

## Allele-Specific Targeting of microRNAs to *HLA-G* and Risk of Asthma

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*HLA-G* is a nonclassic, class I HLA molecule that has important immunomodulatory properties. Previously, we identified *HLA-G* as an asthma-susceptibility gene and discovered that the risk of asthma in a child was determined by both the child's *HLA-G* genotype and the mother's affection status. Here we report a SNP in the 3' untranslated region of *HLA-G* that influences the targeting of three microRNAs (miRNAs) to this gene, and we suggest that allele-specific targeting of these miRNAs accounts, at least in part, for our earlier observations on *HLA-G* and the risk of asthma.

The class Ib HLA gene, *HLA-G* (MIM 142871), is primarily expressed by fetal cells at the maternal-fetal interface, where it displays immunosuppressive properties, and is thought to contribute to the maternal tolerance of the genetically foreign fetus.<sup>1</sup> We previously identified *HLA-G* as an asthma (MIM 600807) gene in a positional cloning study in white Chicago families.<sup>2</sup> In the Chicago families and in a Dutch population, the risk genotype at *HLA-G* varied depending on whether the mother had asthma or bronchial hyperresponsiveness (BHR) or was unaffected. For example, in both populations, the GG genotype at SNP -964G/A in the promoter region of *HLA-G* was associated with asthma in children of affected mothers, whereas the AA genotype was associated with asthma in children of unaffected mothers. A similar interaction was observed with +1489C/T, the SNP that explained nearly the entire linkage signal in the Chicago families.<sup>2</sup> We then focused our studies on the highly polymorphic promoter region and showed that the -964G/A SNP tagged two major haplotype clades with evidence of longstanding balancing selection,<sup>3</sup> but we could not demonstrate functional differences between these two promoter clades by use of luciferase reporter assays.<sup>4</sup> Last, we showed expression of soluble (s)HLA-G protein in bronchial epithelial cells in two individuals with asthma but not in one unaffected control individual, suggesting that this gene was up-regulated in the asthmatic lungs.<sup>2</sup>

Because of the strong linkage disequilibrium (LD) between SNPs in the *HLA-G* gene (fig. 1), we turned our attention to other regions of the gene to identify variation that contributes to asthma risk. As part of these studies, we identified in the 3' UTR of *HLA-G* a putative target site for three microRNAs (miRNAs): *miR-148a*, *miR-148b*, and *miR-152*. A C/G SNP at +3142 in the *HLA-G* mRNA is located in the seed region of this target site.

miRNAs are a family of endogenous, small, noncoding RNAs, ~22 nt in length, that negatively regulate gene expression by suppressing translation or degrading mRNA.<sup>5</sup> They have important and diversified functions—including development and differentiation,<sup>6</sup> apoptosis,<sup>7</sup> hematopoiesis,<sup>8</sup> and tumorigenesis,<sup>9</sup> as well as insulin secretion<sup>10</sup> and viral defense<sup>11</sup>—and have been shown to be involved in various human diseases, including cancer<sup>12</sup> and fragile X syndrome.<sup>13</sup> Although polymorphisms in miRNA genes may be scarce,<sup>14</sup> polymorphisms in miRNA target sites are abundant. Some of these polymorphisms influence the binding of miRNAs and have been associated with muscularity in sheep and with Tourette syndrome.<sup>15,16</sup> Here we demonstrate that +3142C/G influences miRNA targeting of *HLA-G* and is associated with risk of asthma.

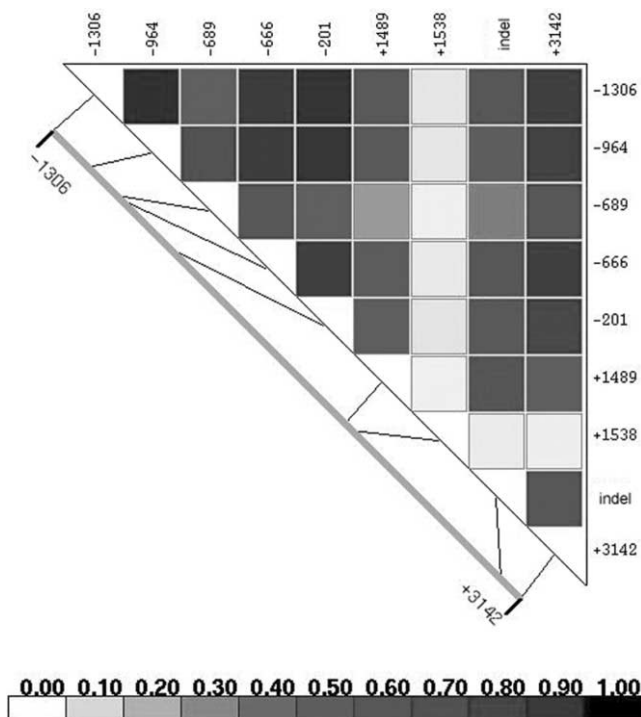
For miRNA target site predictions in *HLA-G*, we used MicroInspector,<sup>17</sup> TargetScan,<sup>18</sup> DIANA-microT,<sup>19</sup> MiRanda,<sup>6</sup> and PicTar,<sup>20</sup> as well as our own algorithm, which is based on the criteria of Watson-Crick base-pair of the 5' "2–8" nucleotides of miRNA to target sequence and more stable binding of miRNA to the target region than to the flanking region. On the basis of the results of those prediction programs, we selected three target sites for further study: target for *miR-148a/148b/152*, target for *miR-143*, and target for *miR-524\**. We used RNAhybrid<sup>21</sup> to analyze the targeting of *miR-148a*, *miR-148b*, and *miR-152* to *HLA-G*. On the basis of the calculation of minimum free energy (MFE), the binding of miRNA to the +3142G allele is predicted to be more stable than binding to the +3142C allele (fig. 2). Thus, +3142 might influence the binding of miRNA to *HLA-G*, and therefore HLA-G protein expression, in an allele-specific manner. To test this hypothesis, we performed luciferase assays. We used firefly luciferase plasmid pLS-HX, which was constructed by inserting firefly luciferase ORF between *SacI* and *XbaI* of pCL-

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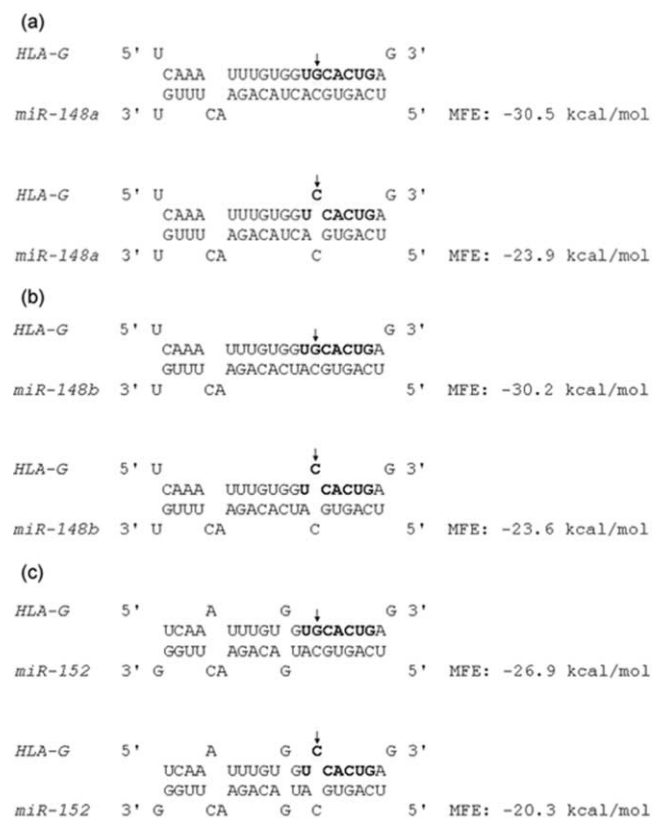


**Figure 1.** Pairwise linkage disequilibrium ( $r^2$ ) map of *HLA-G* in the Chicago families. The LD plot was made by LDPlotter.

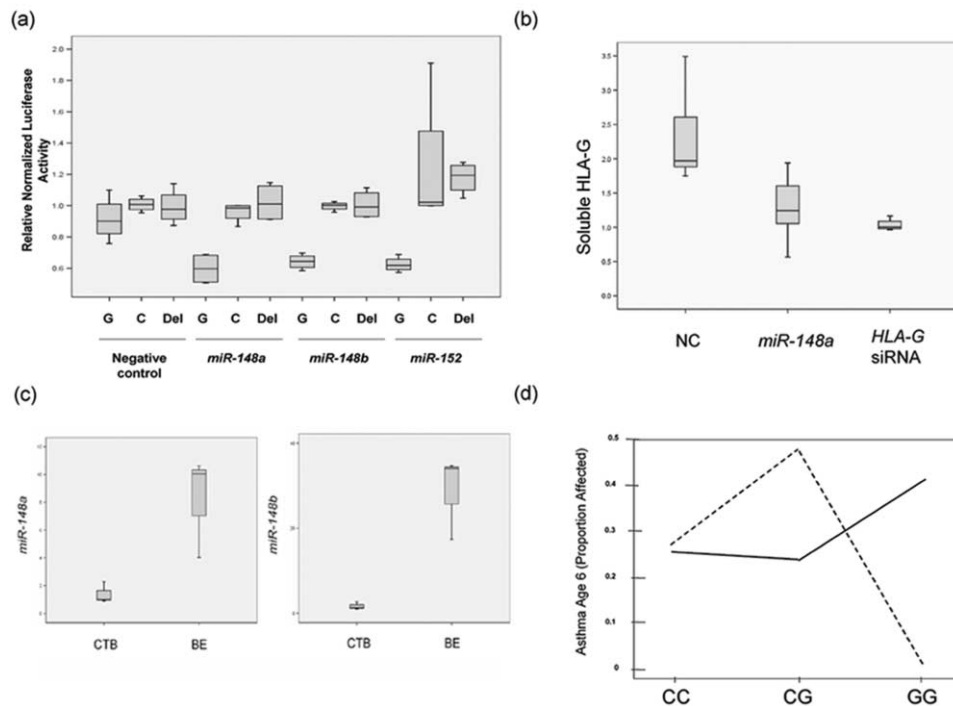
con,<sup>22</sup> and renilla plasmid pRL, which was constructed by inserting renilla luciferase between *SacI* and *EcoRI* of pCL-con. A 365-bp region of *HLA-G* 3' UTR, containing the insertion allele at the exon 8 insertion/deletion (indel),<sup>23</sup> was amplified using forward (CTAGAAGCTTTGTGAAACAGCTGCCC) and reverse (CGCTAGTCTAGATGTCTCTCAAATTC) primers, was digested with *HindIII* and *XbaI*, and was ligated into pLS-HX. Site-directed mutagenesis was performed using QuikChange II XL kit (Stratagene) to generate pluc-HLAG-G and pluc-HLAG-C that carry either C or G at *HLA-G* +3142, and pluc-HLA-G-Del, which has the miRNA target site (22 bp) deleted. We then co-transfected pluc-HLAG-G, pluc-HLAG-C, or pluc-HLA-Del and pRL with either *miR-148a*, *miR-148b*, *miR-152*, or a negative control miRNA (NC miRNA) (Dharmacon) into a human bronchial epithelial cell line, 16HBE14o.<sup>24</sup> For each well, 50 ng luciferase plasmid, 2 ng renilla plasmid, and 20 nM (final concentration) miRNA were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were lysed 40 h after transfection, and luciferase and renilla activities were determined by use of Dual-Luciferase Reporter kit (Promega) and Veritas Microplate Luminometer (Turner Biosystems). We found that, although normalized luciferase activity of either plasmid was not significantly different when cells were cotransfected with NC miRNA, the activity of pluc-HLAG-G in the presence of each of the three miRNAs was significantly lower than those of pluc-HLAG-C and pluc-

*HLA-G-Del*. This indicates allele-specific targeting of the three miRNAs to *HLA-G* (fig. 3a). We were not able to confirm the target for *miR-143* or the one for *miR-524\** by luciferase experimentation.

To validate the presence of the miRNA target site in the endogenous *HLA-G* gene, we used a choriocarcinoma cell line JEG3 (ATCC), which constitutively expresses high levels of *HLA-G* and has the GG genotype at +3142. We transfected JEG3 cells with *miR-148a*, NC miRNA, or *HLA-G* small interfering RNA (siRNA) (positive control), and we measured soluble *HLA-G* level in cell supernatants 5 d after transfection by use of an *HLA-G* ELISA kit (Biovendor & Exbio). s*HLA-G* levels were significantly decreased by *miR-148a* and by *HLA-G* siRNA, compared with the NC miRNA ( $P = .019$  and  $P = .0048$ , respectively), confirming the presence of a functional target site on the +3142G allele (fig. 3b). Next, we measured expression of the three miRNAs by real-time PCR in mRNA from primary cytotrophoblast (CTB) cells, which have high constitutive expression of *HLA-G*, and in primary bronchial epithelial cells,<sup>25</sup> which have low constitutive expression of *HLA-G*. miRNA was extracted by *mirVana* miRNA isolation kit (Ambion), and miRNA real-time PCR was performed with 20 ng miRNA and *mirVana* miRNA detection kit and



**Figure 2.** Predicted binding of *miR-148a*, *miR-148b*, and *miR-152* to *HLA-G*. The seed region of the target site is shown in bold letters, and +3142C/G is indicated by an arrow. The minimum free energy (MFE) of the RNA duplex was analyzed by RNAhybrid.



**Figure 3.** *HLA-G* +3142C/G affects the targeting of *miR-148a*, *miR-148b*, and *miR-152* to *HLA-G* and interacts with mother's asthma status to determine risk of asthma in the child. *a*, Luciferase assays showing the allele-specific targeting of the three miRNAs to *HLA-G* in 16HBE14o- cells. pluc-HLAG-G (G), pluc-HLAG-C (C), or pluc-HLA-G-Del (Del) luciferase plasmid was cotransfected with negative control miRNA, *miR-148a*, *miR-148b*, or *miR-152*. At least six replicate assays were performed for each transfection. For each sample, luciferase activity was normalized by renilla activity and then normalized again by the median value of the pluc-HLAG-C plasmid within each miRNA transfection group. The *P* values for the difference in luciferase activity of the three plasmids are as follows. For NC miRNA transfection: G versus C,  $P > .05$ ; G versus Del,  $P > .05$ . For *miR-148a* transfection: G versus C,  $P = .0002$ ; G versus Del,  $P < .0001$ . For *miR-148b* transfection: G versus C,  $P < .0001$ ; G versus Del,  $P < .0001$ . For *miR-152* transfection: G versus C,  $P = .0002$ ; G versus Del,  $P = .0016$ . *b*, Endogenous *HLA-G* expression is inhibited by *miR-148a* in JEG3 cells. JEG3 cells, which are +3142GG, were transfected with either negative control miRNA, *miR-148a*, or *HLA-G* siRNA as a positive control. A total of seven replicate assays were performed for each RNA. *miR-148a* and *HLA-G* siRNA significantly reduced the level of sHLA-G in JEG3 supernatant compared with the NC miRNA. sHLA-G level was normalized to the median of the *HLA-G* siRNA group. *c*, *miR-148a* and *miR-148b* levels in primary cytotrophoblast (CTB) cells from six individuals and primary bronchial epithelial (BE) cells from three individuals, determined by miRNA real-time PCR. *miR-148a* ( $P = .0016$ ) and *miR-148b* ( $P = .0006$ ) levels were significantly lower in CTB cells than in BE cells. *d*, +3142C/G interacts with maternal asthma status and is associated with asthma in the COAST children. Dashed lines are the children of mothers with asthma ( $N = 58$ ), and solid lines are the children of mothers without asthma ( $N = 119$ ). The interaction  $P = .0011$ .

primer sets (Ambion). For normalization, *U6* real-time PCR was also performed for these samples, and comparative  $C_t$  method was used to calculate the relative abundance of the miRNAs in these samples. The expression of *miR-148a* and *miR-148b*, but not of *miR-152*, was detected in bronchial epithelial cells and to a lesser extent in CTB cells (fig. 3c). The higher expression of the miRNAs in bronchial epithelial cells compared with cytotrophoblast cells is consistent with the inverse expression pattern of *HLA-G*, which is low in the former and high in the latter cells (unpublished data).

*HLA-G* +3142C/G is in high LD with the *HLA-G* promoter polymorphism that was associated with asthma in the Chicago and Dutch families (fig. 1). Thus, it is possible that +3142C/G could account for at least some of the previously observed association. To further explore this

possibility, we studied a third population consisting of children participating in Childhood Origins of ASThma (COAST), a birth cohort study. Couples in which at least one parent had asthma or allergies were recruited into the COAST study in Madison, WI, during pregnancy.<sup>26</sup> Their children are at high risk for developing asthma and have been followed from birth through age 6 years, at which time asthma was diagnosed. We genotyped 180 white COAST children for four polymorphisms in *HLA-G*: -1306G/A, which is in perfect LD with the promoter SNP (-964G/A) that was associated with asthma in the Chicago and Dutch families<sup>2</sup> (fig. 1); +1489C/T, a synonymous SNP in exon 3 that explained most of the evidence for linkage in the Chicago families<sup>2</sup>; a 14-bp indel in the 3' UTR that has been associated with mRNA stability,<sup>23</sup> and +3142C/G. We tested for interaction effects between

mother's affection status (asthma vs. no asthma) and the child's genotype on the development of asthma by age 6 years in this cohort by logistic regression, as described elsewhere.<sup>26</sup> To assess the significance of this interaction, children's asthma status was permuted 10,000 times, whereas mothers' status and children's genotypes were unchanged. For each generated data set, a log likelihood-ratio test was performed to compare the model with interaction to the one without interaction. Permuted  $P$  values ( $P_{\text{interaction}}$ ) were determined by the number of data sets that had smaller log likelihood  $P$  values than the original data set. Significant interactions ( $P < .01$ ) were observed at all SNPs tested except the exon 3 synonymous +1489C/T SNP (table 1); however, the association with +3142C/G was the most significant (permutation  $P_{\text{interaction}} = 0.0011$ ). In the COAST cohort, the GG genotype is protective against asthma among children of asthmatic mothers (0 of 20 asthmatic children were GG;  $P = .0036$ , Fisher's exact test) but is associated with modest risk of asthma among children of nonasthmatic mothers (7 of 32 asthmatic children were GG;  $P = .39$ , Fisher's exact test) (fig. 3d and table 1). Thus, consistent with our earlier report in two other white populations, the +3142G allele, or promoter alleles in strong LD with +3142G ( $r^2 = 0.76$  between +3142 and either -964 or -1306), is a susceptibility allele among children of asthmatic mothers, and the C allele may be a risk allele (albeit more modest) among children of nonasthmatic mothers. Interestingly, the three miRNA genes are located at regions that have been linked to asthma or IgE: 7p15 (*miR-148a*), 12q13 (*miR-148b*), and 17q21 (*miR-152*),<sup>27,28</sup> raising the possibility that variation in the miRNA genes themselves might

contribute to asthma risk, perhaps by interacting with *HLA-G*.

It has become evident in recent years that gene-environment interactions play an important role in the etiology of common diseases,<sup>29,30</sup> and it has even been suggested that genes with main effects on disease risk are more the exception than the rule.<sup>31</sup> Identification and characterization of such interactions is critical for both basic research and clinical practice by helping to explain some of the inconsistencies of disease-association studies and by identifying individuals with genotype-specific risks for detrimental environmental exposures. Here we observed that a functional polymorphism in *HLA-G* interacts with mother's asthma status, which represents an "in utero" environmental exposure. This interaction has now been observed in three white populations ascertained using three different study designs: families ascertained through affected sib pairs in the Chicago study,<sup>2</sup> families ascertained through an affected parent originally studied 20 years earlier in the Dutch study,<sup>32</sup> and children participating in a birth cohort study.<sup>26</sup> Thus, the finding of an interaction between maternal affection status and child's *HLA-G* genotype seems robust to study design and is particularly intriguing for two reasons. First, maternal asthma remains the most significant and best replicated risk factor for asthma in children,<sup>26,33</sup> and second, *HLA-G* is most highly expressed during pregnancy, when it is thought to play a key role in modulating immune tolerance toward the genetically foreign fetus by enhancing the Th2 arm of the immune system.<sup>1</sup> Asthma and allergic disease are also characterized by a skewing toward Th2 immunity. Our study suggests that maternal asthma influences chil-

**Table 1. Interaction of *HLA-G* Genotypes and Maternal Asthma Status on Asthma Risk in COAST Children**

SNP and Genotype	No. of Children, by Affection Status				$P_{\text{interaction}}$
	Mothers Affected		Mothers Unaffected		
	Children Affected	Children Unaffected	Children Affected	Children Unaffected	
<i>HLA-G</i> -1306 ( <i>rs1736936</i> ):					
GG	6	10	14	35	.0045
AG	14	15	11	43	
AA	0	11	5	9	
<i>HLA-G</i> +1489 ( <i>rs1130356</i> ):					
CC	9	16	18	44	.0978
CT	10	12	9	28	
TT	0	3	4	4	
<i>HLA-G</i> exon 8 indel ( <i>rs16375</i> ):					
DelDel	6	15	14	38	.0142
InsDel	14	14	14	44	
InsIns	0	9	4	8	
<i>HLA-G</i> +3142 ( <i>rs1063320</i> ):					
CC	3	8	10	29	.0011
CG	17	18	15	48	
GG	0	12	7	10	

NOTE.—The minor-allele frequencies at each polymorphism in the COAST children are 0.395 (-1306A), 0.293 (+1489T), 0.372 (exon 8 ins), and 0.461 (+3142G).



dren's risk in an allele-specific manner and that the immunosuppressive (Th2-skewing) properties of HLA-G promote asthma pathogenesis.

Although the mechanism of this interaction is still unknown, the results of this study suggest that fetal miRNA regulation might differ in pregnancies of asthmatic and nonasthmatic mothers. The fetal lung is bathed in amniotic fluid that is swallowed by the fetus, and this could provide a direct route for maternal factors to modulate fetal gene expression in the lung as an epigenetic phenomenon. Regardless of mechanism, however, the discovery that a 3' UTR polymorphism affects miRNA targeting reveals a novel functional mechanism by which noncoding polymorphisms can contribute to the risk of common disease. Because ~30% of human genes are regulated by miRNAs,<sup>18</sup> the involvement of miRNA regulation in human diseases may be quite prevalent. This might be particularly relevant to common diseases, to which many susceptibility loci with small effects on gene expression likely contribute. Our study suggests that allele-specific miRNA targeting may indeed be a common mechanism of human disease pathogenesis.

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### Web Resources

Accession numbers and URLs for data presented herein are as follows:

dbSNP, <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp> (for *HLA-G* —1306A/G [accession number *rs1736936*], *HLA-G* +1489C/T [accession number *rs1130356*], *HLA-G* exon 8 indel [accession number *rs16375*], and *HLA-G* +3142C/G [accession number *rs1063320*])

LDPlotter, <http://innateimmunity.net/IIPGA2/Bioinformatics/>  
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for *HLA-G* and asthma)

RNAhybrid, <http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html>

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