Human microRNA-155 on Chromosome 21 Differentially Interacts with Its Polymorphic Target in the *AGTR1* 3′ Untranslated Region: A Mechanism for Functional Single-Nucleotide Polymorphisms Related to Phenotypes

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Animal microRNAs (miRNAs) regulate gene expression through base pairing to their targets within the 3′ untranslated region (UTR) of protein-coding genes. Single-nucleotide polymorphisms (SNPs) located within such target sites can affect miRNA regulation. We mapped annotated SNPs onto a collection of experimentally supported human miRNA targets. Of the 143 experimentally supported human target sites, 9 contain 12 SNPs. We further experimentally investigated one of these target sites for hsa-miR-155, within the 3′ UTR of the human *AGTR1* gene that contains SNP *rs5186*. Using reporter silencing assays, we show that hsa-miR-155 down-regulates the expression of only the 1166A, and not the 1166C, allele of *rs5186*. Remarkably, the 1166C allele has been associated with hypertension in many studies. Thus, the 1166C allele may be functionally associated with hypertension by abrogating regulation by hsa-miR-155, thereby elevating *AGTR1* levels. Since hsa-miR-155 is on chromosome 21, we hypothesize that the observed lower blood pressure in trisomy 21 is partially caused by the overexpression of hsa-miR-155 leading to allele-specific underexpression of *AGTR1*. Indeed, we have shown in fibroblasts from monozygotic twins discordant for trisomy 21 that levels of AGTR1 protein are lower in trisomy 21.

MicroRNAs (miRNAs) are ~21-nt small RNAs involved in posttranscriptional gene regulation. They have been shown to guide the RNA-induced silencing complex of proteins to specific target sites within mRNAs to induce immediate cleavage, localization to P-bodies, or translational repression.[1] These target sites are thought to be most prevalent in the 3′ UTR of mRNAs.[1]

SNPs are DNA sequence variations that occur at a rate of ~1 in every 1,000 bp in the human genome. SNPs that occur in the 3′ UTR can affect gene regulation by interfering with posttranscriptional activity, such as protein binding, polyadenylation, and miRNA binding. Two recent studies reported SNPs that alter the gene expression level by modifying miRNA targeting activity.[2,3] The first showed that a 3′-UTR SNP in human *SLITRK1* strengthens an existing miR-189 target site, thereby amplifying the down-regulation of *SLITRK1,* which is implicated in Tourette syndrome.[2] The second demonstrated that a 3′-UTR SNP in the sheep *Gdf8* gene creates a new illegitimate miRNA target site, which leads to significant down-regulation of *Gdf8* and contributes to muscular hypertrophy.[3]

miRNA target sites can be categorized into two classes: 5′-dominant and 3′-compensatory. 5′-dominant target sites have perfect base pairing with at least 7 nt at the 5′ end of the miRNA, which is also referred to as the "seed" or "nucleus."[4,5] Such binding is considered in most cases to be sufficient for a functional miRNA-mRNA interaction.[4,5] 3′-compensatory target sites are characterized by either a <7-nt stretch of perfect base pairing or an imperfect 7-nt base pairing with the miRNA 5′ end, followed by an extended base pairing with the miRNA 3′ end.[6–9] There are currently three published data sets that provide a mapping of all known SNPs onto a set of computationally predicted miRNA target sites.[3,10,11] Since the introduction of computational miRNA target–prediction programs in 2003, significant progress has been made in the field.[12,13] However, current algorithms still simulate only a limited part of all the biochemical processes that are responsible for a functional miRNA-mRNA interaction. As a consequence, such algorithms often lack specificity.[12] Therefore, we limit our study to experimentally supported target sites.

An up-to-date and comprehensive collection of experimentally supported miRNA target sites can be found in TarBase 4.0, a manually curated database that includes >600 miRNA–target gene interactions in eight different species.[14] For each supported miRNA target site, the da-

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tabase describes the location within the 3′ UTR where it occurs, the nature of the experiments that were conducted to validate it, and the sufficiency of the site to induce translational repression and/or cleavage. Version 4.0 of TarBase contains 143 experimentally supported human target sites for which the exact genomic location is known.

We mapped the human SNPs from dbSNP build 127 onto the experimentally supported human miRNA target sites mentioned above. This mapping revealed nine human target sites that harbor 12 SNPs (fig. 1). A recently published study employing a similar approach also reports one of these target sites (*EZH2*).[11] For three genes (*KCNH2, CAT-1,* and *ESR1*), the target sites are of the 3′-compensatory class, and, for six genes (*AGTR1, EZH2, HOXA7, SMAD1, DLL1,* and *GJA1*), they are of the 5′-dominant class. Two of the latter six, *AGTR1* and *EZH2,* harbor SNPs that occur in a region that interrupts 5′-dominant base pairing. The *AGTR1* target site harbors three SNPs, one of which, *rs5186* (1166A/C), is located in the region that interacts with the miR-155 seed and is also reported in the Allele Frequency Database (ALFRED). Because the 1166C allele interrupts the interaction with the miR-155 seed (fig. 1), we hypothesized that it may severely impair miR-155 targeting.

To validate our hypothesis, we proceeded with in vitro experiments. TarBase shows that this target site gained experimental support via reporter-silencing assays. More specifically, Martin et al. showed that miR-155 represses *AGTR1* in lung fibroblasts via specific binding to this site.[15] We inserted three different *AGTR1* 3′ UTRs downstream of luciferase reporter genes to create three distinct reporter gene constructs: (1) full-length *AGTR1* 3′ UTR with the major allele (1166A), (2) full-length *AGTR1* 3′ UTR with the minor allele (1166C), and (3) full-length *AGTR1* 3′ UTR with the miR-155 target site deleted. The *AGTR1* 3′ UTR with the 1166A allele was amplified by PCR with use of sense (5′-CATGTTCGAAACCTGTCCATAAAG-3′) and antisense (5′-ATAAAATTATTTTATTTTAAAGTAAAT-3′) primers. The PCR products were inserted at the *Xba*I site in the 3′ UTR of the luciferase gene of the pTAL-Luc vector (Promega). The pTAL-Luc *AGTR1* 3′ UTR with the 1166C allele was constructed similarly with genomic DNA containing the polymorphic *AGTR1* 1166C allele. Finally, the pTal-Luc *AGTR1* deletion 1163–1169 construct was generated using the Quickchange XL site-directed mutagenesis kit (Stratagene), with use of a forward mutagenic deletion primer (5′-TCTGCAGCACTTCACTACCAAATGGCTACT-TTTCAGAATTGAAGG-3′) and a complementary reverse mutagenic deletion primer (5′-CCTTCAATTCTGAAAAGT-AGCCATTTGGTAGTGAAGTGCTGCAGA-3′).

293T cells were seeded in 96-well plates (10^4 cells/well) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. 293T cells were then transfected with pTAL-Luc *AGTR1* 3′ UTR A1166/C1166/deletion 1163–1169 (800 ng), pRL-SV40 *Renilla* (40 ng [Promega]), and 2.5 nM, 10 nM, and 20 nM of miR-155 precursor or 20 nM of miR-Let7c as control (double-stranded RNA oligonucleotide [Ambion]) with the use of Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's protocol. After 48 h, firefly and *Renilla* luciferase activities were measured using a dual luciferase assay (Promega). The relative reporter activity was obtained by normalization to the *Renilla* activity.

In the presence of miR-155, the expression of the luciferase containing the 1166A allele is significantly reduced (fig. 2), confirming the function of miR-155 on the *AGTR1* target gene. In contrast, the expression of the luciferase containing the 1166C allele remains unchanged in the presence or absence of miR-155 (fig. 2). Furthermore, this result is similar to that obtained with the ΔmiR-155 target-site luciferase construct, implying that the 1166C allele abolishes the target site and impairs the ability of miR-155 binding, thereby elevating the levels of *AGTR1*. When we repeated the same experiments with a higher (20 nM) and lower (2.5 nM) amount of miR-155, the observation did not change (fig. 2).

Hsa-miR-155 maps onto human chromosome 21 and therefore is triplicated in trisomy 21. Remarkably, it has been reported that individuals with trisomy 21 have lower levels of diastolic and systolic blood pressure than do age- and sex-matched control individuals.[16,17] We hypothesize that overexpression of miR-155 in trisomy 21 excessively suppresses the *AGTR1* common alleles and that this may be one mechanism contributing to the lower blood pressure in individuals with trisomy 21.

To evaluate the expression of miR-155 in trisomy 21, we performed real-time quantitative PCR in fibroblasts from an MZ twin pair discordant for trisomy 21— that is, one twin was unaffected, and the other had a trisomy 21. Both twins were homozygotes for the 1166A *AGTR1* allele, which is the target of miR-155. Twins were chosen so that any gene-expression differences could be attributed only to the supernumerary chromosome 21 and not to polymorphic variability in the rest of the genome. Fibroblast cells from these MZ twins were grown in DMEM with Glutamax I medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin and streptomycin mix (Invitrogen). Total RNA was extracted by Trizol reagent (Invitrogen). RNA quality was assessed using an Agilent 2100 BioAnalyzer with the RNA 6000 Nano LabChip. miRNA expression levels were detected by the SYBR Green I real-time PCR miRNA detection kit (*mir*vana [Ambion]), with the use of SuperTaq (Enzyme Technologies). Reactions were set up, by use of a Biomek 2000 robot (Beckman), in a 10-μl volume in 384-well plates. Each RNA sample was analyzed in six technical replicates and in three independent experiments, with the use of primer sets specific for miR-155 and four different miRNAs used for normalization: miR-124, miR-130b, miR-24, and miR-26a. PCRs were run in an ABI 7900 Sequence Detection System (SDS [Applied Biosystems]).

Raw cycle-threshold (C_T) values were obtained using SDS 2.0 software (Applied Biosystems). From the six replicates, values outside the median ± 0.5 were classified as outliers

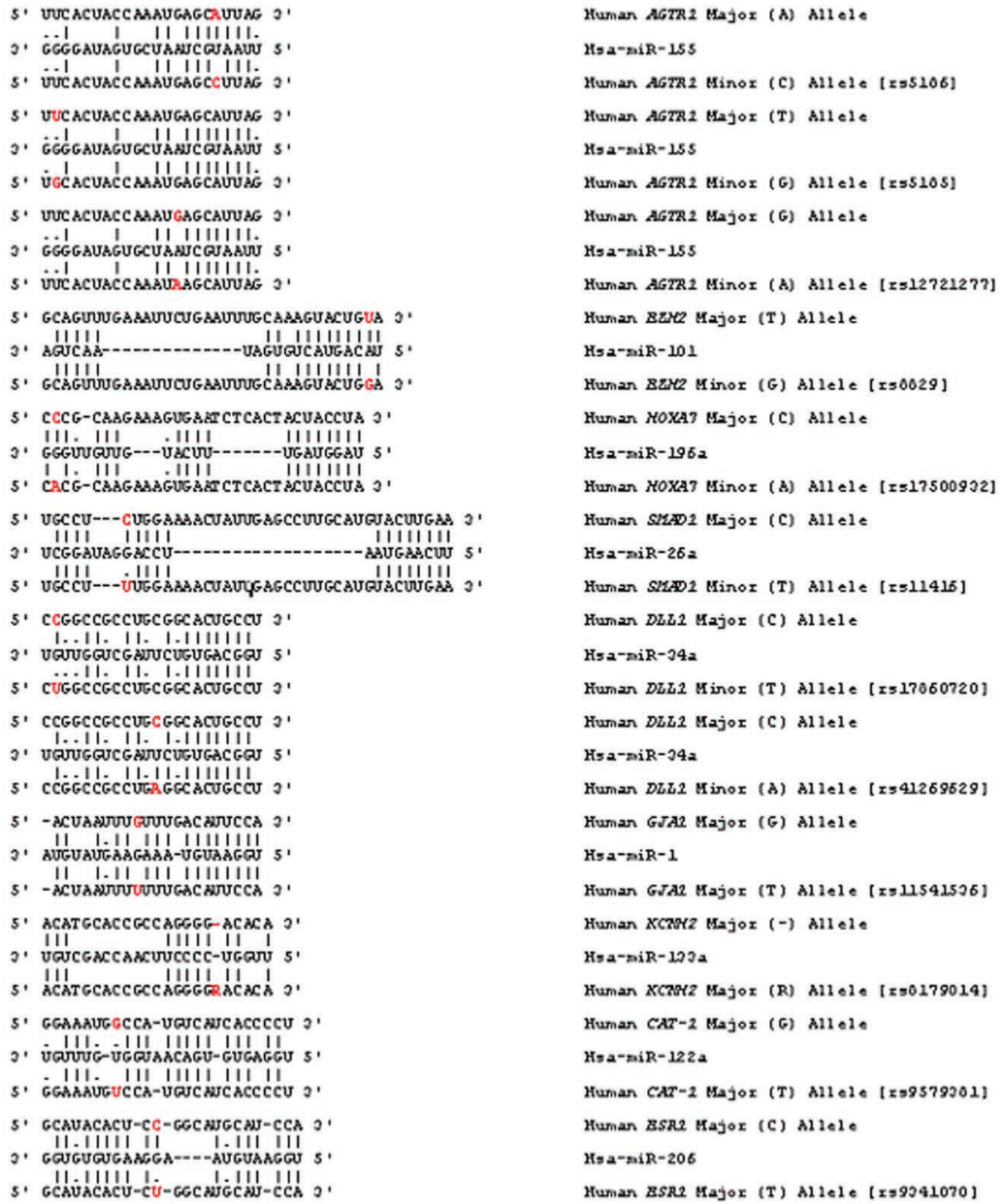

Figure 1. Nine experimentally supported miRNA target sites harbor 12 SNPs. The studies that reported the nine miRNA-target interactions shown here used one or more of the several publicly available computational target-prediction programs to compute the putative binding diagrams. Seven of the nine SNPs (*rs8179014*, *rs17500932*, *rs41269629*, *rs17860720*, *rs11416*, *rs5185*, and *rs12721277*) occur in a region of a 5′-dominant target site that does not affect 5′-dominant base pairing. Three SNPs (*rs11541536*, *rs9579381*, and *rs9341070*) occur in a region of a 3′-compensatory target site that can affect 3′-compensatory base pairing. Two SNPs (*rs5186* and *rs8829*) occur in a region of a 5′-dominant target site that does affect 5′-dominant base pairing.

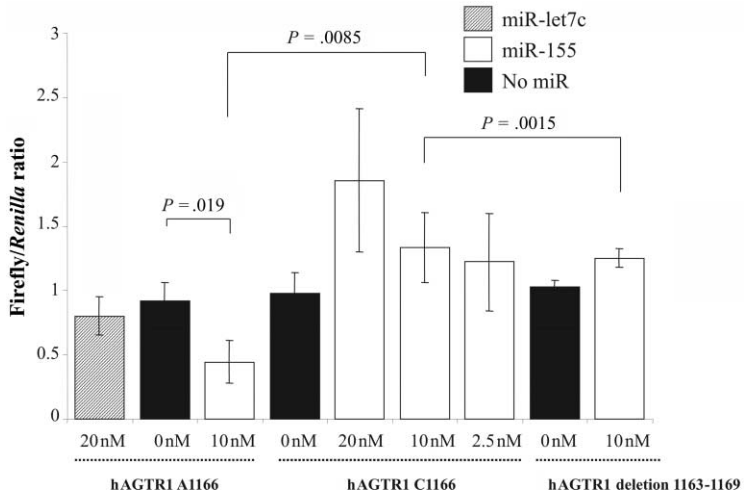

Figure 2. Effects of miR-155 on the luciferase reporter genes bearing 3′ UTR segments from hAGTR1 3′-UTR. 293T cells were cotransfected with pTAL-Luc hAGTR1 3′-UTR 1166A or pTAL-Luc hAGTR1 3′ UTR 1166C, pTAL-Luc hAGTR1 3′ UTR deletion 1163–1169, and different concentrations (20, 10, or 2.5 nM) of miR-155 (*white bars*) or no miRNA (*black bars*) or 20 nM of Let-7c (unrelated miRNA [*dashed lines*]). Values are means of firefly/*Renilla* ratio from three independent experiments (each with three culture replicates). Error bars indicate 1 SD of three independent experiments. *P* values were calculated from a two-sided, two-sample *t* test.

and were excluded. For all calculations, C_T values were converted to quantity (q) with the formula $q = 2^{-C_T}$. The relative expression level of mature miRNAs was normalized according to geNorm,[18] with the use of miR-124, miR-130b, miR-24, and miR-26a as references to determine the normalization factor. Each miRNA was then mean normalized across the two individuals. Normalized relative expression values thus have a median of 1. The results shown in figure 3 indicate that miR-155 is indeed significantly overexpressed in fibroblasts of individuals with trisomy 21. miR-155 is conserved in mouse, and it maps within the region of mouse chromosome 16 that is triplicated in the TS65Dn partial mouse model of Down syndrome.[19,20] However, this and other such mouse models could not be used to test the hypothesis, because the miR-155 target sequence in mouse *AGTR1* is not conserved, and, thus, it is not expected that mmu-miR-155 interacts with mouse *AGTR1*.

The increased levels of miR-155 expression in trisomy 21 predict that the AGTR1 protein levels in fibroblasts isolated from the MZ twins discordant for trisomy 21 should be lower in the twin with trisomy 21 than in the unaffected euploid twin. Since commercially available AGTR1 antibodies are not reliable, we performed whole-cell AGTR1 binding assays as described elsewhere.[15] In brief, the fibroblasts were allowed to reach confluence, the cell medium was aspirated and was replaced with monoiodinated 125I-[Sar1,Ile8] AngII (2–3 × 105 cpm [Peptide Radioiodination Service]) in Hank's Balanced Salt Solution, 20 mM Hepes, and 0.1% BSA). After incubation at room temperature for 60 min, unbound ligand was removed by washing each well twice with 1 ml ice-cold PBS. Bound ligand was recovered by dissolving the protein in each well with 1 ml 0.5-M NaOH and 0.01% SDS. Nonspecific binding was determined by performing the binding assay in the presence of 1 μM unlabeled AngII. The quantity of 125I-[Sar1,Ile8] AngII present in each sample was determined using a Cobra γ-spectrophotometer (Packard Bell). Protein content in wells was assessed using the BioRad Protein Assay dye reagent (BioRad). Values shown in figure 4 represent specific (total minus nonspecific) binding. Consistent with the prediction, the trisomy 21 fibroblasts showed reduced protein levels of AGTR1 compared with that of the euploid fibroblasts (~30% less; $P < .01$).

ALFRED reports that the minor allele, 1166C, occurs with much higher frequency in white populations (20%–

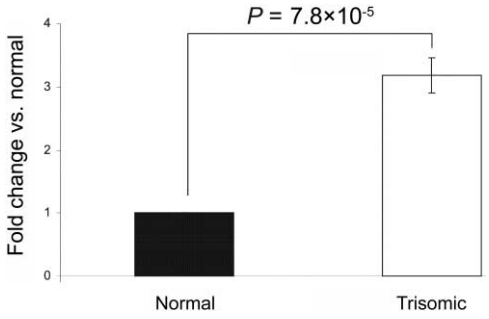

Figure 3. Real-time quantitative PCR for mature miR-155 expression in fibroblast cells from MZ twins discordant for trisomy 21. Data are means (±SD) from three independent experiments analyzed in six replicates. Data are normalized with reference microRNAs, as mentioned in the text. *P* values were calculated from a two-sided, one-sample *t* test.

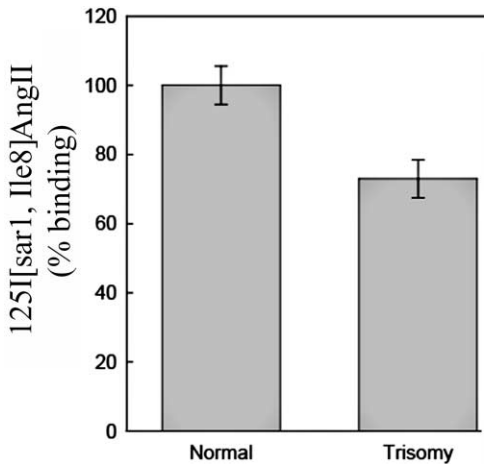

Figure 4. AGTR1 radioligand binding studies in fibroblasts from MZ twins discordant for trisomy 21. There is a lower amount of AGTR1 protein in trisomy 21 fibroblasts than in the unaffected euploid twin fibroblasts.

30%) compared with African or Asian populations (5%–6%). We performed a literature search and identified 40 studies that investigated the association of the 1166C allele with hypertension (MIM 145500) in various populations (table A1). Of these studies, 18 reported that 1166C is a risk allele for hypertension. Type I error, ethnic admixture, and linkage disequilibrium (LD) are highly unlikely to explain the large number of studies with positive findings (table A1). Furthermore, the discrepancies between studies are usually greatly decreased in subpopulations defined by ethnic origin—for example, all six studies with Japanese subjects failed to find significant association, whereas 9 of 13 studies with white subjects showed significant association. Hirschhorn et al. indicate gene-environment factors as a likely source of variable results, and this could play a significant role in this case, since there is a strong ethnic bias in the studies with positive findings.[60] Therefore, although the results must be interpreted as a function of age, sex, and ethnic origin, the literature clearly indicates an association of the 1166C allele with hypertension in several populations.

Despite this literature evidence, the molecular mechanism of the association has remained uncertain. Interestingly, it has been independently shown that elevated levels of *AGTR1* contribute to cardiovascular disease.[61–63] Accordingly, antagonists of AGTR1 have been developed and are now widely used in the treatment of hypertension.[64] This suggests that at least one compelling mechanism for the association of 1166C with cardiovascular disease is the abrogation of miR-155 binding, which elevates *AGTR1* levels (fig. 5). According to expression studies, two human organs—the spleen and kidney—concomitantly express *AGTR1* and miR-155. Since the kidney is a critical organ for the regulation of blood pressure, we postulate that the abrogation of miR-155 binding to *AGTR1* is most detrimental in the kidney. We note here that the mouse and rat genomes preserve only one of either miR-155 or its *AGTR1* target site. Therefore, rodents are not useful model organisms for this study.

A recent study provided convincing evidence that miRNA target sites are under negative selective pressure

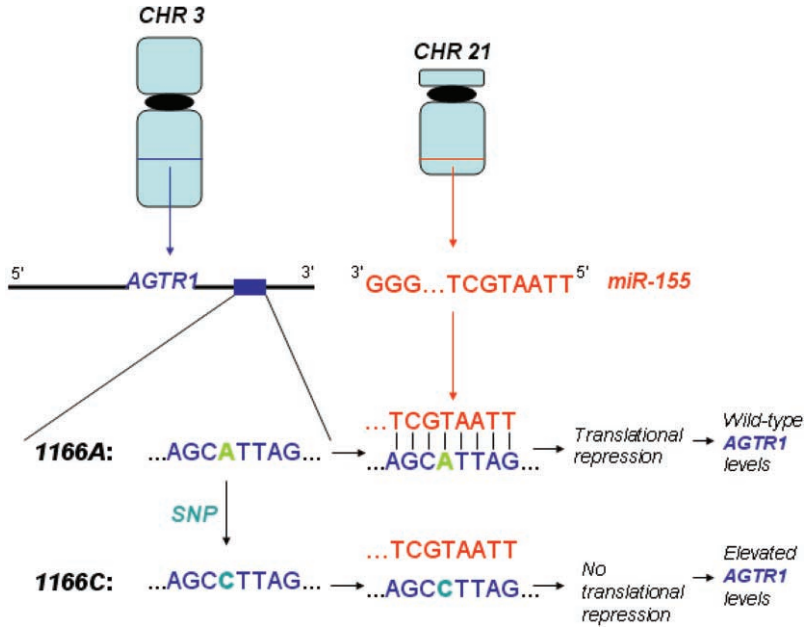

Figure 5. Model for molecular mechanism of 1166C association with hypertension. The 1166C allele in the 3′ UTR of *AGTR1* abrogates miR-155 binding, which induces elevated levels of *AGTR1*.

to harbor SNPs.[10] These studies include the ~450 experimentally verified human miRNAs in the miRBase database.[65] The number of miRNAs in miRBase has grown exponentially during the past few years, and there is little expectation that this growth is complete. A recent massively parallel sequencing effort identified 447 novel miRNAs, many of which are primate specific.[66] Furthermore, current computational/experimental studies conjecture that there are at least 1,000 functional miRNAs encoded within the human genome.[67,68] Further characterization of these miRNAs and improvement of target-prediction programs to enable the specific prediction of nonconserved target sites will be critical for a more complete analysis of SNP abundance in miRNA target sites.

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Appendix A

Literature Review

Of the 40 studies we examined in the literature, 18 had positive results and 22 had negative results. Discrepancies among the study results are greatly reduced in subpopulations defined by ethnic origin. Each study used a 5% significance level for type I error in the association tests. Therefore, on the basis of statistical error, we would expect, on average, only two false-positive associations among 40 studies. However, given 18 positive associations among 40 studies, the type I error alone cannot explain the discrepancy in the results, barring the unlikely possibility that there are hundreds of negative associations that have not been published. Furthermore, the discrepancy is usually greatly decreased in subpopulations defined by ethnic origin—for example, all six studies with Japanese subjects failed to find significant association, whereas 9 of 13 studies with white subjects showed significant association. Some studies on other non–ethnically defined subpopulations also produced more consistent results. For example, the three studies on pregnancy-induced hypertension all found significant association. It must be noted that some studies that used the same population type produced inconsistent results. For example, Reich et al.[27] found association in males but not in females; meanwhile, Tiret et al.[57] found association in females but not in males. Such discrepancies are not surprising in association testing, which is highlighted by the fact that the Hirschhorn et al.[60] review found that only 6 of 166 associations that had been studied at least three times were positive >75% of the time.

The work of Hirschhorn et al.[60] lists the two factors that can result in false-positive associations as ethnic admixture and LD. Ethnic admixture is unlikely to cause the observed high number of positive associations, because the studies comprised a large number of diverse populations. Furthermore, not all studies with positive results used the case-control design that is susceptible to stratification—for example, Kainulainen et al.[59] used a family-based study resulting in a highly significant association. LD can result if the subjects tend to descend from a recent common ancestor. However, most of the studies used populations that were sufficiently heterogeneous to avoid this issue, and, furthermore, no markers have been found in any of the studies to be in LD with the A1166C polymorphism. Therefore, LD is unlikely to explain the large number of positive results. Hirschhorn et al.[60] indicate gene-environment factors as a likely source of variable results, and this could play a significant role in our case, since there is a strong ethnic bias in the positive studies. Therefore, any association of *rs5186* with cardiovascular risk must be interpreted as a function of population—in particular, age, sex, and ethnic origin.

Hirschhorn et al.[60] also discuss the likelihood of obtaining false-negative results when the genetic effect is relatively weak and there is a lack of power in the study to detect it. Given that the association is real, this is likely the cause of some of the observed false-negative results, because the A1166C polymorphism has a fairly low allele frequency in the population (~10% to ~20% in the 40 studies considered). Moreover, when an association is found, it is not at an extreme level in any case—for example, an allele frequency of 28% versus 16% in cases versus controls in the work of Kainulainen et al.[59] Indeed, a number of the studies found positive trends or *P* values that are on the threshold of significance. Therefore, we conclude that the literature provides a very strong case for a true association of the A1166C polymorphism and hypertension in several major subpopulations.

Table A1. Results of 40 Studies that Tested the Association of *AGTR1* 1166C with Hypertension

Study	Association
Ono et al.[21]	None in Japanese
Takami et al.[22]	None in Japanese
Sugimoto et al.[23]	None in Japanese
Kato et al.[24]	None in Japanese
Katsuya et al.[25]	None in elderly Japanese
Bonnardeaux et al.[26]	In whites
Reich et al.[27]	In white males
Castellano et al.[28]	In Italians with "clinical" but not "ambulatory" blood pressure and homozygosity
Schmidt et al.[29]	None in whites
Thomas et al.[30]	None in Chinese
Thomas et al.[31]	None in Chinese
Zhu et al.[32]	In Chinese
Barbalic et al.[33]	None in Croatians
Kaidashev et al.[34]	In Ukrainians
Zhang et al.[35]	None in Chinese (Han)
Spiering et al.[36]	None in whites
Abdollahi et al.[37]	None
Henskens et al.[38]	Association
Gardier et al.[39]	None
Kobashi et al.[40]	With pregnancy hypertension
Agachan et al.[41]	In Turks
Liu et al.[42]	In Tibetans but not in Han or Yi
Porto et al.[43]	None with young-onset hypertension
Petrovic et al.[44]	None with young-onset hypertension
Stankovic et al.[45]	In Serbian males but not in females
Hindorff et al.[46]	In whites but not in large white and black mixed study sample
Jiang et al.[47]	In Chinese (Han)
Giner et al.[48]	None in Spanish
Dzida et al.[49]	In Polish
Davis et al.[50]	None
Li et al.[51]	None in Chinese
Xiang et al.[52]	None in Chinese
Nalogowska et al.[53]	With pregnancy hypertension
Seremak-Mrozikiewicz et al.[54]	With pregnancy hypertension in Polish
Liyou et al.[55]	None in Australian elderly
Berge et al.[56]	Trend seen in three Norwegian populations but not to level of statistical significance
Tiret et al.[57]	In French females but not in males
Wang et al.[58]	In whites
Kainulainen et al.[59]	In Finns

Web Resources

The URLs for data presented herein are as follows:

ALFRED, http://alfred.med.yale.edu/alfred/
dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for hypertension)
TarBase, http://diana.pcbi.upenn.edu/tarbase (for the database of experimentally supported targets)

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