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## Differential microRNA regulation of *HLA-C* expression and its association with HIV control

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### Abstract

The *HLA-C* locus is distinct relative to the other classical *HLA* class I loci in that it has relatively limited polymorphism<sup>1</sup>, lower expression on the cell surface<sup>2,3</sup>, and more extensive ligand-receptor interactions with killer cell immunoglobulin-like receptors (KIR)<sup>4</sup>. A single nucleotide polymorphism (SNP) 35Kb upstream of *HLA-C* (rs9264942; termed -35) associates with control of HIV<sup>5-7</sup>, and with levels of *HLA-C* mRNA transcripts<sup>8</sup> and cell surface expression<sup>7</sup>, but the mechanism underlying its varied expression is unknown. We proposed that the -35 SNP is not the causal variant for differential *HLA-C* expression, but rather is marking another polymorphism that directly affects levels of *HLA-C*<sup>7</sup>. Here we show that variation within the 3' untranslated region of *HLA-C* regulates binding of the microRNA Hsa-miR-148a to its target site, resulting in relatively low surface expression of alleles that bind this microRNA and high expression of *HLA-C* alleles

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### Author Contributions

S.K., R.S. performed and evaluated the microRNA experiments. S.K., R.S., H.A.Y., and M.C. designed the study. M.C. directed the study. S.K., R.S., and M.C. wrote the manuscript. X.G., Y.Y., S.B. and M.M. genotyped *HLA*. Statistical analysis was performed by Y.Q. The clinical samples and data were contributed by P.H., D.D., A.T., D. G, S.W., F.P. and B.W. Intellectual input was provided by all authors.

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that escape post-transcriptional regulation. The 3'UTR variant associates strongly with control of HIV, potentially adding to the effects of genetic variation encoding the peptide-binding region of the *HLA* class I loci. Variation in *HLA-C* expression adds another layer of diversity to this highly polymorphic locus that must be considered when deciphering the function of these molecules in health and disease.

MicroRNAs (miRNA) are a class of non-protein coding RNAs that are estimated to regulate 30% of all genes in animals<sup>9</sup> by binding to specific sites in the 3' untranslated regions (3'UTR), resulting in posttranscriptional repression, cleavage or destabilization<sup>10–12</sup>. The 3'UTR of the *HLA-C* gene is predicted to be a target for 26 distinct human miRNAs using three miRNA-target prediction programs (Supplementary Fig. 1), of which three (miR-148a and miR-148b, which bind the same target site, and miR-657) were shown to have the greatest likelihood of binding. We sequenced the 3'UTRs of the common *HLA-C* alleles (Supplementary Fig. 2) and show that the two binding sites of these three miRNAs are polymorphic (Supplementary Fig. 3a). The binding site for miR-148a/miR-148b contains a single base pair insertion/deletion at position 263 downstream of the *HLA-C* stop codon (rs67384697G representing the insertion [263ins] and rs67384697- representing the deletion [263del]) along with other precisely linked variants (259 C/T, 261T/C, 266C/T). These variants are likely to impose a restriction in miR-148a/miR-148b binding, as prediction algorithms indicate that the binding of these miRNAs to the alleles marked by 263ins (e.g. *Cw\*0702*, a low expression allotype) is more stable than to alleles with 263del (e.g. *Cw\*0602*, a high expression allotype) (Supplementary Fig. 3b). Similarly, alleles with 307C within the miR-657 target site are predicted to be better targets of miR-657 than those with 307T (Supplementary Fig. 4). Thus, variation in the 3'UTR of *HLA-C* may influence the interaction between these miRNAs and their putative binding sites in an allele specific manner, potentially leading to differential levels of *HLA-C* allotype expression.

To test directly whether the variation in the *HLA-C* 3'UTR affects levels of protein expression, the full length 3'UTRs containing intact miR-148a/miR-148b and miR-657 binding sites (i.e. 263ins and 307C, respectively; *Cw\*0702*, *Cw\*0303*, *Cw\*0401*, *Cw\*0701*) and disrupted binding sites (i.e. 263del and 307T, respectively; *Cw\*0602*, *Cw\*0802*, *Cw\*1203*, *Cw\*1502*) were each cloned downstream of the luciferase gene in a pGL3 reporter construct (Fig. 1a). The constructs were then transfected into HLA class I negative B721.221 cells, and the level of luciferase activity was measured (fold increase of relative light units [RLU]). Although the *Cw\*0602* 3'UTR repressed luciferase activity as compared to the control containing no 3'UTR, the constructs containing intact miRNA binding sites (i.e. 263ins and 307C; *Cw\*0702*, *Cw\*0303*, *Cw\*0401*, *Cw\*0701*) produced significantly lower luciferase activity relative to the construct containing the 3'UTR of *Cw\*0602*, which contains 263del and 307T (Fig. 1b). However, 3'UTRs from other alleles with the 263del and 307T variants (*Cw\*0802*, *Cw\*1203*, *Cw\*1502*) did not show significant variation in luciferase activity as compared to *Cw\*0602* (Fig. 1b). Psicheck2 reporter constructs containing 3'UTRs of *Cw\*0602* also produced significantly higher luciferase activity as compared to those with *Cw\*0702* 3'UTR (Supplementary Fig. 5a), indicating that this effect was reproducible in a distinct reporter construct. Further, pGL3 constructs containing 3'UTRs of *Cw\*0602* and *Cw\*0702* in three additional cell lines showed the same pattern as

that seen in B721.221, indicating a consistent difference of these 3'UTRs in the regulation of HLA-C expression that is independent of cell type (Supplementary Fig. 5b–e). Thus, *HLA-C* 3'UTR alleles characterized by variation at positions 263 and 307 within miRNA binding regions differentially regulate gene expression.

The expression of endogenous mature miR-148b and miR-152, another miR-148 miRNA family member, was very low as compared to miR-148a, and miR-657 was undetectable in *HLA-C* homozygous BLCL and B721.221 (Supplementary Fig. 6). These data point to the involvement of miR-148a rather than miR-148b, miR-152, or miR-657 in regulation of *HLA-C* expression. Additionally, disruption of the miR-657 binding site by site directed mutagenesis had no effect on luciferase activity (Supplementary Fig. 7a & b), indicating that miR-657 does not affect *HLA-C* expression.

To test whether variants in the miR-148a binding site account for the differential gene expression patterns, we swapped positions 256–266 of the 3'UTR of *Cw\*0602* to match those of *Cw\*0702* and vice versa, thereby providing an intact miR-148a binding site to the 3'UTR of *Cw\*0602* (*06mut*) and disrupting the binding site for miR-148a in the *Cw\*0702* 3'UTR (*07mut*), but leaving the remainder of the 3'UTR sequences intact (Fig. 2a). The luciferase activity of *06mut* was significantly lower than that of *07mut* (Fig. 2b), indicating that the polymorphisms between positions 256–266 in the miR-148a binding region account for the difference in luciferase expression between constructs containing the 3'UTRs of *Cw\*0602* vs. *Cw\*0702*. Two other polymorphic sites, A256C and A267G, in the miR-148a binding site (Supplementary Fig. 3a) distinguish different sets of alleles as compared to 263del/ins, but these two variants had no effect on miRNA-mediated suppression (Supplementary Fig. 7c & d).

Further validation of the differential regulation of *HLA-C* alleles by miR-148a was achieved by co-transfection of B221.227 cell lines with either a mimic or an inhibitor of miR-148a along with a luciferase reporter construct that contained the 3'UTR with 263ins (*Cw\*0702* or *06mut*) or with 263del (*Cw\*0602* or *07mut*). The normalized luciferase activity in cells transfected with the constructs containing the 263del allele (*Cw\*0602* and *07mut* 3'UTR) was not significantly altered by co-transfection with either the mimic or the inhibitor (Fig. 2c and 2d). However, the mimic of miR-148a further repressed luciferase activity in cells transfected with the 263ins allele (*Cw\*0702* and *06mut* 3'UTR), whereas co-transfection with inhibitor rescued the suppression significantly (Fig. 2c and 2d). These data provide further support for allele-specific miR-148a targeting of the *HLA-C* 3'UTR.

B Lymphoblastoid Cell Lines (BLCL) from individuals homozygous for either *Cw\*0602* (BLCL-*Cw\*0602*Hom) or *Cw\*0702* (BLCL-*Cw\*0702*Hom) were used to determine whether the variation in the miR-148a binding site affected endogenous *HLA-C* expression on the cell surface. As described previously, overall *HLA-C* expression on a *Cw\*0602* homozygous cell line was higher than that on a *Cw\*0702* homozygous cell line. As expected, transfection with mimics or inhibitors of miR-148a (Fig. 3a) and miR-148b (Fig. 3b) had no significant effect on cell surface expression of *Cw\*0602*, an allele containing 263del in the 3'UTR that disrupts miR-148a/miR-148b binding. However, transfection of either miR-148a or miR-148b mimic resulted in decreased expression of *Cw\*0702* relative

to cells transfected with a negative control (NC; Fig. 3c & d, respectively), indicating that increased levels of either of these miRNA can further downregulate HLA-C expression of alleles that contain an intact binding site for miR-148a/miR-148b (263ins), such as *Cw\*0702*. The inhibitor of miR-148a significantly increased the level of endogenous *Cw\*0702* expression, but the inhibitor of miR-148b had no effect on expression of *Cw\*0702* (Fig. 3c & d, respectively), confirming the very low levels of miR-148b endogenous expression (Supplementary Fig. 6).

The differential miR-148a regulation of expression across *HLA-C* alleles was precisely reflected in experiments involving: i) additional *HLA-C* homozygous BLCLs (Supplementary Fig. 8a & b), ii) use of another form of miR-148a inhibitor (peptide nucleic acid inhibitor; Supplementary Fig. 8c), and iii) analysis of total cellular HLA-C protein as determined by Western blot (Supplementary Fig. 9–10 and Supplementary note 1). We conclude that miR-148a regulates the expression of HLA-C in an allele-specific manner that is dependent on variation in the miR-148a binding site of the *HLA-C* 3'UTR.

The miR-148a binding site of the *HLA-C* 3'UTR is in strong LD with the -35 SNP that was shown to associate with control of HIV and HLA-C expression levels<sup>5–7</sup> ( $D'=0.75$ ,  $r^2=0.74$ ;  $p<0.0001$ ,  $N=1760$ ). While there is no explanation for a direct causal effect of -35 variation on HLA-C expression<sup>7</sup>, the interaction between miR-148a and its polymorphic binding site in the 3'UTR of *HLA-C* presents a clear rationale for variable levels of HLA-C expression. We determined the frequencies of the 263del/ins genotypes in a cohort of 2527 HIV infected European American (EA) individuals. Subjects with mean plasma viral loads of <2000 copies of viral RNA/ml of plasma (controllers) were enriched for 263del, whereas those with viral loads of >10,000 copies of viral RNA/ml of plasma (noncontrollers) had a significantly higher frequency of 263ins (Supplementary Table S1). Because of the strong LD across the *HLA-C* and *HLA-B* genes (Supplementary Table S2 and S3), we determined whether the 3'UTR variant has an effect on HIV control that is independent of individual *HLA-A*, *-B*, or *-C* alleles. A logistic regression approach with stepwise selection of the *HLA-C* 3'UTR 263 variant along with all *HLA-A*, *-B*, and *-C* alleles that have 1% frequency in our cohort (63 alleles) was employed. In this analysis, the 263del/del vs. 263ins/ins comparison remains significant along with five of the 63 *HLA* alleles (*B\*5701*, *B\*5703*, *B\*2705*, *B\*5801* and *Cw\*1402*; Table 1; for frequencies, see Supplementary Table S1). While these data suggest that the 3'UTR del/ins variant has an independent effect on HIV control (see Supplementary note 2 for potential mechanisms that could explain the association), we still cannot completely rule out the possibility that the strong LD in the region is confounding the results (Supplementary Table S4; Supplementary note 3).

The extensive number of disease associations with *HLA* class I and II genes has largely been ascribed to the polymorphic peptide binding amino acid positions of these molecules. Some reports have speculated that gene expression<sup>13</sup> and/or splicing patterns of the *HLA* genes<sup>14,15</sup> may play a role, but convincing data is missing. Of interest, the *HLA-G* 3'UTR was shown to encode a polymorphic target site for miR-148a/b<sup>16,17</sup>. Levels of HLA-G have been suggested to alter risk of asthma in children of mothers with asthma<sup>17</sup>, though specificity of assays reporting HLA-G expression beyond implanting placental cells has been questioned<sup>18</sup>. Recently, a variant 35Kb upstream of the *HLA-C* gene was shown to

associate with differential HLA-C mRNA expression, cell surface expression, and outcome after HIV infection<sup>5–8</sup>. We have now established a very convincing case that this –35 SNP is marking a functional insertion/deletion variant in the 3'UTR of *HLA-C* that directly determines expression of the various HLA-C allotypes differentially through miR-148a recognition. These data indicate another tier of diversity to the polymorphic *HLA-C* locus beyond that encoding variation in the peptide-binding region of the gene. We suggest that disease associated haplotypes may exert their effects through multiple mechanisms, including the type of peptides they bind and their level of expression, and that it is the combination of these that then determines the overall susceptibility status of the haplotype.

Expression levels of different HLA-C allotypes occur as a continuous gradient rather than the bimodal expression pattern that would be expected if miR-148a regulation were the sole mechanism involved. Thus, additional *cis* acting factors may fine tune HLA-C expression in an allotype-specific manner. *Trans* acting factors unlinked to the *HLA-C* locus may also affect expression levels in a manner that is independent of HLA-C allotype, leading to some degree of variation in expression levels of a given HLA-C allotype. Although the system regulating *HLA-C* expression is multifactorial, the significant involvement of miRNA in this process provides new approaches for manipulation of the immune system in the treatment of human disease.

## Methods Summary

DNA from 2527 HIV<sup>+</sup> patients of European descent was used to determine the effect of the *HLA-C* 3'UTR variation on control of HIV viral load. Viral load measurements were obtained from participants of the Multicenter AIDS cohort study (MACS)<sup>19</sup>, Swiss HIV Cohort (<http://www.shcs.ch>), the SCOPE cohort<sup>20</sup>, and the International HIV Controllers Study Cohort ([www.hivcontrollers.org](http://www.hivcontrollers.org)). Individuals were grouped into those who maintain mean VL<2000 (controllers) and those who have mean VL>10,000 (noncontrollers).

Complete *HLA-C* 3'UTR fragments were amplified, inserted into *XbaI* site downstream of the luciferase gene in a pGL3-control vector (Promega), and transfected into B721.221, BLCL and Jurkat cells using AMAXA nucleofector (Lonza) and into 293T cells using Fugene6 (Roche). Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) and presented as fold change of relative light units<sup>21</sup>. For studies of miR-148a/miR-148b mimics and inhibitors (Dharmacon), 20 pmole/well of oligonucleotide mimics or inhibitors of miR-148a and miR-148b were transfected into the cells. Surface expression of HLA-C on BLCLs was analyzed using staining with DT9 antibody (kindly provided by V. Braud)<sup>22</sup>.

Total RNA was extracted using the Total RNA Purification Kit (Norgen). Relative quantification of miR-148a and miR-148b was performed using a *Taqman* real time PCR assay (Applied Biosystems) and RNU48 served as an endogenous RNA control.

SAS9.1 (SAS Institute) was used for data management and statistical analyses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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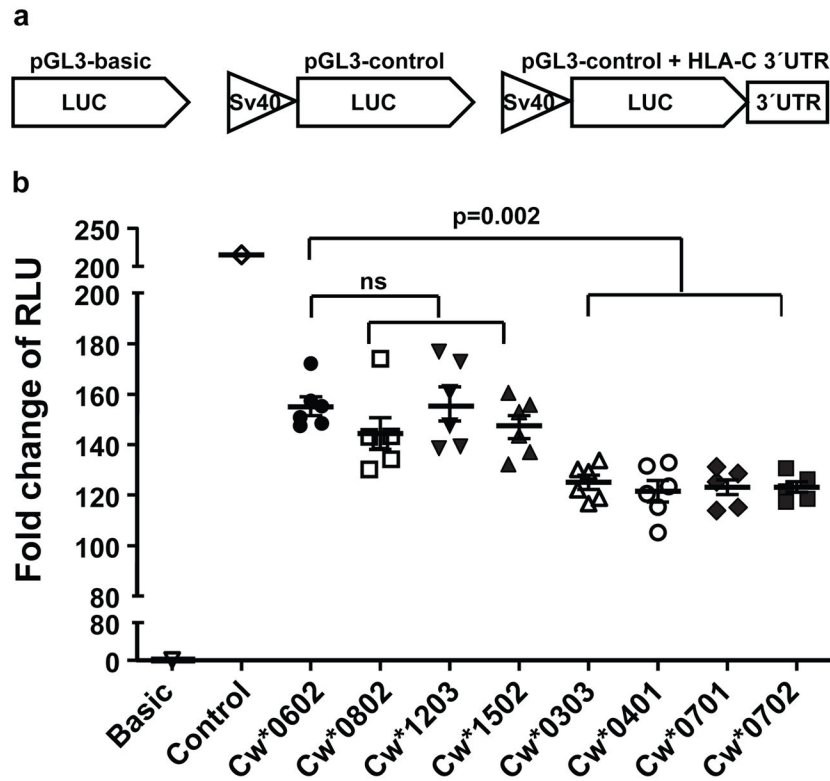
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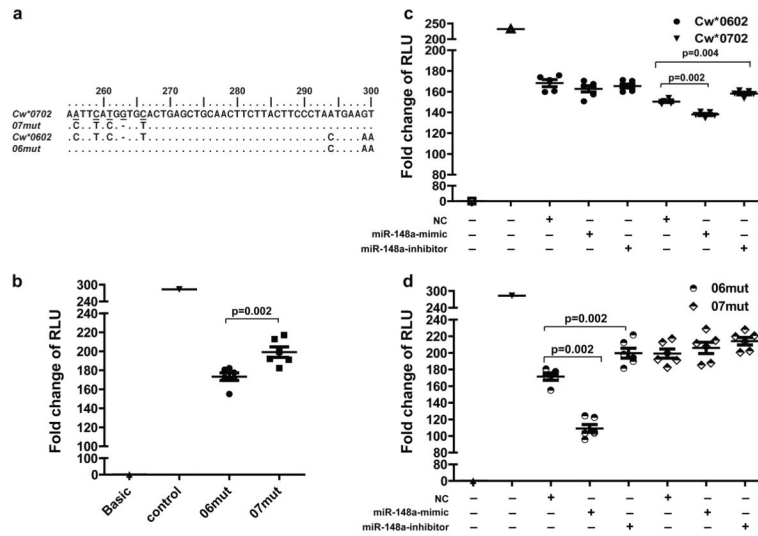
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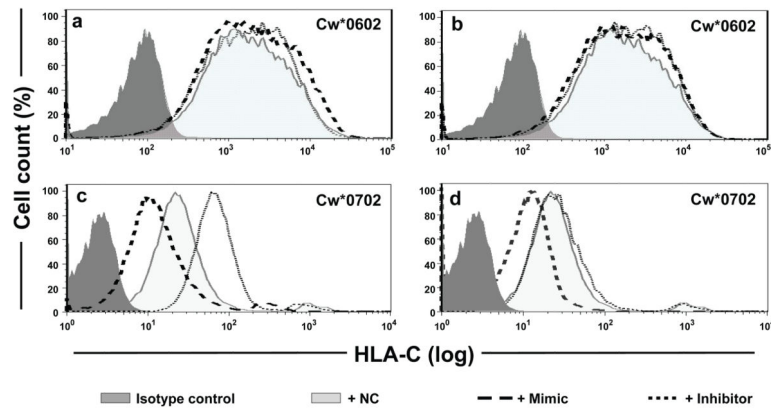


**Figure 1. Variation in the *HLA-C* 3'UTR differentially affects the expression of a reporter gene** Full length 3'UTRs of various *HLA-C* alleles cloned into luciferase reporter constructs were transfected into B721.221 cell lines and the stability of the mRNA was estimated by dual luciferase reporter assays. The normalized luciferase activity is presented as fold change of relative light units (RLU). The data represent six replicates in each experimental group, the Mean  $\pm$ SE are depicted as horizontal and vertical bars for each group, respectively, and one of three comparable experiments performed is shown. Non-parametric Wilcoxon-Mann-Whitney tests were used for statistical comparisons and two tailed p values are indicated. ns = not significant. **a.** Schematic representations of the luciferase reporter constructs used in this study. **b.** Fold change in luciferase activity of 3'UTRs of *HLA-C* alleles as compared to that of *Cw\*0602*.



### Figure 2. Disruption of miR-148a target site rescues suppression

**a.** Partial sequence of mutated 3'UTRs of *Cw\*0602* and *Cw\*0702* (*06mut* and *07mut*, respectively) are aligned to 3'UTR sequences of native *Cw\*0602* and *Cw\*0702*. Identical nucleotides are shown as dots, altered nucleotides are underlined, and deletions are indicated by hyphens (-) for optimal alignment. **b.** Fold change in luciferase activity of the modified 3'UTR (*06mut* and *07mut*). **c.** Fold change in luciferase activity of reporters containing wild type *Cw\*0602* or *Cw\*0702* 3'UTR sequences upon introduction of miR-148a mimic and inhibitor. **d.** Fold change in luciferase activity of reporters containing *06mut* and *07mut* 3'UTR sequences upon introduction of miR-148a mimic and inhibitor. Presence (+) or absence (-) of each variable, including a negative control (NC) miRNA, a mimic of miR-148a, or an inhibitor of miR-148a is shown. The data represent six replicates in each experimental group, the Mean  $\pm$ SE are depicted as horizontal and vertical bars for each group, respectively, and one of three comparable experiments performed is shown. Non-parametric Wilcoxon-Mann-Whitney tests were used for statistical comparisons and two tailed p values are indicated.



**Figure 3. miR-148a affects cell surface expression of HLA-C**

Histograms of HLA-C cell surface expression on HLA-C homozygous BLCL using flow cytometry are illustrated. In each plot, a NC miRNA that does not bind to the 3'UTR of *HLA-C* was included. **a–b.** *HLA-Cw\*0602* homozygous cells (BLCL-*Cw\*0602Hom*) transfected with either a mimic or an inhibitor of miR-148a (**a**) or miR-148b (**b**). **c–d.** *HLA-Cw\*0702* homozygous cells (BLCL-*Cw\*0702Hom*) transfected with either a mimic or an inhibitor of miR-148a (**c**) or miR-148b (**d**).

**Table 1**

Effect of *HLA-C* 3'UTR 263 on mean viral load using a logistic regression model by stepwise selection of *HLA-A*, *-B* and *-C* alleles

Significant independent variables	p value	OR	95% CI
del/del vs ins/ins	$2 \times 10^{-14}$	0.33	0.25–0.43
<i>B*2705</i> vs others	$3 \times 10^{-6}$	0.34	0.22–0.54
<i>B*5701</i> vs others	$1 \times 10^{-12}$	0.21	0.14–0.32
<i>B*5703</i> vs others	$3 \times 10^{-5}$	0.01	0.002–0.10
<i>B*5801</i> vs others	$9 \times 10^{-4}$	0.27	0.12–0.59
<i>Cw*1402</i> vs others	$1 \times 10^{-4}$	0.26	0.13–0.52

OR = odds ratio; CI = confidence interval; N = 2527. A logistic regression analysis with stepwise selection using the *HLA-C* 3'UTR 263 del/del vs. ins/ins comparison and 63 *HLA-A*, *-B* and *-C* alleles with 1% frequency as independent variables in the model was performed using PROC LOGISTIC (SAS 9.1 version, SAS Institute). Significance level for selecting variables shown in the table was  $p < 0.05$ .

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