that a polymorphism at 11525 of the hAGT gene occurs in the seed binding sequence of miR-584 and miR-31 (Fig. 1). Computational modeling suggested that the nucleotide sequence of the hAGT gene containing the 11525C allele has perfect Watson-Crick base pairing with miR-584 and miR-31 seed sequence. On the other hand, presence of the 11525A allele leads to a destabilization of the Watson-Crick base pairing with the seed sequence. We therefore hypothesized that, due to decreased binding of miR-584 and miR-31, human subjects having the 11525A allele may have increased hAGT expression compared with human subjects with the 11525C allele. This may lead to increased plasma or tissue hAGT level, ultimately resulting in increased blood pressure in human subjects with the 11525A allele compared with human subjects with the 11525C allele.

hAGT +11525A allele reduces miR-31 and miR-584 induced luciferase expression in HEK293 cells. To test the hypothesis that miR-31 and miR-584 regulate the expression of

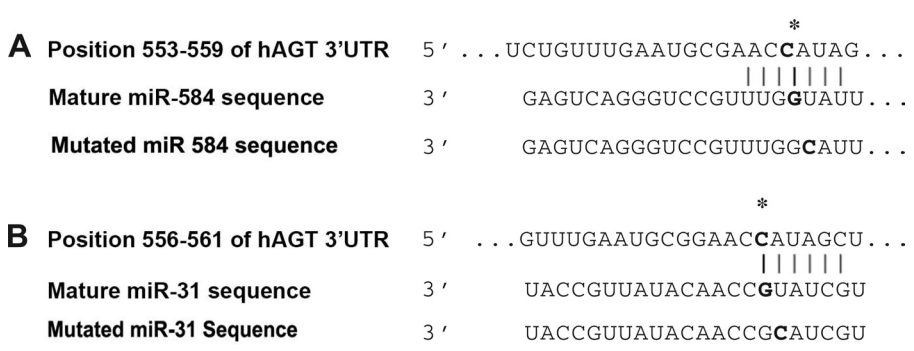

Fig. 1. microRNA (miR)-584 and miR-31 bind to the 11525C allele of the human angiotensinogen (hAGT) gene. In silico Target Scan-predicted complementarity between miR-584 and hAGT 3′-untranslated region (UTR) is shown in *A*, and miRanda-predicted complementarity between miR-31 and hAGT 3′-UTR is shown in *B*. Solid lines represent the Watson-Crick base pairing, and the 11525 C/A polymorphism is marked in boldface. The nucleotide sequence of mutated miR-584 and miR-31 mimics is also shown.

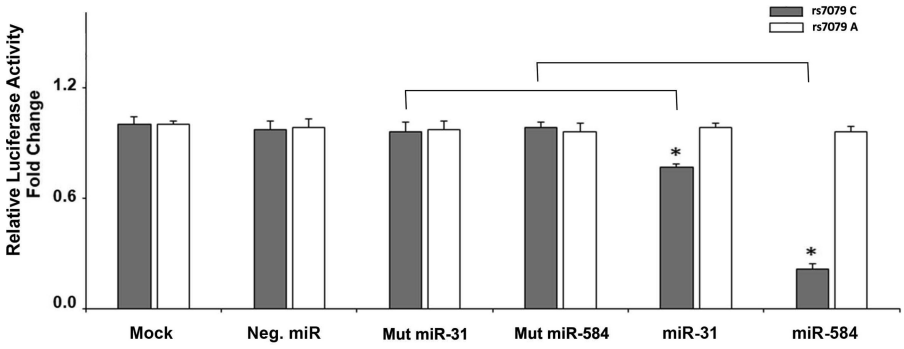

Fig. 2. Effect of microRNA (miRNA) mimics miR-31 and miR-584 on 11525 C/A polymorphism of the hAGT by dual luciferase (Luc) assay in HEK293 cells. Human embryonic kidney cells (HEK293) were cotransfected either in the absence of miRNA (mock) or with plasmid construct hAGT-pMIR/Luc-11525C or hAGT-pMIR/Luc-11525A along with negative miR, mut miR-31, mut miR-584, miR-31, or miR-584 miRNA mimics (50 nM). Luc activity was measured after 48 h of transfection. Firefly Luc activity was normalized to β-galactosidase expression, and the mean activities ± SE from 3 independent experiments are shown. $*P < 0.05$. Luc activity of the 11525C allele is shown by dark bar and of the 11525A allele is shown by light bar.

hAGT, the entire 632 bp 3′-UTR of the hAGT gene (containing either the 11525C allele or 11525A allele) was subcloned in an expression vector immediately downstream from the firefly luciferase coding region. The resulting plasmid constructs were designated as hAGT-pMIR/Luc-11525C (containing +11525C allele) and hAGT-pMIR/Luc-11525A (containing +11525A allele). To test the effect of miR-31 and miR-584 on luciferase gene expression, either hAGT-pMIR/Luc-11525C or hAGT-pMIR/Luc-11525A was cotransfected in HEK293 cells along with either miR-31 or miR-584, and luciferase activity was determined after 48 h of transfection. We also performed control experiments in the absence of miRNA (mock), in the presence of nontargeting miRNA (Neg. miR), and in the presence of mutated miR mimics. We used HEK293 cells in this experiment because they are easily transfected and do not express the hAGT gene. (Initial experiments were performed in the presence of 10, 20, 50, and 100 nM miRNA, and since 50 nM miRNA gave the optimum result, this concentration was used in all experiments.) Results of this experiment show that luciferase expression remained the same either in the absence of miRNA (mock) or in the presence of nontargeting miRNA. On the other hand luciferase expression was downregulated in the presence of either miR-31 or miR-584 (Fig. 2). The luciferase expression was downregulated by ~80% in the presence of miR-584 and by ~30% in the presence of miR-31.

hAGT +11525A allele reduces miR-31 and miR-584 induced luciferase expression in Hep3B cells. We next performed the luciferase assay by transient transfection of the abovementioned reporter constructs in human liver (Hep3B) cells since AGT gene is expressed in the liver cells. Results of this experiment show that luciferase expression remained the same either in the absence of miRNA or in the presence of nontargeting miRNA. On the other hand luciferase expression was downregulated by ~20% in the presence of miR-31 and ~40% in the presence of miR-584 in Hep3B cells (Fig. 3). Taken together, results of these experiments showed that miR-31 and miR-584 bind to +11525C allele of the hAGT gene and downregulate the luciferase gene expression either in human kidney or human liver cells.

Anti-miRNAs relieve the miR-584- and miR-31-induced downregulation of the luciferase gene-containing +11525C allele of the 3′-UTR of hAGT gene. Since the above experiments suggested that miR-584 and miR-31 bind to the +11525C allele and downregulate the expression of luciferase gene, we argued that anti-miRNAs should relieve this miRNA induced downregulation. In the next set of experiments, we therefore examined the effect of increasing amount of anti-miRNA in the presence of a fixed amount of either miR-584 or miR-31 by measuring the luciferase expression. Results of this experiment using 10, 20, and 50 nM anti-miR-584 in the presence of 50 nm miR-584 showed that anti-miR-584 relieves the miR-584-induced downregulation of luciferase expression in a dose-dependent manner (Fig. 4). Thus miR-584-induced downregulation of the luciferase gene is completely attenuated when 50 nM anti-miR-584 is used in transient transfection in Hep3B cells. Results of a similar experiment showing the effect of anti-miR-31 on miR-31-induced downregulation of the luciferase gene are presented in Fig. 5.

miR-584 and miR-31 reduce human AGT mRNA level in human liver (Hep3B) cells. Since the abovementioned experiments suggested that miR-31 and miR-584 selectively bind to +11525C in the reporter construct containing 3′-UTR of the hAGT gene and downregulate the luciferase expression, we next wanted to examine whether these miRNAs reduce the hAGT mRNA level in human liver cells. Before performing this experiment, we determined the nucleotide sequence of 3′-UTR of the AGT gene in Hep3B cells. Results of this

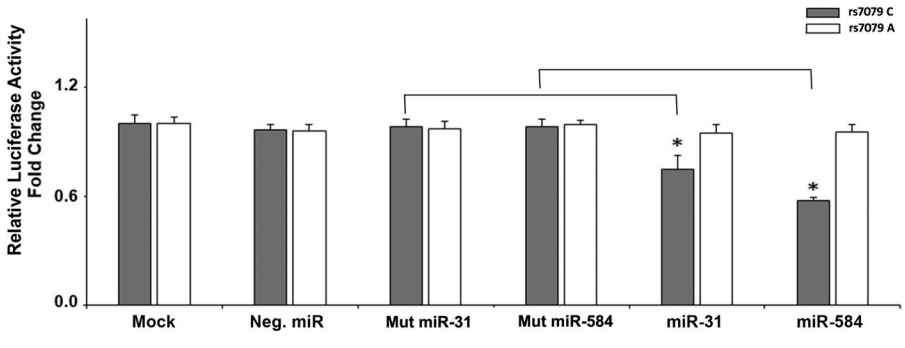

Fig. 3. Effect of miRNA mimics miR-31 and miR-584 on 11525 C/A polymorphism of the hAGT by dual luciferase assay in human hepatoma (Hep3B) cells. Hep3B cells were cotransfected with hAGT-pMIR/Luc-11525C or hAGT-pMIR/Luc-11525A and miRNA mimics and Luc activity was determined as described in Fig. 2. $*P < 0.05$.

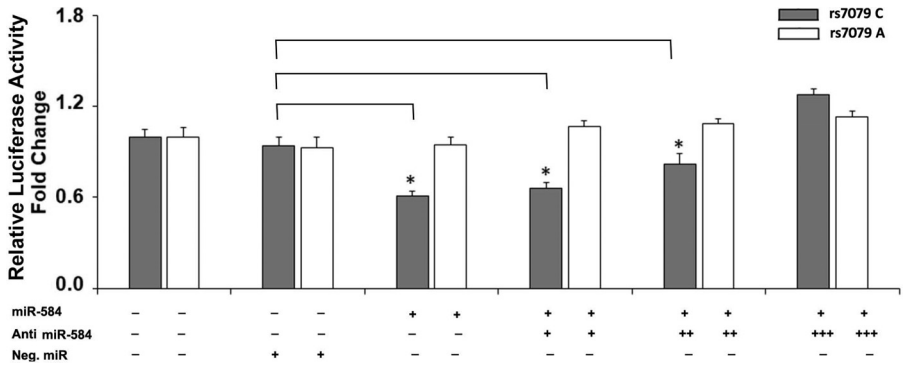

Fig. 4. Anti-miR-584 mimics reduce the repression capacity of miR-584 in a dose-dependent manner in a dual luciferase assay system in Hep3B cells. Hep3B cells were cotransfected with plasmid construct hAGT-pMIR/Luc-11525C or hAGT-pMIR/Luc-11525A, negative miR or miR-584 (50 nM), and the anti-miR-584 (10, 20, or 50 nM shown by +, ++, and +++, respectively). Mock transfections were performed without transfecting with any miRNA. Luc activity was measured after 48 h of transfection. Firefly Luc activity was normalized to β-galactosidase expression, and the mean activities ± SE from 3 independent experiments are shown. *$P < 0.05$. Luc activity of the rs7079C allele is shown by dark bar, and of rs7079A allele is shown by light bar.

experiment suggested that Hep3B cells have the +11525C allele of the hAGT gene (data not shown). To analyze the effect of miRNAs on hAGT mRNA level, we transfected nontargeting miR, miR-31, and miR-584 in Hep3B cells and measured the mRNA level by Q-RT-PCR. Results of this experiments show that hAGT mRNA levels remained same in the absence of miRNA (mock) or in the presence of nontargeting miRNA in Hep3B cells. However, hAGT mRNA level was reduced to 67% in the presence of 50 nm miR-31 and to 61% in the presence of 50 nm miR-584 (Fig. 6).

miR-584 and miR-31 reduce human AGT protein level in human hepatoma (Hep3B) cells. Since the abovementioned experiments showed that Hep3B cells have the +11525C allele of the hAGT gene and transfection of these cells with miR-584 and miR-31 reduces the hAGT mRNA level, our next objective was to see whether miR-584 and miR-31 transfection reduces the AGT protein level in human hepatoma cells. To perform this experiment, we transfected 50 nM miR-31 and miR-584 in Hep3B cells and analyzed AGT protein level by Western blot assay. Results of the Western blot are shown in Fig. 7*A,* and quantitation of the protein level is shown in Fig. 7*B*. The AGT protein level remained the same when Hep3B cells were transfected in the absence of miR (mock) or in the presence of nontargeting miRNA. On the other hand AGT protein level was reduced to 55% in the presence of miR-31 and to 36% in the presence of miR-584. Results of this experiment suggest that hAGT protein level is reduced by miR-31 and/or miR-584 transfection.

DISCUSSION

In this study, we have analyzed the role of the C/A polymorphism at +11525 of the hAGT gene (rs7079) on its expression. We have earlier shown that SNPs in the promoter region of the hAGT gene alter the binding of transcription factors, which leads to modulation of the promoter activity in transient transfection assays. These polymorphisms modulate the expression of hAGT gene in different tissues of transgenic animals, which leads to altered plasma AGT level and modulation of blood pressure (12, 13). However, the C/A polymorphism at 11525 is in the 3′-UTR of the hAGT gene, and its role in the expression of AGT gene is not known. The recent discovery of miRNAs that may bind to the 3′-UTR and posttranscriptionally regulate the expression of a gene prompted us to examine the role of the C/A polymorphism at 11525 of the hAGT gene on its expression. Computer alignment of the hAGT 3′-UTR sequence by TargetScan and miRanda showed that miR-584 and miR-31 are able to bind the nucleotide sequence around 11525 region of the hAGT gene. The nucleotide sequence of the 3′-UTR of the hAGT gene harboring the 11525C allele has a perfect 7 bp Watson-Crick base pair complementary seed sequence of miR-584 and miR-31. On the other hand, if the hAGT gene has the 11525A allele in its 3′-UTR region, the seed sequence will be interrupted, resulting in a thermodynamically less stable complex. As a result, expression of the 11525C allele of the hAGT gene may be downregulated by posttranscriptional modification of this gene by miR-584 and miR-31. On the other hand, expression of the 11525A allele of the hAGT gene should not be affected by these miRNAs due to a mismatch in the seed sequence.

In support of this hypothesis, we show that transfection of miR-584 and miR-31 reduces the hAGT mRNA level of the hAGT gene in human hepatoma cells (Hep3B cells). We chose Hep3B cells for this experiment because hAGT gene is predominately expressed in liver cells. Moreover, Hep3B cells

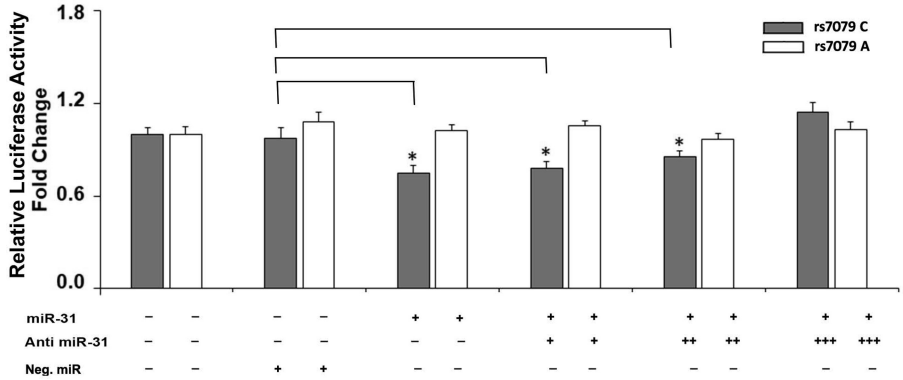

Fig. 5. Anti-miR-31 mimics reduce the repression capacity of miR-31 in a dose-dependent manner in Hep3B cells. Hep3B cells were cotransfected with plasmid construct hAGT-pMIR/Luc-11525C or hAGT-pMIR/Luc-11525A along with negative miR or miR-31 (50 nM), and the anti-miR-31 (10, 20 or 50 nM), and Luc activity was determined as described in Fig 4. Firefly Luc activity was normalized to β-galactosidase expression, and the mean activities ± SE from 3 independent experiments are shown.*$P < 0.05$. Luc activity of the rs7079C allele is shown by dark bar and of rs7079A allele is shown by light bar.

harbor the 11525C allele of the hAGT gene, and therefore miR-31 and miR-584 should bind to the 3′-UTR of hAGT gene in these cells. To further confirm that miR-31 and miR-584 differentially bind to C/A polymorphism at 11525 of the hAGT gene and modulate the expression of this gene, we synthesized reporter constructs where the entire 632 bp of the 3′-UTR of the hAGT gene containing either the 11525C or 11525A allele was ligated in front of the luciferase gene. These reporter constructs were then transfected in either Hep3B or human embryonic kidney (HEK293) cells in the presence of miR-31 or miR-584 mimics. Results of these experiments show that transfection of miR-31 or miR-584 downregulated the luciferase activity of reporter construct containing only the 11525C allele and not the 11525A allele. Our experiments also show that downregulation of the luciferase activity containing the 11525C allele of the hAGT gene in Hep3B and HEK293 cells was relieved in the presence of antagomir of miR-31 and miR-584. Finally, we have shown that miR-31 and miR-584 not only modulate the hAGT mRNA levels but also modulate hAGT protein levels. Thus transfection of either miR-31 or miR-584 reduces the hAGT protein level in Hep3B cells as shown by Western blot analysis.

The frequency of the 11525A allele (rs7079A) is 0.20 and of the 11525C allele (rs7079C) is 0.8 in humans. It is important to mention that the seed sequence AACCAUA that binds to miR-584 and miR-31 in human AGT gene is also present in the monkey AGT gene. Thus it seems that 11525C is the ancient allele and was mutated to the 11525A allele in some human populations. The physiological benefit for this mutation (if any) is not clear at this time.

Recent advances in the synthesis and chemistry of nucleic acids have allowed efficient methods to inhibit specific miRNAs in vitro and in vivo (15). Locked nucleic acid (LNA)-modified anti-miRNAs (anti-miR) have been shown to be very effective for inhibiting miRNAs in nonhuman primates. Thus, Elmen et al. (5, 6) have shown that simple systemic delivery of a unconjugated, PBS-formulated LNA-anti-miR effectively antagonizes the liver-expressed miR-122 and lowers plasma cholesterol in African green monkeys. Efficient silencing of miR-122 was achieved by three doses of 10 mg/kg of LNA-anti-miR without any evidence of toxicity or histopathological changes. Putta et al. (24) have recently demonstrated specific and efficient reduction of miR-192 in vivo in mouse kidneys injected with LNA-modified anti-miR-192 (LNA-anti-miR-192). The specific reduction of renal miR-192 decreases renal fibrosis and improves proteinuria, lending support for the possibility of an anti-miRNA-based therapeutic approach to the treatment of diabetic nephropathy. These studies suggest that it may be possible to use miRNA-based strategies to reduce blood pressure and related cardiovascular diseases in future.

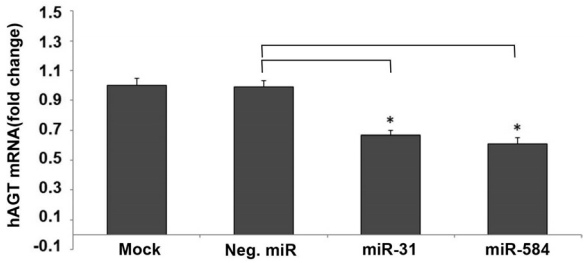

Fig. 6. miR-31 and miR-584 downregulate the endogenous hAGT mRNA levels in Hep3B cells. Hep3B cells were transfected either in the absence of miRNA (mock) or in the presence of negative miR, miR-31, or miR-584 microRNA mimics (50 nM). After 48 h, transfected cells were washed twice with ice-cold 1× PBS, and total RNA was isolated and reverse-transcribed into cDNA. Quantitative RT-PCR was performed, and cycle threshold (ct) values for hAGT were determined and normalized to GAPDH expression. Relative ct values for each group were determined and compared with those for mock transfections taken as 1 for fold change. Each group represents the mean of at least 3 independent experiments. $*P < 0.05$.

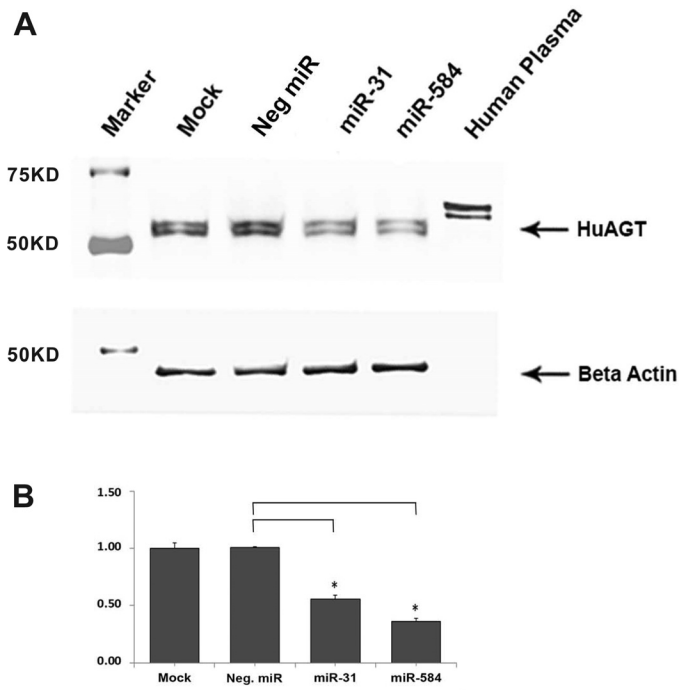

Fig. 7. AGT level is reduced in human liver cells after miR-31 or miR-584 treatment. *A*: Hep3B cells were transfected either in the absence of miRNA (mock) or in the presence of negative miR, miR-31 or miR-584 (50 nM). After 48 h of transfection, cell extract was subjected to SDS-PAGE followed by Western blot analysis using anti-human AGT antibody or anti-β-actin antibody. Human plasma was used as a positive control. hAGT and β-actin-specific bands are shown by arrows. *B*: hAGT and β-actin protein levels of each individual band were measured and normalized to β-actin levels. The downregulation of hAGT protein after transfection with miR-31 and miR-584 was calculated from 3 independent experiments. $*P < 0.05$.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B.M. and M.P. performed experiments; B.M. and M.P. analyzed data; B.M. interpreted results of experiments; B.M. prepared figures; B.M. drafted manuscript; A.K. conception and design of research; A.K. edited and revised manuscript; A.K. approved final version of manuscript.

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