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A Common Polymorphism in TLR3 Confers Natural Resistance to HIV-1 Infection

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TLR3 recognizes dsRNA and activates antiviral immune responses through the production of inflammatory cytokines and type 1 IFNs. Genetic association studies have provided evidence concerning the role of a polymorphism in TLR3 (rs3775291, Leu412Phe) in viral infection susceptibility. We genotyped rs3775291 in a population of Spanish HIV-1–exposed seronegative (HESN) individuals who remain HIV seronegative despite repeated exposure through i.v. injection drug use (IDU-HESN individuals) as witnessed by their hepatitis C virus seropositivity. The frequency of individuals carrying at least one 412Phe allele was significantly higher in IDU-HESN individuals compared with that of a matched control sample (odds ratio for a dominant model $= 1.87; 95\%$ confidence interval, 1.06–3.34; $p = 0.023$). To replicate this finding, we analyzed a cohort of Italian, sexually HESN individuals. Similar results were obtained: the frequency of individuals carrying at least one 412Phe allele was significantly higher compared with that of a matched control sample (odds ratio, 1.79; $95\%$ confidence interval, 1.06–3.34; $p = 0.029$). In vitro infection assays showed that in PBMCs carrying the 412Phe allele, HIV-1 replication was significantly reduced ($p = 0.025$) compared with that of Leu/Leu homozygous samples and was associated with a higher expression of factors suggestive of a state of immune activation (IL-6, CCL3, CD69). Similarly, stimulation of PBMCs with a TLR3 agonist indicated that the presence of the 412Phe allele results in a significantly increased expression of CD69 and higher production of proinflammatory cytokines including IL-6 and CCL3. The data of this study indicate that a common TLR3 allele confers immunologically mediated protection from HIV-1 and suggest the potential use of TLR3 triggering in HIV-1 immunotherapy. The Journal of Immunology, 2012, 188: 818–823.

Toll-like receptors recognize pathogen-associated molecular patterns and activate innate immune responses. In humans, four TLR family members (TLR3, TLR7, TLR8, and TLR9) are specialized in sensing viral-derived components and mainly localize to the endosomes (1). Among these, TLR3 recognizes dsRNA, an almost universal intermediate of viral replication, and triggers immune responses against both RNA and DNA viruses (1). A synthetic ligand, polyinosinic-polycytidylic acid [poly(I:C)], can also mediate immune responses through activation of TLR3. TLR3 signals through TIR domain-containing adaptor-inducing IFN-β (also known as TICAM1) and modulates the production of inflammatory cytokines and type I IFNs through NF-κB activation (1).

The TLR3 ectodomain has a horseshoe-like shape that possibly increases the available surface for dsRNA binding (2). Previous studies have described a human polymorphism in TLR3, Leu412Phe (rs3775291), that changes a highly conserved residue on the surface of the horseshoe structure. The minor 412Phe allele occurs with a frequency of $\sim$30% in populations with European and Asian ancestry, whereas it is virtually absent in Africans (3). In vitro analyses have suggested that TLR3 molecules carrying the 412Phe allele display reduced activation after poly(I:C) stimulation and allow increased coxsackievirus replication compared with 412Leu TLR3 receptors (4, 5). Genetic association studies have provided mixed evidence concerning the role of Leu412Phe in viral infection susceptibility. In fact, the minor allele has been shown to predispose to entero viral myocarditis (5) but to be protective against tick-borne encephalitis virus infection (6).

Recently, stimulation of distinct TLRs, including TLR3, with specific agonists was shown to elicit more vigorous immune activation in HIV-1–exposed seronegative (HESN) individuals compared with that in healthy controls (7). The notion that a subset of human subjects remains uninfected despite multiple exposures to HIV-1 has been known for years (8, 9). Natural resistance to HIV-1

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is thought to result from a favorable genetic and immunologic milieu that generates systemic and mucosal cell-mediated immune responses (10–13). In this study, we analyzed the role of the TLR3 Leu412Phe variant in HIV-1 infection. Our data indicate that the 412Phe allele is significantly more common among HESN subjects compared with controls, irrespective of the infection route. Consistently, cells from individuals carrying at least one 412Phe allele sustain lower HIV-1 replication compared with that of Leu/Leu homozygotes and display more vigorous immunological responses upon TLR3 stimulation.

Materials and Methods

Human subjects

We recruited 102 white males exposed to HIV-1 infection by injection drug use enrolled in prospective cohort studies in Spain (Valme Hospital, Seville), who had shared needles for >3 mo. Concurrent markers of hepatitis C virus (HCV) infection, one of the most prevalent blood-borne viruses, was present in 100% of individuals who were HIV-1–exposed seronegative despite repeated exposure through i.v. injection drug use (IDU-HESN). These values are significantly higher than the reported HCV prevalence of 1–2% for the general population in Spain. In addition, a group of 124 healthy white males recruited from anonymous blood donors from City of Jaen Hospital who tested negative for HIV-1 and HCV was used as a healthy control (HC) sample. The main epidemiological and clinical characteristics of the series studied were described previously (14). HC and HESN diagnoses were performed as previously described (14). All IDU-HESN individuals and their respective controls were Spanish of Caucasian origin.

The study was designed and performed according to the Declaration of Helsinki and was approved by the ethics committees of the participating hospitals and the University of Jaen. All patients and healthy blood donors provided written informed consent to participate in this study. As for sexually HESN individuals, inclusion criteria were a history of unprotected sexual contacts per year. These subjects are part of a well-enrolled since 1997 (7). HESN and healthy controls were recruited at the two Spanish cohort centers (Valme Hospital, Seville) and the University of Jaen. The study was reviewed and approved by the Institutional Review Board of the University of Jaen. Written informed consent was obtained from all subjects.

Genotyping

rs3775291 was genotyped in the HESN and control samples through PCR amplification and direct sequencing (primer sequences: forward, 5'-TGGAGCACCTTAACATGGAAGA-3'; reverse, 5'-TCTTGGTGTTG-3'). PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH), directly sequenced on both strands with a Big Dye Terminator sequencing Kit (v3.1; Applied Biosystems), and run on an Applied Biosystems ABI 3130 XL Genetic Analyzer. Sequences were assembled using AutoAssembler version 1.4.0 (Applied Biosystems) and inspected manually by two distinct operators.

HIV infection assay

Whole blood was collected from 46 healthy volunteers by venipuncture in Vacutainer tubes containing EDTA (Becton Dickinson), and PBMCs were separated on lymphocyte separation medium (Organon Teknica, Malvern, PA). PBMCs (10 × 10⁶ cells/ml) were cultured for 2 d at 37°C and 5% CO₂ in RPMI 1640 containing FBS (20%), PHA (7.5 μg/ml), and IL-2 (15 ng/ml). After 60 h, 10⁶ PBMCs were resuspended in medium containing 1 ng HIV-1aGag p24 viral input and incubated for 3 h at 37°C. Cells were then washed and resuspended in 3 ml complete medium with IL-2 (15 ng/ml). Cells were plated in 24-well tissue culture plates and incubated at 37°C and 5% CO₂. After 3 d, 2.5 × 10⁶ PBMCs were collected for gene expression analyses. After 5 d, supernatants were collected for ELISA of p24 Ag. Absolute levels of p24 were measured using a sandwich ELISA (Promega, Madison, WI). cDNA quantification for IL-1β, IL-6, IL-10, TNF-α, CCL3, RANTES, IFN-γ, IFN-β, CD69, and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, NJ). Primer sequences are listed in Supplemental Table I. Reactions were performed using a SYBR Green PCR mix (5 prime, Gaithersburg, MD). Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

Cytokine analyses after HIV infection assay and TLR3 stimulation

PBMCs (2.5 × 10⁵) freshly isolated from healthy volunteers (six with CC and eight with CT genotype) were incubated for 6 h with medium or 10 μg/ml poly(I:C). RNA was extracted from cultured PBMCs and from HIV-infected PBMCs by using the acid guanidium thiocyanate–phenol–chloroform method. The RNA was dissolved in RNase-free water and purified from genomic DNA with RNase-free DNase (New England Biolabs, Ipswich, MA). One microgram of RNA was reverse transcribed into first-strand cDNA in a 20-μl final volume containing 1 μM random hexanucleotide primers, 1 μM oligonucleotide, and 200 U Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). cDNA quantification for IL-1β, IL-6, IL-10, TNF-α, CCL3, RANTES, IFN-γ, IFN-β, CD69, and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research, Ramsey, NJ). Primer sequences are listed in Supplemental Table I. Reactions were performed using a SYBR Green PCR mix (5 prime, Gaithersburg, MD). Results were expressed as ΔΔCt and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

TLR3 expression in basal condition and after specific agonist stimulation

PBMCs isolated from six CC and eight CT subjects were analyzed under basal conditions and after 24-h stimulation with 10 μg/ml poly(I:C). PBMCs were resuspended in PBS and stained for surface mAbs CD14PECY7 and CD4PECY7 (Beckman-Coulter, Fullerton, CA). After 15-min incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldehyde in PBS. Cells were then permeabilized with saponin 0.5% (Sigma-Aldrich, St. Louis, MO) and stained with an mAb for TLR3 FITC (1 μg; R&D Bioscience). Cells were incubated for 45 min at 4°C in the dark, washed, and fixed in 1% paraformaldehyde in PBS.

Flow cytometric analysis

All the cytometric analyses were performed using an FC500 flow cytometer (Beckman-Coulter, Miami, FL) equipped with a double 15-mW argon ion laser operating at 456 nm and 488 nm and interfaced with an Intercorp computer. For each analysis, 20,000 events were acquired and gated on CD4 or CD14 expression and side-scatter properties. Green fluorescence from FITC (FL1) was collected through a 525-nm band-pass filter, and far red fluorescence from PC7 (FL5) was collected through a 770-nm band-pass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1 and FL5.

Statistical analysis

Genetic association analyses were performed using PLINK (15). Wilcoxon rank sum tests were used to evaluate differences in p24 levels, cytokine expression, and cell counts. All tests were two-tailed and were performed with R (http://cran.r-project.org/).

Results

Association analysis with HIV-1 infection susceptibility

In populations of European ancestry, rs3775291 (Leu412Phe) is the only non-synonymous variant in TLR3 that segregates at appreciable frequency (3). This variant was shown to be functional and to be associated with different human phenotypes, including viral infection susceptibility (5, 6). Thus, we wished to verify whether rs3775291 modulates the predisposition to HIV-1 infection. Most humans are susceptible to the virus, but a minority of individuals do not seroconvert despite multiple exposures. We genotyped rs3775291 in a cohort of 102 Spanish IDU-HESN individuals and in 131 age- and sex-matched healthy controls (HCs) with the same geographic origin. We observed a slight deviation from Hardy–Weinberg equilibrium (HWE) in IDU-HESN individuals with an excess of heterozygotes (HWE p value = 0.020); HCs complied with HWE. A significant difference was observed in the genotypic distribution of rs3775291 in the two cohorts (Table I), and the frequency of individuals carrying at least one T (412Phe) allele was significantly higher in IDU-HESN individuals (67.6%) compared with that in HCs (52.6%). The odds ratio (OR) for a dominant model with the T allele protecting from HIV-1 in-

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TLR3 POLYMORPHISM CON芙ERS RESISTANCE TO HIV-1 INFECTION

Cytokine production after HIV infection in PBMCs isolated from subjects with different rs3775291 genotype

To establish whether the above-noted increases in cytokine/chemokine expression levels after TLR3 stimulation of PBMCs were associated with enhanced TLR3 expression in these sub-populations of cells from subjects carrying a CT genotype for rs3775291, we analyzed TLR3 expression in CD4+ and CD4+ by phocytes, was significantly upregulated in CT versus CC individuals (p = 0.033) (Fig. 2A).

Responsiveness to TLR3 stimulation in PBMCs isolated from subjects with different rs3775291 genotype

To determine whether the cytokine profile characterizing heterozygous individuals after HIV infection could be directly dependent on TLR3 activation, we compared the cytokine/chemokine profiles in TLR3 agonist-stimulated PBMCs from the same 14 volunteers described earlier. Stimulation with poly(I:C) increased expression levels of mRNA specific for IL-1β, IL-6, IL-10, TNF-α, CCL3, RANTES, IFN-β, and IFN-γ in PBMCs from both CC and CT individuals, but the induction was significantly higher in CT compared with CC cells (CCL3, p = 0.014; IL-1β, p = 0.007; IL-6, p = 0.011) (Fig. 2B), resembling the cytokine milieu observed in response to HIV infection. Similarly, increased expression levels of CD69 in response to TLR3 triggering was observed in CT versus CC individuals (p = 0.028) (Fig. 2B).

Table I. Association of rs3775291 with HIV-1 infection susceptibility

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype Counts (TT/CT/CC)</th>
<th>Genotype Counts (Dominant) (TT+CT/CC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HESN</td>
<td>HC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P_{genotypic}^a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDU-HESN (Spain)</td>
<td>96/60/33</td>
<td>16/53/62</td>
</tr>
<tr>
<td></td>
<td>11/38/34</td>
<td>15/91/132</td>
</tr>
</tbody>
</table>

*Fisher’s exact test p value for a genotypic model.

^bCombined p value for the two samples.

^cFisher’s exact test p value for a dominant model.

^dOR for a recessive model with 95% CI.

FIGURE 1. p24 levels measured from HIV-1_{Ba-L}–infected cells. PBMCs from healthy volunteers were infected with HIV-1_{Ba-L}; the levels of viral p24 were measured after 5 d and are plotted as a function of rs3775291 genotype.

HIV-1 infection assay

To verify whether the Leu412Phe variant affects HIV-1 replication in vitro, we performed an infection assay. Specifically, PBMCs from 46 healthy volunteers were cultured and infected with HIV-1_{Ba-L}. Viral replication was assessed after 5 d by measuring viral p24 levels produced by the infected cells. Genotyping of these subjects for rs3775291 indicated that most of them were CC homozygotes (Leu/Leu, n = 31), and only two had a TT genotype; thus, heterozygotes and TT homozygotes were analyzed together (n = 15), as also suggested by the dominant effect of the 412Phe allele. Analysis of p24 level as a function of TLR3 genotype indicated that cells from CC individuals sustain higher HIV-1_{Ba-L} replication compared with that of PBMCs derived from CT plus TT individuals (two-tailed Wilcoxon rank sum test, p = 0.025) (Fig. 1).

Cytokine production after HIV infection in PBMCs isolated from subjects with different rs3775291 genotype

To establish whether the reduced susceptibility to HIV infection observed in PBMCs from individuals carrying at least one 412Phe allele could be secondary to a peculiar immunological profile, we analyzed the cytokine/chemokine expression levels in HIV-infected PBMCs from 14 healthy volunteers, 8 of them heterozygous for rs3775291 (CT genotype) and 6 CC homozygotes. Three days postinfection, expression rates of mRNA specific for IL-6, IL-10, TNF-α, CCL3, and IFN-γ were raised in PBMCs from both CC and CT individuals. However, the mean responses were higher in CT compared with CC subjects for all parameters analyzed, and mainly for CCL3 (p = 0.033) and IL-6 (p = 0.040) (Fig. 2A). Besides, after HIV infection, mRNA expression levels of CD69, a surface marker specifically induced on early activated T lymphocytes, was significantly upregulated in CT versus CC individuals (p = 0.033) (Fig. 2A).
flow cytometry. The results showed no appreciable differences in the percentage of CD4+/TLR3+ and CD14+/TLR3+ cells in the PBMCs of CT compared with CC subjects under basal conditions. After poly(I:C) stimulation, the percentage of TLR3-expressing CD4+ and CD14+ cells increased in the PBMCs of both CT and CC individuals, without showing significant differences between the two groups.

The mean fluorescence intensity (MFI) indicated that TLR3 densities on CD4+ and CD14+ cells were comparable among the groups examined (Table II). The similarities observed between the two groups were maintained even after TLR-specific stimulation (Table II).

Discussion

The ability of TLR3 to sense dsRNA and stimulate the production of type 1 IFNs and inflammatory cytokines suggests that the receptor plays a central role in antiviral defense. Yet, studies in mice and humans have indicated that TLR3 may act as a double-edged sword during viral infection and subsequent pathology. In fact, Tlr3<sup>-/-</sup> mice display increased mortality after infection with murine CMV, encephalomyocarditis virus, coxsackievirus B, and intracranially inoculated West Nile virus (WNV) (17–20). Conversely, animals lacking a functional Tlr3 gene display milder pathology when challenged with phelebovirus, influenza A virus, and i.p.-administered WNV (21–23). The reasons for these conflicting results are currently unknown, although it has been speculated that sustained activation of TLR3 might result in increased permeability of the hematocerebral barrier to WNV and that overproduction of proinflammatory cytokines in animals with functional TLR3 activity might be toxic and eventually lead to death (21, 23). In humans, rare mutations in TLR3 have been associated with susceptibility to herpes simplex virus encephalitis and to enteroviral myocarditis (5, 24). As mentioned earlier, this latter phenotype has also been shown to occur with increased frequency in subjects homozygous for the 412Phe allele. Consistently, in an in vitro system, cells expressing TLR3 412Phe molecules allow increased replication of coxsackievirus B3 compared with 412Leu receptors (5). Similarly, our data are consistent in showing a role for the Leu412Phe variant in HIV-1 infection susceptibility, but the allelic effect is exactly the opposite of that described in enteroviral infection. We analyzed two populations of HESN subjects with different geographic origin and distinct routes of exposure to the virus. Results were consistent in showing that the 412Phe allele protects from infection with a dominant effect. The replication of an association result in distinct cohorts is regarded as a gold standard for genetic studies; thus, we consider that, despite the relatively small sample size of our HESN cohorts, the current data provide strong support to the role of rs3775291 in HIV-1 infection susceptibility. As a further confirmation, infection assays with HIV-1Ba-L indicated that PBMCs from subjects carrying at least one 412Phe allele sustain lower viral replication compared with that of cells derived from CT heterozygous (black bars) or CC homozygous (white bars) individuals. Results were calculated relative to a housekeeping gene and are shown as fold-change expression from the unstimulated sample. Mean values ± SEs are shown.

Table II. Percentage and MFI of TLR3 expressing CD4+ and CD14+ cells in basal condition and after stimulation of PBMCs with poly(I:C) for 24 h

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Genotype (rs3775291)</th>
<th>Basal (Mean ± SE)</th>
<th>Poly(I:C) (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; Cells</td>
<td>CD14&lt;sup&gt;+&lt;/sup&gt; Cells</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; Cells</td>
</tr>
<tr>
<td>Percentage</td>
<td>CT</td>
<td>50.1 ± 2.5</td>
<td>6.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>48.3 ± 3.9</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>MFI</td>
<td>CT</td>
<td>27.3 ± 3.1</td>
<td>25.2 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>26.2 ± 1.9</td>
<td>26.3 ± 3.1</td>
</tr>
</tbody>
</table>

PBMCs were derived from subjects with different genotype for rs3775291.
Leu/Leu homozygotes. This effect may at least partially be mediated by the increased production of proinflammatory cytokines by innate and activated CD69+ lymphocytes that we observed in PBMCs derived from individuals carrying the 412Phe allele after HIV-infection assay. Recently it has been reported that after HIV exposure in HESN individuals, TLR engagement results in the induction of enhanced innate and adaptive immune responses, which in turn interfere with HIV replication and productive infection (7). In line with these observations, the cytokine milieu derived from TLR3-specific stimulation in PBMCs carrying the 412Phe allele resembles the one detected after HIV infection assay. Thus, the huge amount of immunological factors released in response to HIV-induced TLR3 stimulation may be able to overwhelm the virus and inhibit the infection process. Again, this latter finding contrasts with previous data indicating that ectopic expression of TLR3 412Phe molecules results in blunted immune responses after agonist stimulation (4, 5). A major difference between the results we report in this study and previous functional characterization of TLR3 variants (4, 5) resides in our using an ex vivo approach rather than relying on cell line transfection experiments. Indeed, previous data on TLR3 activity have been obtained using HEK293 or COS7 cells that were driven to express the receptor from an exogenous promoter (4, 5). Thus, some tiers of physiological regulation might be lost in cells that overexpress the two TLR3 variants. Another possibility is that one or more genetic elements in HEK293 and COS7 cells interact with the TLR3 Leu412Phe polymorphism, resulting in an epistatic effect. Otherwise, this amplified sensitivity to TLR3 stimulation may result from an increased expression of TLR3 in cells carrying the 412Phe allele. Indeed, several reports emphasize the relation between TLR expression and responsiveness in HIV infection (25), as well as in other physiological or pathological conditions (26–28). However, our results underscore a comparable TLR3 expression in CD4+ and CD4+ cells from subjects carrying at least one 412Phe allele compared with that in cells derived from Leu/Leu homozygotes, which is maintained even after TLR3 stimulation, ruling out the possibility to establish a correspondence between TLR3 expression and responsiveness. Finally, the possibility exists that the results we obtained are not directly an effect of the Leu412Phe single nucleotide polymorphism (SNP) but of a variant in linkage disequilibrium (LD) with it. We consider this to be an unlikely explanation for the following reasons: 1) rs3775291 is the only non-synonymous variant in TLR3 segregating at appreciable frequency in European populations (3); 2) LD analysis of HapMap data for a 20-kb region surrounding rs3775291 revealed no SNP in strong LD with it (r2 values <0.6); 3) resequencing data for TLR3 (3) indicated that only three variants in TLR3, two noncoding and one synonymous, have a frequency similar to rs3775291 in Europeans. Although these variants might in principle represent regulatory SNPs, our data showing no difference in TLR3-expressing cells depending on rs3775291 genotype argue against this possibility. Thus, the results of this study suggest that information obtained through in vitro expression of antiviral response genes may not be readily generalized to predict viral infection susceptibility in living organisms. Similar considerations have recently been reported for another antiviral response gene involved in HIV-1 infection, namely APOBEC3H (29). Indeed, a haplotype that generates APOBEC3H products with limited stability and antiviral activity in vitro was shown to confer resistance against sexually transmitted HIV-1 infection (29). It is also worth noting that the TLR3 412Phe allele has previously been associated with increased resistance against tick-borne encephalitis virus (6), although the mechanism underlying this observation is currently unknown.

Thus, data in both mouse models and humans have remained elusive. Although our data suggest that TLR3 stimulation might be associated with an amplified defensive responsiveness in subjects carrying at least one 412Phe allele, the function of the TLR3 pathway in resistance to HIV infection is still debated. In fact, TLR3 is not directly implicated in HIV-1 genome detection, but its triggering has been consistently coupled with the generation of a robust and defensive anti–HIV-1 response. It is likely that after HIV-1 exposure, the TLR3 pathway is stimulated through an indirect mechanism that is turned into an enhanced and protective immune response in subjects carrying the 412Phe allele. These data suggest the potential use of TLR3 triggering in HIV-1 immunotherapy.

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Disclosures
The authors have no financial conflicts of interest.

References
a tool set for whole-genome association and population-based linkage analyses. 


### Table S1. Forward and reverse sequences of each primer used for Real Time PCR analyses

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<thead>
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