Human parainfluenza virus serotypes differ in their kinetics of replication and cytokine secretion in human tracheobronchial airway epithelium

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Human parainfluenza viruses (PIVs) cause acute respiratory illness in children, the elderly, and immunocompromised patients. PIV3 is a common cause of bronchiolitis and pneumonia, whereas PIV1 and 2 are frequent causes of upper respiratory tract illness and croup. To assess how PIV1, 2, and 3 differ with regard to replication and induction of type I interferons, interleukin-6, and relevant chemokines, we infected primary human airway epithelium (HAE) cultures from the same tissue donors and examined replication kinetics and cytokine secretion. PIV1 replicated to high titer yet did not induce cytokine secretion until late in infection, while PIV2 replicated less efficiently but induced an early cytokine peak. PIV3 replicated to high titer but induced a slower rise in cytokine secretion. The T cell chemoattractants CXCL10 and CXCL11 were the most abundant chemokines induced. Differences in replication and cytokine secretion might explain some of the differences in PIV serotype-specific pathogenesis and epidemiology.

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Introduction

PIV infections are a common cause of acute respiratory illness (ARI) in all age groups. In healthy young adults, illness is typically mild, self-limited, and restricted to the upper respiratory tract. In infants and young children, the elderly, and patients with cardiopulmonary disease or immunodeficiency, however, PIVs can cause LRTI, including croup, bronchiolitis, and pneumonia (Karron and Collins, 2007). Globally, LRTI is the most common cause of under-five mortality, and viral LRTI represents a large share of this burden of disease (Nair et al., 2010). PIVs and respiratory syncytial virus (RSV) are the most frequently detected viruses in lung tissue specimens from infants who died of LRTI (do Carmo Debur et al., 2010).

The PIVs are enveloped, cytoplasmic viruses of Family Paramyxoviridae with single-stranded negative-sense RNA genomes of approximately 15 kb. Four different PIV serotypes exist, but PIV4 is generally thought to be infrequently associated with severe disease and its epidemiology is less well characterized (Weinberg et al., 2009). PIV3, like RSV (another member of the family), causes bronchiolitis and pneumonia in young infants while PIV1 and PIV2 are best known for epidemics of croup (Marx et al., 1997). Although PIV1 and PIV2 disease is seen most commonly in 1- to 5-year-olds, hospitalization rates for all three PIVs are highest in the first six months of life, with bronchiolitis, fever/possible sepsis, URI, pneumonia, and apnea as the most frequent discharge diagnoses (Weinberg et al., 2009).

PIV mortality is highest in bone marrow transplant (BMT) patients, and PIVs and RSV are reported to be the most frequent viral etiologies of respiratory illness in both pediatric and adult bone marrow transplant patients (Campbell et al., 2010; Srinivasan et al., 2011). Immunohistochemistry (IHC) and virology studies provide evidence that PIV replicates predominantly in respiratory epithelial cells and that, in general, infection is restricted to the respiratory tract (Bartlett et al., 2008; Schaap-Nutt et al., 2010c; Zhang et al., 2005). Only in severely immunocompromised patients, such as patients with severe combined immunodeficiency or following BMT, has systemic spread reproducibly been detected by IHC (Madden et al., 2004).

The histopathology of viral bronchiolitis and pneumonia is not known to have clear virus-specific differences, and there is wide overlap between PIV and RSV lung pathology (do Carmo Debur et al., 2010). Fatal RSV LRTI is dominated by mononuclear cell infiltrates and strong neutrophil movement toward the
bronchiolar epithelium (Johnson et al., 2007; Welliver et al., 2008). In a child who died from trauma one day after being diagnosed with RSV LRTI, inflammatory infiltrates were found around bronchial and pulmonary arteries and consisted mostly of monocytes, neutrophils, and double-negative T cells. Neutrophils were concentrated between arteries and airways whereas mononuclear cells were found in airways and lung parenchyma (Johnson et al., 2007). Although RSV has often been described as unique in that it induces a weak T helper type 1 (Th1) response and a bias towards Th2, more recent studies suggest that this observation might not be RSV specific but a consequence of infection very early in life. For example, mucosal interleukin (IL)-4, CCL4, and eotaxin concentrations were reported not to differ significantly between RSV and PIV or influenza-infected infants ≤3 months of age (Kristjansson et al., 2005).

PIVs encode one or more proteins that function to block cellular innate responses to viral infection and gene expression, thus helping the virus remain undetected in epithelial cells for as long as possible and facilitating efficient virus replication. PIV1 and PIV3 (both of genus *Respirovirus*) encode a nested set of C proteins, whereas PIV2 (genus *Rubulavirus*) encodes a V protein bearing a characteristic cysteine-rich zinc finger. Although the V and C proteins are unrelated in sequence or mechanism of action, both proteins block the induction of type I interferons (IFN) as well as IFN-induced signaling (Bartlett et al., 2010; Boonyaratanakornkit et al., 2011, 2009; Schaap-Nutt et al., 2010a,b; Schomacker et al., 2012). The innate immune response to PIV infection helps restrict viral replication but also is thought to contribute to disease. Therefore, an understanding of the cytokine response of epithelial cells—the cells that support PIV replication—is important to understanding PIV pathogenesis. Local production of inflammatory cytokines has been described in case series of children with PIV disease, and nasal wash samples were found to contain elevated concentrations of IFN (Hall et al., 1978) and chemokines such as IL-8/CXCL8, MIP1α/1β/CCL3/CCL4, RANTES/CCL5, and CXCL9 (El Feghaly et al., 2010; Gern et al., 2002). IL-8 and IP-10 concentrations were found to have a positive correlation with PIV disease (El Feghaly et al., 2010; Gern et al., 2002). PIV viral load also has been reported to correlate with severity of illness (Utokaparch et al., 2011). However, the kinetics of PIV replication and cytokine secretion have not, to our knowledge, been compared in a single study in primary respiratory epithelial cells. While increases in nasal wash (i.e., lumenal or apical) cytokine concentrations are consistent with the neutrophil and monocyte infiltrations seen in histopathology, chemotaxis and transmigration of white cells from the blood stream likely depend on local basolateral (rather than apical) cytokine concentrations, which cannot be measured in vivo in humans. In addition, the timing of the inflammatory response relative to virus replication is impossible to establish in naturally acquired infection, and experimental primary infection of PIV-naïve infants and young children with wild-type PIV would be unethical. An additional layer of complexity is that nasal wash and tracheal lavage fluids do not nasal wash (i.e., lumenal or apical) cytokine concentrations to the immune response and to PIV pathogenesis, and to test whether the PIV serotypes differ in the inflammatory response they induce.

2. Results and discussion

**Side-by-side comparison of PIV replication in tracheobronchial epithelium**

PIV1, 2, and 3 replication kinetics were compared in parallel in human tracheobronchial airway epithelium cultures. For each virus, three cultures from each of two individual donors were infected apically, i.e., from the side that would correspond to the lumenal surface. Virus stocks used in these infections were inoculated at low multiplicity of infection (MOI) from low passage virus stocks to minimize contamination by defective interfering (DI) particles. Virus particles were purified from the cell culture supernatant by centrifugation and banding in discontinuous sucrose gradients to remove cytokines and other molecules produced by the infected cultures. The ratio between the infectivity titer and the titer of physical particles measured by hemagglutination assay (Section 3) was similar for each of the viruses, indicating that none of the viruses contained a disproporionate content of inactivated particles or DI particles.

Virus release into the apical and basolateral compartments was monitored daily over the course of seven days after infection (Fig. 1A). For all three viruses, apical wash titers peaked on day 2 or 3 post-infection (pi) and then plateaued or decreased slightly over the next several days (Fig. 1A). PIV1- and PIV3-infected epithelium yielded much higher apical wash titers (mean peak values of 10^8.9 and 10^7.9 TCID50/ml, respectively) than PIV2-infected cells (10^5.3 TCID50/ml). Previous studies in HAE suggested that PIV1 replicated better than PIV2, but those studies involved different donors and could not be directly compared (Bartlett et al., 2008; Schaap-Nutt et al., 2010c). PIV3 replication in HAE was previously shown to peak at day 2 pi, though at a lower peak titer than in the present experiment (Palermo et al., 2009). PIV1, 2, and 3 exhibit similar growth kinetics in standard cell lines, reaching peak titers of 10^7–10^8 TCID50/ml (see Supplemental Fig. S1). Since high titers of PIV and RSV replication in vivo generally correlates with the severity of disease and contributes to transmissibility (Karron et al., 1997; Utokaparch et al., 2011), the lower level of replication observed with PIV2 could explain why this virus causes the least burden of disease of these three serotypes (Weinberg et al., 2009). For any serotype, virus replication was similar in the two donors examined (Fig. 1A).

Fig. 1. (A) Replication of PIV1, PIV2, and PIV3 in HAE. HAE were infected via the apical surface and titers of virus shed into the apical compartment were determined at the indicated times post-infection (pi). Virus titers shown are the means of three cultures per individual tissue donor ± SE (standard error). The limit of detection was 1.2 log10 TCID50/ml. (B) Apical cytokine secretion patterns of PIV-infected HAE. After apical infection, samples from the apical compartment were analyzed for protein concentrations of secreted IFN-α/β, IFN-β, IL-6, MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), and I-TAC (CXCL11) by sandwich immunoassay using MesoScale Discovery cytokine detection assays. Mean cytokine concentrations are expressed in pg/ml ± SE (triplicate cultures from each donor). Solid lines (■) show mean cytokine concentrations for PIV-infected cultures, and dashed lines (○) show cytokine concentrations from mock-infected cultures from the same donor. The lower limit of detection was 2.4 pg/ml. Cytokine concentrations in infected culture fluids were compared to those in mock-infected cultures using two-way analysis of variance (ANOVA) with Benjamini post-test; an asterisk (*) indicates a significant difference, P < 0.05.
En face immunostaining for PIV antigens showed a significant number of cells staining positive for each of the three PIVs through day 7 pi (25% HPIV1+, 9% HPIV2+, and 14% HPIV3+), and the ranking of PIV positive cells for each serotype correlated with the ranking of virus titers in the apical compartment (PIV1 > PIV3 > PIV2, data not shown). However, the difference in the number of infected cells (3-fold between PIV1 and PIV2) did not explain the much larger difference in peak virus titers (4000-fold between PIV1 and PIV2), suggesting that the virus yield per infected cell is the major determinant of the peak virus titer.

Previous studies of PIV infection of HAE indicated that PIVs are primarily released from the apical surface of ciliated HAE, and this directional budding was proposed to help limit PIV replication to the respiratory tract (Bartlett et al., 2008; Palermo et al., 2009; Schaap-Nutt et al., 2010c; Zhang et al., 2005, 2011). Our study confirmed that apically infected HAE cultures release virus from the apical/luminal surface only. None of the three PIVs could be detected in the basolateral compartment (data not shown). Although our data suggest that polarized budding and possibly the innate epithelial immune response contribute to the absence of viremia and systemic spread in infected individuals, it remains to be elucidated whether the systemic spread seen in severely immunodeficient patients is due to a leaky epithelium (possibly as a result of the adaptive immune response) or the ability of the virus to leave the respiratory tract within leukocytes, e.g. within mucosal dendritic cells (Le Nouen et al., 2009).

2.1. Cytokine secretion from tracheobronchial epithelium in response to PIV infection

One of the principal triggers of the cellular innate response to viral infection is the detection of viral nucleic acid by cytosolic pattern recognition receptors such as RIG-I and MDA5, which initiate signaling cascades that lead to the activation of cellular transcription factors and expression of type I IFNs, resulting in the induction of an antiviral state in the infected cell as well as neighboring cells, and expression of pro-inflammatory cytokines (Boonyaratankornkit et al., 2011; Schaap-Nutt et al., 2011). Side-by-side comparison of cytokine secretion showed that PIV2 induced significant apical and basolateral IFN-α secretion by day 2 pi, while PIV3 took three days to induce basolateral IFN-α, and PIV1 did not induce much apical IFN-α at all (Fig. 1B and Fig. 2). This was particularly surprising because PIV2 titers on day 2 pi were approximately 1000-fold lower than those of PIV1 and PIV3 (Fig. 1A). Whether the relative restriction of PIV2 is a viral property (i.e., a lack of viral fitness), or an effect of the early IFN-α response on PIV2 replication, or both, cannot be deduced from this study. However, using PIV1 mutants that cannot inhibit the IFN response as well as wild-type PIV1, we previously observed that the type I IFN response can restrict PIV1 replication in HAE approximately 100-fold (Bartlett et al., 2008). Similar to our results, PIV3 was previously shown to induce type I IFN release at the basolateral surface, while UV-inactivated PIV3 did not induce IFN (Zhang et al., 2011).

IFN-β induction was modest in HAE, regardless of the PIV serotype (Fig. 1B and Fig. 2). PIV1 did not induce a significant amount of IFN-β in either donor. Compared to mock-infected cells, PIV2 and PIV3 induced 3.5-fold and 2.3-fold rises in apical IFN-β, respectively, but only in donor 2 was this rise statistically significant. We and others had previously reported that the PIV1 C proteins inhibit the induction of type I IFNs in HAE (Bartlett et al., 2008) and showed that in the respiratory A549 cell line this phenotype is due to the inhibition of viral dsRNA synthesis, which results in delayed activation of the MDA5/IFN pathway (Boonyaratankornkit et al., 2011). Although viral dsRNA accumulation was not examined here, the lack of type I IFN induction by PIV1 suggests that the underlying mechanism in HAE might be the same (Bartlett et al., 2008). For PIV2, the binding of the viral V protein to MDA5 is known to play a role in inhibiting IFN-β expression (Schaap-Nutt et al., 2011).

IL-6 plays an important role in acute and chronic inflammation since it controls the shift between the recruitment of neutrophils (early) and monocytes (late) to the site of inflammation (Silver and Hunter, 2010). In children with PIV-positive ARI, IL-6 concentrations were found to be significantly elevated in nasal wash fluid (El Feghaly et al., 2010), and elevated serum IL-6 has been associated with prolonged hospital stays for RSV bronchiolitis (Vieira et al., 2010). In our study, IL-6 was significantly elevated only in HAE infected with PIV2 or PIV3 (except for a single time point for PIV1), and the kinetics of basolateral IL-6 secretion were similar to those observed for IFN-α: in PIV2 infected cells, basolateral IL-6 rose early and then waned whereas concentrations in PIV3 infected cells increased gradually through day 5 pi (Fig. 2). For PIV1, we previously observed that the viral C proteins strongly inhibit the activation of transcription factors such as IRF3 and NF-κB that are needed for both IL-6 and IFN-α expression (Boonyaratankornkit et al., 2011; Van Cleve et al., 2006).

Since the peak apical IL-6 secretion in donor 2 HAE was significantly higher than in donor 1 HAE, and because a polymorphism in the IL-6 promoter at position –174 was reported to be associated with IL-6 expression and with a number of inflammatory diseases (Fishman et al., 1998), we genotyped the two donors for this polymorphism. Donor 1 had a G/C genotype, while donor 2 (with higher levels of IL-6 in the apical compartment) had a G/G genotype (data not shown). The C allele at –174 has been associated with lower IL-6 expression, more severe symptoms following rhinovirus and RSV infection, and an increased frequency of otitis media (Alper et al., 2009; Doyle et al., 2010). In general, HAE from donor 2 secreted higher levels of most of the cytokines examined than HAE from donor 1 did, suggesting a stronger pro-inflammatory response by donor 2. Although the genotype of donor 1 could explain the lower apical secretion of IL-6 by HAE cells of donor 1, it is important to point out that our results do not determine an association between PIV-induced IL-6 secretion and the examined polymorphism: a study of that type would require a very large sample size and would likely not be feasible.

We also examined the kinetics of expression of two CC chemokines that are thought to play a role in viral ARI (McNamara et al., 2005): MCP-1/CC12, which acts as a macrophage chemoattractant, and RANTES/CC15, which attracts monocytes, T-helper cells and eosinophils (Fig. 1B and Fig. 2). Apically, MCP-1 was not significantly induced by any of the PIVs (Fig. 1B), similar to what was reported for MCP-1 in PIV-infected children (El Feghaly et al., 2010). Basolateral MCP-1 concentrations were elevated on days 2 and 3 in PIV2-infected epithelium and through day 5 in PIV3-infected HAE (Fig. 2). RANTES was secreted by the
HAE in response to all three of the viruses, both apically and basolaterally. Basolateral RANTES secretion kinetics followed the kinetics of the majority of cytokines, i.e., it peaked early in PIV2 infected HAE, increased steadily in PIV3 infected cells, and was not significantly elevated in PIV1-infected cells until day 5 pi (Fig. 2). Peak RANTES concentrations ranged from 580 to 2130 pg/ml apically (10- to 30-fold over mock) and from 660 to 3220 pg/ml (17- to 85-fold over mock) basolaterally. Previously, a six-fold rise in RANTES levels was detected basolaterally in RSV-infected HAE at 2 day pi, while no significant rise was detected in the apical supernatants (Mellow et al., 2004). A separate study in RSV-infected bronchial epithelium found much lower peak concentrations than in the present study (Oshansky et al., 2010), but this difference is likely due to the different time periods examined (1 day versus 5 days). In our hands, most cytokines did not peak until 2 day pi, and for PIV1 and PIV3 the concentrations increased until the last day of sampling (day 5). We feel that the longer follow-up is meaningful since severe PIV and RSV disease is generally not seen until 2 to 3 days past the onset of URI symptoms, which is likely 5–7 days pi. Our results do agree with findings in the nasal wash fluid of children naturally infected with RSV (Becker et al., 1997; Noah et al., 1995).

Finally, we examined the kinetics of expression of three CXC chemokines (IL-8/CXCL8 [not shown], IP-10/CXCL10, and I-TAC/CXCL11), based on previous clinical reports on their induction during viral ARI (McNamara et al., 2005) (Fig. 1B and Fig. 2). IP-10 and I-TAC are structurally and functionally related chemokines that attract CXCR3-positive activated Th1 cells to the mucosa (Groom and Luster, 2011). In addition to attracting Th1 cells, these CXCR3 ligands also antagonize CCR3-mediated Th2 cell migration, further enhancing Th1 polarization of effector T cell recruitment (Loetscher et al., 2001; Sallusto et al., 1998; Xanthou et al., 2003). With regards to respiratory illness, IP-10 has been suggested as a biomarker for viral (as opposed to non-viral) ARI, and our data indicate that all three PIV serotypes are potent inducers of IP-10 (Bafadhel et al., 2011; Sumino et al., 2010). CXC chemokine concentrations were much higher than those of the CC chemokines, with basolateral IP-10 and I-TAC concentrations above 100 and 50 ng/mL, respectively. Again, the kinetics of basolateral secretion differed by PIV serotype, with PIV2 inducing an early peak, PIV3 causing a slow rise, and PIV1 remaining undetected until day 5 pi. IP-10 concentrations as high as we observed were reported in BAL fluid from RSV-infected infants with severe bronchiolitis, and it was speculated that neutrophils might be the source of this cytokine since they were the predominant cell type in BAL fluid (McNamara et al., 2005). Our data indicate that the airway epithelium itself is an important source of IP-10 following PIV infection, and that neither neutrophils nor IFN-γ (the best known inducer of IP-10) from immune cells are needed for induction. IP-10 and I-TAC were predominately secreted into the basolateral compartment (Fig. 1B and Fig. 2), in line with the idea that CXCR3 ligands facilitate transmigration of Th1 cells across the endothelium and into inflamed tissue (Xie et al., 2003). High levels of IP-10 and I-TAC induction were reported in response to infection with either RSV or influenza A viruses in human airway epithelial cells, also with marked basolateral polarization (Ioannidis et al., 2012). Potent I-TAC induction was also recently reported for influenza A virus infection of primary, differentiated type II alveolar cells in culture (Wang et al., 2011), supporting the notion that CXCR3 ligands are the most abundant chemokines in viral ARI and an important determinant of a Th1-dominated adaptive immune response.

IL-8, the third CXC chemokine examined here, is a neutrophil chemoattractant and is elevated during RSV and PIV ARI in children (El Feghaly et al., 2010; Larranaga et al., 2009; McNamara et al., 2005). Unfortunately, in our study, IL-8 concentrations were very high in all cultures, infected or not, from day 1 pi onwards (not shown), which might have been induced by manipulation of the cultures as has been previously reported (Mellow et al., 2004).

In summary, our study represents the first side-by-side comparison of PIV serotype-specific induction of cytokines in primary well-differentiated human airway epithelium. We detected serotype-specific differences in virus replication as well as in the kinetics of cytokine induction. The kinetics of the innate immune responses suggested that (i) PIV1 is better able than PIV2 and PIV3 to stay undetected for several days post-infection; (ii) PIV2 is less able than PIV1 and PIV3 to inhibit an early immune response, and (iii) PIV3 induces a slow but steady increase in basolaterally-secreted cytokines. We report that IFN-γ from immune cells is not necessary for the induction of IP-10 in PIV infection, and our findings confirm previous clinical reports that CXCR3 ligands such as IP-10 and I-TAC are dominant chemokines in PIV infection, and likely mediate recruitment of Th1 cells into the infected epithelium. With regard to the potential clinical implications of the differences in cytokine secretion kinetics, one could speculate that the ability of PIV1 to stay undetected for so long contributes to its ability to re-infect PIV1-experienced children and adults more efficiently than PIV2 and PIV3 (Clements et al., 1991; Smith et al., 1966; Tremonti et al., 1968). PIV2 stands out in that an early innate immune response is followed by restricted replication, a finding that could explain why PIV2 circulates less widely and is responsible for less disease than PIV1 and PIV3. PIV3 was notable for its ability to induce a steadily increasing inflammatory response over several days, which fits well with the observation that PIV3 is a more frequent etiology of bronchiolitis and pneumonia than PIV1 and 2.

3. Materials and methods

3.1. Cells and viruses

Human tracheobronchial epithelial cells were isolated from airway specimens of patients without underlying lung disease by the UNC Cystic Fibrosis Center Tissue Culture Core, which obtained airway specimens with informed consent under University of North Carolina at Chapel Hill Institutional Review Board-approved protocols from the National Disease Research Interchange (NDRI, Philadelphia, PA) or as excess tissue following lung transplantation. Primary cells were expanded on plastic for one passage and plated at a density of 3 × 10^5 cells per well on permeable Transwell-Col (12 mm-diameter) supports. HAE cultures were grown at an air-liquid interface for 4–6 weeks to form differentiated, polarized cultures, as previously described (Pickles et al., 1998).

Recombinant (r) PIVs were derived from clinical isolates: rPIV1 was from strain Washington/20993/1964 (Bartlett et al., 2005); rPIV2 was from strain V9412-6 (V94) (Skiaiopoulos et al., 2003); and rPIV3 was from the JS strain (Durbin et al., 1997). rPIV1 was constructed from the biologically-derived PIV1 wild-type (wt) strain Washington/20993/1964, which was isolated in primary African green monkey (AGM) kidney cells from a clinical sample from a child with upper respiratory tract illness and subsequently passaged two additional times in primary AGMs kidney cells (Murphy et al., 1975) and once in LLC-MK2 cells (Bartlett et al., 2005). rPIV1, previously referred to as HPIV1LCLI (Genbank ID: AF457102), has a wt phenotype in AGMs (Bartlett et al., 2005). rPIV2 was constructed from the biologically-derived HPIV2 strain V9412-6 (V94), kindly provided by Peter Wright of Vanderbilt University. The V94 strain (Genbank ID: AF533010) was isolated in Vero cells from the nasal wash specimen of an infected
The infectivity to HA ratios were similar for all three viruses at 4 h post-infection. Viruses were diluted 1/2 in 0.5% guinea pig RBCs and incubated for 2 h at 37°C. Stocks were 7.7 log10 TCID50/ml (HPIV1), 8.1 log10 TCID50/ml (HPIV2), and 9.8 log10 TCID50/ml (HPIV3). The ratio between infectious viral particles and total viral particles was also calculated to confirm that the virus preparations did not have high concentrations of DI particles, as previously described (Johnston, 1981; Youn et al., 2006). To determine total particle titers in virus preparations, we used a hemagglutination (HA) assay based on the binding of gp RBCs to the external HN protein of HPIV particles. Viruses were diluted 1/2 in 0.5% gp RBCs and incubated 1 h at 4°C. HA of RBCs indicates the presence of virus particles. The infectivity to HA ratios were similar for all three viruses (1.6 × 105 for HPIV1, 2.0 × 105 for HPIV2, and 3.1 × 105 for HPIV3) and indicate a low level of DI particle contamination.

3.4. Analysis of IL-6 G–174C polymorphism

Genomic DNA was isolated from HAE cells, and Custom Taq-Man Genotyping Assays were designed using primer and probe sequences 5’–TAGCTCTAATGAGCCAATGCTT (forward primer), 5’–GGGTGAGTGTGAAAACCTTATAG (reverse primer), TGTCTTGCGATGCTA (VIC probe, G allele), and TGTCTTGCCATGCTA (FAM probe, C allele). Genotyping was performed using the TaqMan Universal PCR Master Mix according to the manufacturer's protocol. Samples were run on a 7900HT fast real-time PCR system and quantitative analysis was performed using the SDS 2.3 software (all from Applied Biosystems).

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Appendix A. Supporting information

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