Severe viral respiratory infections in children with IFIH1 loss-of-function mutations

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Viral respiratory tract infections are the most common childhood infections worldwide, with close to 100% of children being infected during the first years of life. Whereas the vast majority of viral respiratory infections are mild and self-limiting, more severe disease leads to the hospitalization of about 3% of children in each birth cohort (1). In-hospital mortality rates are limited to <1% with intensive care support; still these infections account for 21% of childhood mortality worldwide (2, 3). The main viral pathogens causing lower respiratory tract infections are human respiratory syncytial virus (RSV), enteroviruses [including human rhinoviruses (HRV)], adenoviruses, human metapneumovirus, coronavirus, influenza, and parainfluenza viruses, with RSV being responsible for the majority of hospitalized pediatric cases (4, 5).

A number of risk factors including socioeconomic and environmental influences, preterm birth, chronic diseases, and immunosuppression are associated with more severe clinical presentation (6). However, ~1 out of 1,000 children without any known risk factor will require intensive care support due to life-threatening manifestations of common viral respiratory infections. In the absence of established differences in pathogen virulence, we hypothesized that human genetic variation contributes to unusual susceptibility to severe disease due to common viruses. Supporting evidence is provided by a recent study, which showed that rare variants in IRF7 resulted in life-threatening influenza in an otherwise healthy child (7).

We combined exome sequencing, transcriptomic analysis, and in vitro functional testing to identify and characterize potentially causal genetic variants in a prospective cohort of previously healthy children requiring intensive care support for common respiratory viral infections. We report the identification of a pathogen-restricted immunodeficiency due to loss-of-function variants in IFIH1, which result in defective innate recognition of RNA viruses, preventing the activation of an efficient antiviral IFN response.

Results

Study Participants. We enrolled 120 previously healthy children admitted to pediatric intensive care units (PICUs) with respiratory failure due to a common viral respiratory infection. The most common clinical presentation was bronchiolitis (n = 105, 88%) and the median age was 78 d (interquartile range, IQR: 37–769). RSV was the most common pathogen, identified in 67 (56%) of the cases, followed by HRV in 31 (26%) of the cases (Table 1).

Exome Sequencing and Analysis. DNA samples were sequenced to a mean coverage of 70x, with 96% of exonic bases achieving at least 10x coverage and 78% achieving at least 30x coverage. The final set of variants included 2,793 stop-gained single-nucleotide variants (SNVs), 297 splice-site SNVs, and 951 frame-shift indels. Among these putative loss-of-function variants (LoFs), we searched for variants that were homozygous in at least one study participant, and with a higher minor allele frequency in our cohort than in the genome Aggregation Database (gnomAD) (8) and in an in-house database.

Significance

Life-threatening susceptibility to common respiratory infections in previously healthy children can be indicative of pathway-specific primary immunodeficiencies due to rare deleterious variants in key genes and pathways of the immune system. These findings have implications for prevention and treatment of susceptible children.
In total, eight study participants carried a rare [gnomAD minor allele frequency (MAF) = 0.064%] splicing variant, rs35732034 (Fig. L4): one in homozygous and three in heterozygous form. We used RNA sequencing to characterize the transcriptomic impact of this variant. We observed that the minor allele T causes skipping of exon 14 (IFIH1-Δ14) (Fig. 1B), which results in a frame shift and an early stop codon in exon 15. The resulting protein lacks the final 153 amino acids of wild-type IFIH1, including the C-terminal regulatory domain (CTD), which is essential for binding to viral dsRNA (10) (Fig. 1C). Western blot analyses of peripheral blood mononuclear cells from the homozygous patient and her heterozygous parents demonstrated that the IFIH1-Δ14 protein is expressed upon in vitro RSV infection (SI Appendix, Fig. S1). We identified two additional rare LoF variants in IFIH1, present in heterozygous form in a total of four study participants (Fig. L4): the splicing variant rs35337543 (n = 3, gnomAD MAF = 0.67%) and the stop-gained variant rs35744605 (n = 1, gnomAD MAF = 0.32%). RNA sequencing showed that the minor allele G at rs35337543 causes skipping of exon 8 (IFIH1-Δ8) (Fig. 1B), which removes 39 amino acids at the end of the helicase 1 domain and in the linker part between helicase 1 and helicase 2, but does not result in a frame shift. rs35744605 is a stop-gained SNV in exon 10 that leads to the loss of 399 amino acids from the C-terminal end of IFIH1 (IFIH1-ΔCTD) (Fig. 1C).

**Description of Study Participants Carrying IFIH1 LoF Variants.** A 16-month-old girl was homozygous for rs35732034. She presented with respiratory failure due to RSV infection requiring invasive ventilation. The disease course was complicated by a pulmonary superinfection with *Staphylococcus aureus*. She had a full recovery and did not develop any other severe infection up to the age of 3. Her phenotype and history was otherwise unremarkable. In particular, she did not develop any complication after live vaccine administration. Full blood count, Ig levels and IgG subclasses, and lymphocyte subclasses were within normal limits. Three infants requiring noninvasive respiratory support for bronchiolitis were heterozygous for rs35732034. One of these had recurrent severe viral lower respiratory tract infections leading to repeated PICU admissions during childhood. Three children were heterozygous for rs35337543 and required noninvasive ventilatory support for RSV bronchiolitis. One infant was heterozygous for rs35744605 and required invasive ventilatory support for HRV-positive bronchiolitis. Parental DNA was available for three of the eight children. Targeted genotyping confirmed that the relevant IFIH1 variant was present in heterozygous form in one of the parents for two heterozygous individuals, and in both parents for the homozygous patient. Parental medical history was unremarkable in all cases.

**Functional Characterization of IFIH1 Variants.** To functionally characterize the identified variants, we first measured the ability of wild-type (IFIH1-wt) and mutant IFIH1 isoforms to induce IFN β (IFNβ) in vitro. We transfected plasmids carrying IFIH1-wt, rs35732034, rs35337543, and rs35744605 into human peripheral blood mononuclear cell (PBMC) lines and measured IFNβ expression upon RSV infection.

**Table 1. Baseline characteristics of the 120 study participants**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Variable</th>
<th>Median (IQR) or N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, d</td>
<td></td>
<td>78 (37–269)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td></td>
<td>5.9 (4.4–9.8)</td>
</tr>
<tr>
<td>Country of recruitment</td>
<td></td>
<td>Australia 100 (83%) Switzerland 20 (17%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td>Caucasian 90 (78%) African 4 (4%) Asian 4 (4%) Australian aboriginal 6 (5%) Pacific Islander 11 (10%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>Male 70 (58%) Female 50 (42%)</td>
</tr>
<tr>
<td>Clinical phenotype</td>
<td></td>
<td>Bronchiolitis 105 (88%) Pneumonia 8 (7%) Laryngotracheobronchitis 5 (4%) Reactive airway disease 2 (2%)</td>
</tr>
<tr>
<td>Virus identified in respiratory sample</td>
<td></td>
<td>RSV 67 (56%) Enterovirus/rotavirus 31 (26%) Adenovirus 17 (14%) Human bocavirus 9 (8%) Influenza 2 (2%) Parainfluenza 6 (5%) Human metapneumovirus 3 (2%) CoV-HKU1 3 (2%) CoV-NL63 1 (1%)</td>
</tr>
<tr>
<td>Length of PICU stay, d</td>
<td></td>
<td>2.7 (1.6–5.0)</td>
</tr>
<tr>
<td>Expected mortality</td>
<td></td>
<td>0.02 (0.01–0.7)</td>
</tr>
<tr>
<td>Observed mortality</td>
<td></td>
<td>No fatal case</td>
</tr>
</tbody>
</table>

CoV-HKU1, coronavirus HKU1; CoV-NL63, coronavirus NL63.

**Table 2. Characteristics of the eight study participants carrying an IFIH1 loss-of-function variant**

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Zygosity</th>
<th>Sex</th>
<th>Ethnicity</th>
<th>Virus</th>
<th>Ventilation required</th>
<th>Clinical presentation</th>
<th>Parental allele</th>
<th>Variant ID</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>gnomAD AC (Wt), MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRI_022</td>
<td>hom</td>
<td>F</td>
<td>White</td>
<td>RSV</td>
<td>I</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>rs35732034</td>
<td>2:163124596 C/T</td>
<td>p.Ile872Ter</td>
<td>1673 (7), 0.006</td>
</tr>
<tr>
<td>PRI_050</td>
<td>het</td>
<td>M</td>
<td>Aboriginal</td>
<td>HRV</td>
<td>NI</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>NA</td>
<td>rs35337543</td>
<td>2:163136505 C/G</td>
<td>p.Leu509_Glu547del</td>
</tr>
<tr>
<td>PRI_960</td>
<td>het</td>
<td>M</td>
<td>41</td>
<td>White</td>
<td>RSV</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>NA</td>
<td>rs35744605</td>
<td>2:163134090 C/A</td>
<td>p.Glu627Ter</td>
</tr>
<tr>
<td>PRI_065</td>
<td>het</td>
<td>M</td>
<td>38</td>
<td>White</td>
<td>RSV</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRI_116</td>
<td>het</td>
<td>M</td>
<td>479</td>
<td>White</td>
<td>RSV</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRI_080</td>
<td>het</td>
<td>M</td>
<td>343</td>
<td>White</td>
<td>HRV</td>
<td>Bronchiolitis</td>
<td>ns</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NA, not available; het, heterozygous; hom, homozygous; I, invasive; NI, noninvasive; AC, allele count.
IFIH1-Δ8, IFIH1-Δ14, and IFIH1-ΔCTD into 293T cells. Overexpression of IFIH1-wt, but not of any of the mutant IFIH1 isoforms, led to IFNβ induction. Cotransfection of IFIH1-wt with each of the mutant IFIH1 isoforms showed interference with IFIH1-wt-induced IFNβ production (*P < 0.05, Fig. 2A). We then tested the ATPase activity of recombinant IFIH1-wt and mutant IFIH1 isoforms. IFIH1-wt was able to hydrolyze ATP and showed a typical dsRNA-dependent increase in enzymatic activity, whereas the mutant isoforms had no detectable ATPase activity, even upon stimulation with polyinosinic-polycytidylic acid (polyI:C), a synthetic analog of dsRNA (Fig. 2B). Furthermore, all mutant isoforms decreased IFIH1-wt ATPase activity in a dose-dependent manner, thus interfering that was specific to mutant IFIH1 isoforms, as demonstrated by the absence of any effect of BSA on IFIH1-wt ATPase activity (*P < 0.05, Fig. 2C and SI Appendix, Fig. S2 A and B). Finally, we checked the stability of the various IFIH1 protein isoforms by performing pulse-chase experiments in transiently transfected 293T cells. The three mutant isoforms were less stable than IFIH1-wt (Fig. 2D) and had a negative impact on the stability of the wild-type isoform when cotransfected (Fig. 2E). Jointly, these experiments demonstrate that the three putative LoF variants identified in our study population lead to severe disruption of IFIH1 signaling function, enzymatic activity, and protein stability in vitro. In addition, the observations that the mutant IFIH1 isoforms interfere with the wild-type protein in terms of IFNβ induction, and enzymatic activity and protein stability suggest a dominant negative role for heterozygous LoF variants in IFIH1.

Role of IFIH1 in RSV and HRV Replication. Viral testing of respiratory samples showed that six of the patients harboring IFIH1 LoF alleles were infected with RSV and two with HRV. To study the effect of IFIH1 on RSV and HRV replication, we used Huh7.5 cells, which lack endogenous expression of IFIH1 and express a mutated, inactive form of RIG-I, and thus are completely unreactive to the RNA pathogen-associated molecular patterns that normally activate these pathways (11). The cells were transduced with an IFIH1-wt-expressing lentiviral vector, which made them highly responsive to polyI:C stimulation (SI Appendix, Fig. S3A) without causing any nonspecific or constitutive activation of the IFN system (SI Appendix, Fig. S3B). We observed a much higher level of viral replication in native than in IFIH1-wt-transduced Huh7.5 cells upon infection with HRV-B14, HRV-
A16, and RSV (Fig. 3 A–D). Furthermore, RSV replication level was higher in cells transduced with the mutant forms of IFIH1 than in IFIH1-wt-transduced cells (P < 0.05, SI Appendix, Fig. S4 A and B). The role of IFIH1 in HRV restriction was further demonstrated by 35S labeling of infected cells, which showed a stronger shutoff of cellular protein synthesis in native than in IFIH1-transduced Huh7.5 cells, due to higher replication of the virus in the absence of IFIH1 (Fig. 3E). We also measured RSV replication in mouse embryonic fibroblasts (MEFs), ifih1(+/−), and in IFIH1-knockout MEFs, ifih1(−/−), and obtained similar results (Fig. 3 F–H). Together, these results affirm the central role of IFIH1 in innate immune recognition of RSV and HRV (12, 13). Therefore, LoF variants in IFIH1 can be reasonably expected to increase susceptibility to these viruses.

Discussion

We hypothesized that extreme susceptibility to common viral respiratory infection in previously healthy children—a rare, potentially lethal phenotype—could reflect an underlying primary immunodeficiency. Using an unbiased exome-wide approach in a prospective cohort of carefully selected individuals requiring intensive care support, we identified a rare monogenic defect predisposing to severe clinical presentations of RSV and HRV infections.

Three deleterious variants were observed in IFIH1, which encodes a cytoplasmic receptor critical for viral RNA sensing. It has been shown previously that IFIH1, alone or in combination with RIG-I, recognizes and limits the replication of many RNA viruses including: positive single-stranded RNA (ssRNA) viruses like picornaviruses (14–16), negative ssRNA viruses like paramyxoviruses (17–19), and dsRNA viruses like reoviruses (20). IFIH1 recognizes viral RNA via interaction of its CTD and helicase domains with long dsRNA molecules. This is an ATP-dependent reaction that leads to polymerization of IFIH1 molecules into a filament and assembly of IFIH1 caspase activation recruitment domains (CARDs) (21, 22). This in turn initiates a signaling cascade that results in type 1 IFN production and activation of antiviral genes (23). Our transfection and transduction analyses show that this process is disrupted in the presence of any of the IFIH1 rare variants found in our study population. Our exome and RNA sequencing data predict that the loss of IFIH1 function is due to loss of the CTD (rs35732034) or to partial loss of the helicase domain (rs35744605) or to partial loss of the helicase domain (rs35337543).

We observed interference between IFIH1-wt and the three mutant proteins in terms of stability, ATPase activity, and capacity to induce IFNβ production, suggesting a dominant negative effect, which provides a rationale for the unusual susceptibility to respiratory viruses observed in heterozygous individuals. The exact interfering mechanism is not known but could involve physical interaction between IFIH1-wt and mutant proteins, preventing the formation of normal, multimeric IFIH1 filaments.

While the revised version of this paper was under review, an independent study showed an association between IFIH1 deficiency and life-threatening infections with HRV and other respiratory viruses in a child carrying another homozygous missense IFIH1 variant (24). This observation further supports a causal role for IFIH1 deficiency in extreme susceptibility to common respiratory viruses.

The three IFIH1 variants described in this study have allele frequencies of 0.32–0.67% in gnomAD. The cumulative frequency of all putative LoF alleles is 1.89% in the same database, which is significantly less than the 3.75% cumulative frequency observed in our study population (P = 0.037, Fisher’s exact test). Nevertheless, the presence of alleles of potentially devastating consequences at such frequency in the general population is intriguing, as they are expected to be removed by purifying selection. Two nonexclusive mechanisms can explain this observation: balancing selection and incomplete penetrance. We here show that some IFIH1 alleles increase susceptibility to viral respiratory infections, but the same LoF variants are known to be protective against type 1 diabetes and other autoimmune diseases (25–30), strongly suggesting a role for balancing selection in their maintenance. In a comparable example, rare nonsynonymous variants in TYK2, a known primary immunodeficiency gene, were shown to be protective against rheumatoid arthritis (31). Incomplete penetrance, on the other hand, could be due to modulating effects of environmental or genetic factors, like compensatory mutations, or to functional redundancy in innate immune response to RNA viruses (32, 33). This hypothesis is in line with several recent publications (34–37), which suggest that incomplete penetrance and genetic heterogeneity are likely to be the rule rather than the exception in severe clinical presentations of infectious diseases.

On top of their associations with autoimmunity, more common IFIH1 variants have also been associated with hepatitis C virus clearance (38). Additionally, rare gain-of-function mutations in IFIH1 dramatically up-regulate type 1 IFN production, resulting in Alcardi–Goutières syndrome or Singleton–Merten syndrome (39–41). At the functional level, Gorman et al. recently studied the effects on viral sensing and autoimmune pathogenesis of rs1990760, a missense IFIH1 variant that is associated with multiple autoimmune diseases (30). They showed that the allele providing better defense against viral infection also bolsters autoimmune responses against self-RNA (42). Together, these results underscore the pivotal role of innate immune recognition and activation in the intricate balance between host defense, inflammation, and autoimmunity.
Our study demonstrates the power of using an unbiased, exome sequencing approach to variant discovery in prospective cohorts of extreme infectious disease phenotypes. Nevertheless, LoF variants in IFIH1 were only found in a minority (n = 8, 6.2%) of the 120 children enrolled in our study, suggesting that other genetic or nongenetic risk factors remain to be discovered. Larger sample sizes will be required to delineate the relevance of other rare potentially causal alleles. Whole genome sequencing will also be needed to obtain a more complete coverage of exonic regions (43), and to explore noncoding and large-scale structural variation.

RSV and rhinovirus infections are the two most common viral respiratory infections in children. The elucidation of the human genetic basis of extreme susceptibility to these viruses provides insight into pathogenesis and innate immune response. An immediate practical implication is the possibility to develop diagnostic assays to identify susceptible individuals who could benefit from specific preventive and interventional measures. By highlighting the genes and pathways that play an essential role in host–pathogen interaction, genetic discovery in individuals with extreme phenotypes also provides the opportunity to design new therapeutic strategies that could be useful for the vast majority of patients with milder clinical presentation.

Materials and Methods

Between December 2010 and October 2013, we prospectively enrolled previously healthy children below 4 y of age suffering from severe lower respiratory tract infection and requiring invasive or noninvasive respiratory support in five specialized PICUs from Australia and Switzerland. The study was approved by the respective institutional Human Research Ethics Committees. Written informed consent was obtained from parents or legal guardians. Exclusion criteria were the presence of any significant underlying disease or comorbidity, including prematurity, congenital cardiac disease, chronic lung disease, sickle cell disease, hepatic, renal, or neurologic chronic conditions, solid and hematological malignancies, and known primary immunodeficiency. Supportive care was delivered using standard PICU practice including high-flow nasal cannulae (HFNC) and continuous or bilevel positive airway pressure (CPAP and BiPAP), or invasive ventilation including conventional and high-frequency oscillation ventilation (HFOV). The following demographic and clinical information was collected: age, gender, weight, ethnicity, type of ventilation, length of ventilation in days, clinical outcome, microbiological diagnostic procedures and results including rapid antigen test for RSV and influenza, respiratory virus PCR panel, and viral cultures. For each study participant, we obtained a nasopharyngeal aspirate or endotracheal tube aspirate, 1 ml EDTA in vacutainer tubes, and 2.5 ml blood in PAXgene blood RNA tubes. Samples were immediately frozen at −70°C until shipment, and all were stored in batches.

We generated high-coverege exome sequencing data for all study participants (SI Appendix, Table S1). We then used a combination of three variant calling methods (GATK, Platypus, and SAMtools) and only kept SNVs and small insertion and deletions (SI Appendix, Table S2). Assuming that causal genetic variants are likely to be highly deleterious, we focused on rare gene knockout events, defined as homozygous, putative LoF variants (stop-gained and splice-site SNVs, frame-shift indels) with an MAF of <1% in the ExAC (SI Appendix, Table S3). We performed RNA sequencing to assess the functional impact of candidate DNA variants, and characterized the infecting viruses using multiplex PCR assays. We then used in vitro functional testing to demonstrate the biological relevance of candidate variants and tested in vivo expression of the affected protein (SI Appendix).

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Asgari et al.