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Short Communication

Fungal colonization with *Pneumocystis* correlates to increasing chloride channel accessory 1 (hCLCA1) suggesting a pathway for up-regulation of airway mucus responses, in infant lungs

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ABSTRACT

Fungal colonization with *Pneumocystis* is associated with increased airway mucus in infants during their primary *Pneumocystis* infection, and to severity of COPD in adults. The pathogenic mechanisms are under investigation. Interestingly, increased levels of hCLCA1 – a member of the calcium-sensitive chloride conductance family of proteins that drives mucus hypersecretion – have been associated with increased mucus production in patients diagnosed with COPD and in immunocompetent rodents with *Pneumocystis* infection. *Pneumocystis* is highly prevalent in infants; therefore, the contribution of *Pneumocystis* to hCLCA1 expression was examined in autopsied infant lungs. Respiratory viruses that may potentially increase mucus, were also examined. hCLCA1 expression was measured using actin-normalized Western-blot, and the burden of *Pneumocystis* organisms was quantified by qPCR in 55 autopsied lungs from apparently healthy infants who died in the community. Respiratory viruses were diagnosed using RT-PCR for RSV, metapneumovirus, influenza, and parainfluenza viruses; and by PCR for adenovirus. hCLCA1 levels in virus positive samples were comparable to those in virus-negative samples. An association between *Pneumocystis* and increased hCLCA1 expression was documented ($P=0.028$). Additionally, increasing *Pneumocystis* burden correlated with increasing hCLCA1 protein expression levels ($P=0.017$). Results strengthen the evidence of *Pneumocystis*-associated up-regulation of mucus-related airway responses in infant lungs. Further characterization of this immunocompetent host-*Pneumocystis*-interaction, including assessment of potential clinical significance, is warranted.

1. Introduction

Mild and asymptomatic infections by the fungal pathogen *Pneumocystis* are of uncertain pathological significance. They are known as "*Pneumocystis* colonization" and are highly frequent in normal immunocompetent infants and adults [1]. Recent evidence of *Pneumocystis*-related pathology in immunocompetent infants with histologically mild and asymptomatic *Pneumocystis* infection, was provided by documenting increased protein levels of the goblet cell mucin MUC5AC, a marker of mucus, associated with *Pneumocystis* in autopsied lungs of infants who died in the community with an autopsy diagnosis compatible with Sudden Unexpected Infant Death (SUID) [2]. Mucus is widely recognized as an aggravating factor of respiratory illnesses, including chronic obstructive pulmonary disease (COPD), where *Pneumocystis* has been associated with increased disease severity [3]. Therefore, the documentation of *Pneumocystis*-related mucus pathology in infant lungs warrants continued research to elucidate whether *Pneumocystis* plays a role in the increased respiratory morbidity of infants characteristic of this age group [2].

Mucus production is stimulated through several intracellular pathways still under investigation; one proposed pathway is mediated by chloride channel accessory 1 (hCLCA1), a member of the calcium-sensitive chloride conductance (*CLCA*) family of genes, whose expression is increased in human airways of asthmatic and COPD patients [4–9]. In general, CLCA proteins mediate airway epithelium immune responses inducing mucous cell metaplasia and airway hyperreactivity [6,7]. More specifically, it has been documented in cell culture models, that *mClca3*/*hCLCA1* stimulates mucus (MUC5AC) production [7,10]. In addition, it has been shown in mouse models, and in human and rodent primary

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cell cultures, that *mClca3*/*hCLCA1* expression occurs through a Stat6-dependent pathway [8].

Important insight into the role of *Pneumocystis* in this pathway has been gained through studies using immunocompetent mouse models which showed that *mClCa3* (or *Gob5*), the murine homolog of *hCLCA1*, is significantly increased in association with *Pneumocystis*[11] . In addition, it has been documented more recently that *Pneumocystis* can induce STAT6-dependent pathways eliciting mouse-strain-dependent responses [12]. The link between CLCA proteins and mucus overproduction is well reported in animal models [6,7]. Studies in infant lungs would be ideal for understanding the link between *Pneumocystis* colonization and mucus overproduction recently reported in infants [2,13]. Moreover, since respiratory viruses are recognized agents of increased mucus production [4] and because their relative contribution to hCLCA1 and MUC5AC with respect to *Pneumocystis* in infant lung samples remains unknown, we also evaluated the presence of common respiratory viruses in infant lungs in this study.

2. Materials and methods

2.1. Subjects and samples

The study, approved by the Ethics Committees of the North Metropolitan Area of Health and of the University of Chile School of Medicine in Santiago, was retrospectively conducted in fresh-frozen stored infant lung specimens previously categorized as *Pneumocystis* negative or positive, blinded to autopsy diagnosis and date of death, by microscopy and n-PCR. Samples were age matched and a 1:2 (negative:positive) ratio was used. They corresponded to 55 legally-required infant autopsies conducted between 1999 and 2004 at the Servicio Medico Legal, the coroner's office in Santiago. Samples stored at −80 °C, were selected from 18 *Pneumocystis*-negative and 37 *Pneumocystis*-positive infants with sufficient tissue left for analyses. Their mean age was 3.19 (1.0–11.9) months; all had died suddenly and unexpectedly (SUID) in the community without hospitalization [2]. One gram of deep lung tissue was extracted with all possible sterile precautions inside a laminar flow biosafety cabinet, flash-frozen pulverized in liquid nitrogen using a mortar and pestle, homogenized, and frozen at −80 °C until nPCR was repeated to re-confirm their *Pneumocystis jirovecii*-status. Quantitative PCR (qPCR) for *P. jirovecii* was performed on all *P. jirovecii*-positive samples; Reverse Transcription PCR (RT-PCR) or PCR for respiratory viruses, and Western blot analyses of hCLCA1 were also performed.

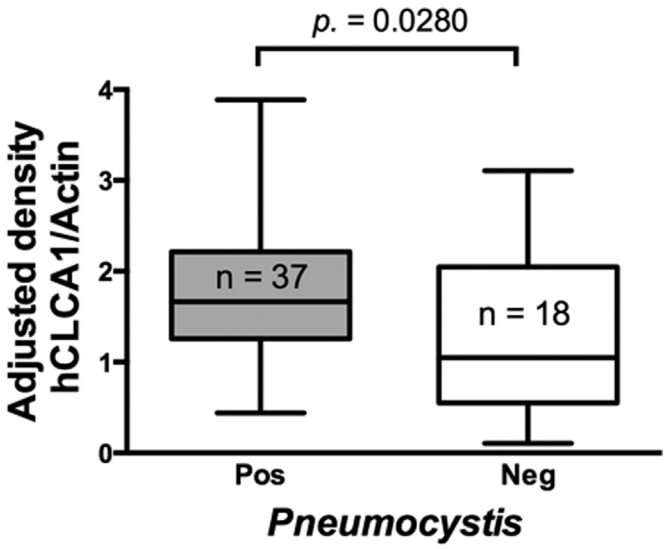

Fig. 1. *Pneumocystis*-associated increased expression of hCLCA1 ($P=0.0280$). Western blot determinations performed in fresh-frozen homogenized autopsy lung samples from *Pneumocystis*-positive ($n=37$) and *Pneumocystis*-negative ($n=18$) infants. Data are presented as median interquartile range. hCLCA1 expression levels were analyzed using Mann–Whitney test. Significance was defined as $P<0.05$.

2.2. Pneumocystis and virus determinations

Pneumocystis status of samples was re-confirmed using a nested-PCR specific for *P. jirovecii* as described [2]. Total DNA extraction was performed using QIAamp®DNA Minikit (Qiagen, Valencia, CA, USA). RNA was extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacture's instructions. *P. jirovecii* burden was quantified by qPCR amplifying the human *Pneumocystis* GpA/MSG gene with specific primers and probe (5′ d FAM-TGCAAACCAACCAAGTGTACGACAGG-BHQ-1 3′) as described [14,15]. These probe quantifications were compared with *Pneumocystis* SYBR green quantifications of the same specimens in our previous study [2]. cDNAs were synthesized to identify Respiratory Syncytial Virus (RSV), Influenza A and B, Parainfluenza virus 1, 2, and 3, and Metapneumovirus, by RT-PCR with specific primers [16–19]. Total DNA was used to evaluate Adenovirus by PCR as described [20]. Viral positive controls were additionally confirmed using standard diagnostic immunofluorescence microscopy. Bacterial cultures are not considered as part of the legal autopsy protocol, and were not done because the samples were received after 24 h post-mortem [2].

2.3. hCLCA1 determinations

Samples for hCLCA1 determinations were processed as described, unless stated otherwise. Western blot were performed from 30 μg protein aliquots, using SDS-PAGE 12% polyacrylamide resolving gels. hCLCA1 was detected using mouse anti-hCLCA1 IgG (1:500 sc-271156, Santa Cruz, USA). Measured values were normalized by human actin-gene expression for inter-sample comparison.

2.4. Statistical analyses

GraphPad Prism 5 software (San Diego, CA, USA) was used for analysis. Comparisons between normalized levels of hCLCA1 protein expression values according to the presence of *Pneumocystis* or of viruses were performed using Mann–Whitney. The correlation between hCLCA1 protein levels with *Pneumocystis* GpA/MSG copies was done using the Spearman test. A P value of <0.05 was considered significant.

3. Results

All selected infants were confirmed to have died suddenly and unexpectedly at home and without being hospitalized, indicating that *Pneumocystis* infection in them was mild. *P. jirovecii* diagnostic status was also re-confirmed by n-PCR in the 37 *Pneumocystis*-positive and 18 *Pneumocystis*-negative infants. Mean *Pneumocystis* burden, as determined using the probe method, was 10,119 (1–299,697; median 120) GpA/MSG copies/ng human DNA. *Pneumocystis* burden determinations using SYBR Green method, reported in a previous study on these same samples [2], were concordant with the probe method determinations in this study.

Analysis of protein extracts documented a significant increase in normalized expression levels of hCLCA1 in *Pneumocystis*-positive samples compared to *Pneumocystis*-negative samples ($P=0.0280$) (Fig. 1), suggesting that *Pneumocystis* is associated