

## SHORT REPORT

# CLCA4 variants determine the manifestation of the cystic fibrosis basic defect in the intestine

Ernst-Wolfgang Kolbe<sup>1</sup>, Stephanie Tamm<sup>1</sup>, Silke Hedtfeld<sup>1</sup>, Tim Becker<sup>2</sup>, Burkhard Tümmler<sup>1,3</sup> and Frauke Stanke<sup>\*,1,3</sup>

The manifestation of the monogenic disease cystic fibrosis results from the cystic fibrosis transmembrane conductance regulator (CFTR)-mediated basic defect defined as an altered chloride transport. An association study using contrasting endophenotypes was conducted with 17 markers to allow fine-mapping of a previously reported association signal within the *CLCA* gene cluster. Markers were analyzed for association with the manifestation of the basic defect in the patient population of the European CF Twin and Sibling Study composed of 101 families with a total of 171 patients. The manifestation of the basic defect was associated with markers rs11807298–rs6684219, encompassing the *CLCA4* promoter ( $P_{\text{raw}} = 0.0013$ ;  $P_{\text{corr}} = 0.0157$ ). Refined analysis of the *CLCA4* association signal among F508del homozygous CF patients who exhibit either no, CFTR-mediated or  $\text{Ca}^{2+}$ -mediated residual chloride conductance revealed that allele distributions for markers rs11807298–rs113894048–rs6684219 differed significantly among these three patient groups. Our data strongly argue that *CLCA4* modulates the capability to express residual chloride secretion in colonic tissue. The latter finding is in consistency with the now favored role of the *CLCA* proteins in signal transduction in epithelial cells.

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## INTRODUCTION

Cystic fibrosis (CF) is the most common severe monogenic autosomal-recessively inherited disease affecting 1 out of 3200 newborns in the Caucasian population.<sup>1</sup> The basic defect in CF is defined as an impaired chloride conductance in epithelia that express the disease-causing gene *CFTR*, a chloride channel localized in the apical membrane of differentiated epithelial cells.<sup>1</sup> Apart from the diagnostic sweat test and transepithelial nasal potential difference measurement, intestinal current measurement (ICM) can be used to characterize the CF basic defect *ex vivo* in excised rectal suction biopsies.<sup>2</sup> Even though the manifestation of the basic defect at the cellular and epithelial levels is less vulnerable to environmental influences than any clinical outcome parameter such as lung function, growth and stature, substantial variability of the basic defect phenotype among sib pairs with shared *CFTR* mutation genotype is observed.<sup>3,4</sup> A subportion of F508del-*CFTR* homozygous patients display residual chloride secretion in ICM, whereby the pleiotropic compound 4,4,8-diisothiocyanostilbene-2,2,8-disulfonic acid (DIDS), which inhibits  $\text{Ca}^{2+}$ -activated chloride channels but not cystic fibrosis transmembrane conductance regulator (CFTR),<sup>5</sup> can be used to distinguish CFTR-mediated chloride secretion from alternative chloride secretion.

We have previously investigated the *CLCA* gene cluster as a modifier in CF, demonstrating that the locus contains a modifier for the manifestation of DIDS-inhibitable residual chloride secretion.<sup>6</sup> Here, we have used a customized SNP map of informative markers and a resequencing strategy for fine-mapping of the modifier in an association study of contrasting phenotypes.

## PATIENTS AND METHODS

### Study population

Genetic data for this study was obtained from 101 CF families with a total of 171 CF patients as described in detail elsewhere.<sup>7</sup> Subsets of case and reference patient populations were selected based on the manifestation of the basic defect in colonic tissue as described elsewhere.<sup>3,4</sup> Briefly, secretagogues that activate or block ion channels, ion exchangers or components of the cellular signal transduction pathways were applied to excised rectal suction biopsies mounted in a micro-Ussing chamber.<sup>8</sup> DIDS, which has been reported to block chloride channels other than CFTR,<sup>5</sup> was used to differentiate between CFTR-mediated residual chloride secretion and chloride secretion through alternative channels.<sup>3</sup> We have enrolled unrelated patients without residual chloride secretion ( $n = 17$ ), chloride secretion mediated by alternative ion channels ( $n = 13$ ) or chloride secretion mediated by CFTR ( $n = 22$ ).<sup>7</sup>

### Genotyping

All SNPs were selected from the database dbSNP and optimized for PCR-RFLP testing in a multiwell-setup.<sup>7</sup> Alleles are named for the absence (allele 1) or presence (allele 2) of the restriction site at all loci as detailed within the Supplementary Table S1.

### Data evaluation

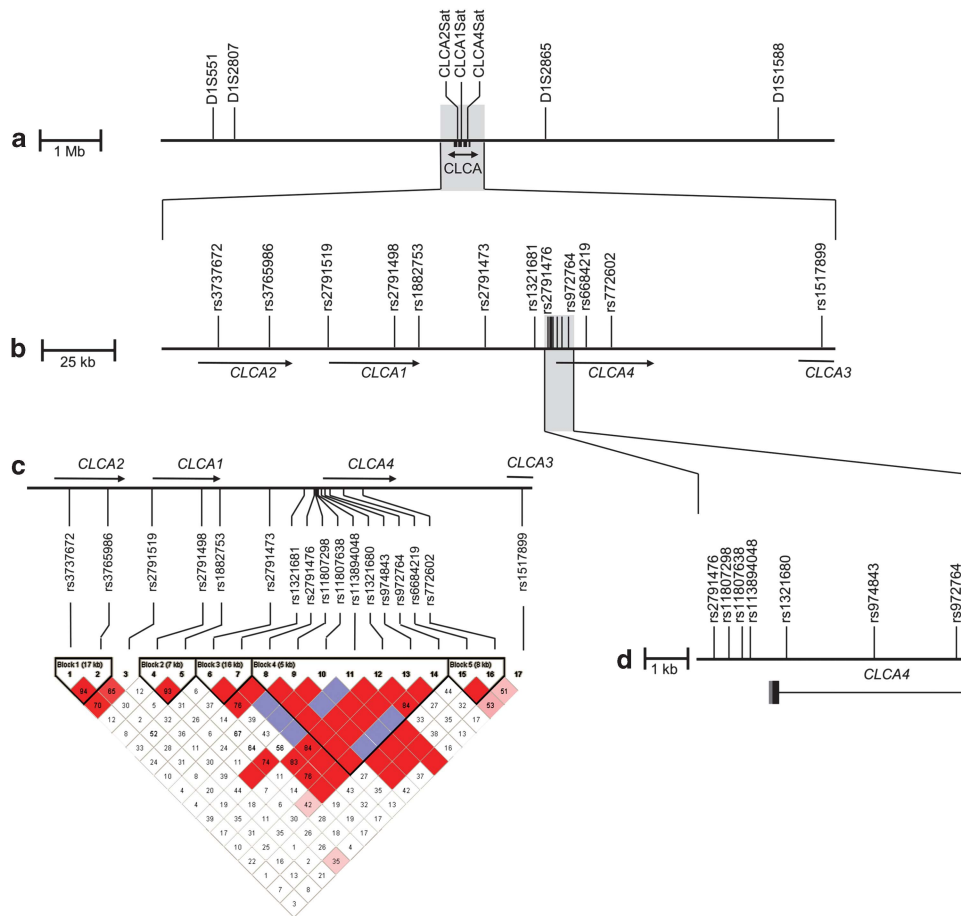
Genotyping data was evaluated with FAMHAP,<sup>9–11</sup> which handles non-informative phases by likelihood-weighted haplotype explanations, whereby all haplotypes consistent with the Mendelian law are considered and their weight is established in accordance with the haplotype distribution observed in the entire data set. To enable a consistent assignment of weighted haplotype explanations within all subgroups, the genotyping data set of the European CF Twin and Sibling study composed of 101 CF families with a total of 171 CF patients was provided as training set to FAMHAP.<sup>7</sup> Analysis was done with

<sup>1</sup>Department of Pediatrics, Hannover Medical School, Hannover, Germany; <sup>2</sup>Institute of Medical Biometry, Informatics and Epidemiology, University of Bonn, Bonn, Germany;

<sup>3</sup>Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research, Hannover, Germany

\*Correspondence: Dr F Stanke, Department of Pediatrics, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany. Tel: +49 511 532 6722; Fax: +49 511 532 6723; E-mail: mekus.frauke@mh-hannover.de

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**Figure 1** The *CLCA* gene cluster. The display shows the location of microsatellite markers within the *CLCA* gene cluster<sup>6</sup> and the surrounding genomic area (a) and the customized SNP map with a total of 17 diallelic markers (b, d) used for fine-mapping. Marker D1S551 is also known as COPD11 (Chronic obstructive pulmonary disease QTL 11).<sup>15</sup> SNPs rs2791476, rs11807298, rs11807638 and rs113894048 in the *CLCA4* promoter area (d) were identified after sequencing of contrasting haplotypes (see text for details). Please note the change in scale by several orders of magnitude from A to D. *CLCA* haplotype blocks (c) were visualized using Haploview.<sup>16</sup>

10000 Monte-Carlo simulated data sets<sup>9–11</sup> and all raw observed  $P$ -values ( $P_{\text{raw}}$ ) were corrected for multiple testing carried out by FAMHAP for the entire data set of 17 SNP markers ( $P_{\text{corr}}$ ).<sup>10</sup>

### Sequencing

The 2965-bp PCR product was generated using the terminal primers 5'-GGGCATCCAGATTGGTAAAG-3' and 5'-CCCGACACAATGCAAAGATA-3'. For generation of 500–800bp reads, the terminal primers and a total of 12 evenly spaced internal primers on the forward and the reverse strand were employed (Qiagen, Hilden, Germany). Raw reads were aligned to the reference sequence using the CodonCodeAligner (CodonCode Corporation, Dedham, MA, USA).

### RESULTS

Previously, we have described an association signal at D1S2807, localized several Mb upstream of the *CLCA* gene cluster (Figure 1a).<sup>6</sup> Next, we could reproduce this finding at marker *CLCA2*-Sat, which was one out of three microsatellite markers placed within the *CLCA* gene cluster to confirm the effect observed at D1S2807 (Figure 1a).<sup>6</sup> To follow up on the previously reported association signal with DIDS-sensitive residual chloride secretion, each *CLCA* gene was now targeted with up to three SNPs in the initial phase of this study. Markers rs3737672, rs3765986, rs2791519, rs2791498, rs1882753, rs772602 and rs1517899 were genotyped

(Figure 1b), six two-marker haplotypes of adjacent markers were constructed and the haplotype distributions were compared between case and reference populations. The best  $P$ -value was observed for fragment rs1882753–rs772602 spanning the intergenic segment between *CLCA1* and *CLCA4* ( $P_{\text{raw}}=0.0387$ ). Hence, our further analyses were directed to the 5' end of *CLCA4*.

We have selected three individuals with contrasting diplotypes and ICM basic defect phenotypes and compared the primary sequence of 2965 bp of the *CLCA4* 5' area including exon 1. In comparison with the NCBI reference sequence, differences at four positions were identified after sequencing (Table 1c). These four markers, another two SNPs within the intergenic region between *CLCA1* and *CLCA4* as well as four intergenic markers in *CLCA4* were typed on the entire patient cohort of 171 individuals from 101 CF families in addition to the initial seven SNPs. LD analysis performed on the data set for the 101 CF families described five haplotype blocks within the *CLCA* gene cluster (Figure 1c).

Two-marker-haplotype distributions comparing patients without chloride secretion and patients with DIDS-sensitive residual chloride secretion revealed best  $P$ -values for three combinations with the *CLCA4* intragenic marker rs6684219 in block 5 ( $P_{\text{raw}}=0.0006$  for rs2791473–rs6684219,  $P_{\text{raw}}=0.0008$  for rs3765986–rs6684219 and  $P_{\text{raw}}=0.0013$  for the two-marker haplotype spanning block 4 and 5 in *CLCA4* at rs11807298–rs6684219), whereby the association signal

**Table 1** Sequence analysis of the *CLCA4* promoter

A Haplotype distribution		ICM – no Res.	ICM – DIDS Res.					
rs1882753–rs772602	1–1	0.667	0.272					
	1–2	0.111	0.283					
	2–1	0.111	0.061					
	2–2	0.111	0.384					
$P_{raw}$			0.0387					
B Selected samples								
Patient 1	ICM – no Res.	Paternal chr.	rs1882753–rs772602 haplotype 1–1					
Patient 2	ICM – DIDS Res.	Maternal chr.	1–1					
		Paternal chr.	1–2					
Patient 3	ICM – DIDS Res.	Maternal chr.	2–2					
		Paternal chr.	1–2					
		Maternal chr.	2–2					
C Haplotypes of the sequenced fragment								
Patient 1	ICM – no Res.	Paternal chr.	rs1882753	rs2791476	rs11807298	rs11807638	rs113894048	rs772602
		Maternal chr.	1	2	1	2	1	1
Patient 2	ICM – DIDS Res.	Paternal chr.	1	1	1	2	1	2
		Maternal chr.	2	2	1	2	2	2
Patient 3	ICM – DIDS Res.	Paternal chr.	1	1	1	2	1	2
		Maternal chr.	2	2	1	2	2	2

$P_{raw}$ : uncorrected *P*-value for marker combination; among the initially typed SNPs rs3737672, rs3765986, rs2791519, rs2791498, rs1882753, rs772602 and rs1517899, best *P*-value among the six combinations of two adjacent markers was observed for the fragment rs1882753–rs772602 (see text for details). Population sizes genotyped for these markers are: patients without residual chloride secretion (ICM no Res.;  $n = 17$ ), chloride secretion mediated by alternative ion channels (ICM – DIDS Res.;  $n = 13$ ). Maternal and paternal chromosomes were reconstructed at heterozygous loci after confirmatory retyping of the family.

retained significance upon correction for multiple testing of 17 SNPs ( $P_{corr} = 0.0157$ ). Furthermore, patients with DIDS-insensitive, ie CFTR-mediated residual chloride secretion, differed from patients who do not exhibit residual chloride secretion at rs113894048–rs6684219 spanning block 4 and 5 in *CLCA4* ( $P_{raw} = 0.0049$ ;  $P_{corr} = 0.181$ ).

We noticed that the same 3'-marker rs6684219 defines the fragment with best *P*-value for both types of chloride secretion. The systematic evaluation of both two-marker haplotypes and the corresponding three-marker haplotype is shown in Table 2. Evidently, the *CLCA4* promoter haplotype distributions are dissimilar for patients with DIDS-sensitive, alternative chloride conductance and DIDS-insensitive, CFTR-mediated chloride conductance.

## DISCUSSION

We were curious about the role of the SNPs identified within the *CLCA4* 5' area identified after resequencing (Figure 1d). First, we used a database of transcription factor-binding consensus sites<sup>12</sup> to inquire whether SNPs rs2791476, rs11807638, rs11807298 or rs113894048 change the predicted binding site of a transcription factor and identified 74 possible candidates. Next, we restricted our analysis to those transcription factors which are expressed in rectal suction biopsies (deposited in the GEO database under accession no. GSE15568; van Barneveld *et al*, personal communication), reducing the list to seven plausible candidates. Strikingly, five of these seven expressed and differential binding transcription factors (the capicua homolog CIC, the HMG-box transcription factor 1 HBP1, the high-mobility group AT-hook 1 HMGA1, the high-mobility group box 3 HMGB3 and the SRY sex-determining region Y-box 9 SOX9) were predicted to be unique to the rs113894048 allele. We hypothesize that the observed association of *CLCA4* 5' haplotypes is at least partially caused by the activation of *CLCA4* gene expression in response to the differentiation state of the tissue, marking *CLCA4* as a developmental surface protein that determines whether none, CFTR-mediated

residual chloride secretion or DIDS-sensitive alternative chloride secretion is maintained by the epithelial cell.

CLCA proteins are most likely expressed as a precursor and cleaved asymmetrically in a soluble and a membrane-bound product.<sup>13</sup> The secreted soluble CLCA peptide is surmised to bind to its receptor and release a signal transduction cascade that ultimately leads to differentiation of the target cell.<sup>13</sup> For CLCA1, the pathway has so far been reconstructed as follows: IL13 promotes via STAT6 the expression of CLCA1.<sup>13</sup> Upon processing and cleavage, the soluble CLCA1 peptide promotes the expression of mucin genes *MUC5AC*, *MUC5B* and *MUC19* and leads to differentiation into a mucus-secreting cell that explains the role of this protein in asthma and COPD.<sup>13</sup> Although similar key players for *CLCA4* have not been identified yet, the highly conserved protein sequence of the CLCAs supports a similar mechanism and function. Our data suggest that effector proteins of a *CLCA4*-mediated signal transduction cascade determine whether or not the colonocyte of F508del homozygous CF patients expresses no chloride channel, retains the residual expression of the chloride channel CFTR or alternatively expresses the molecular entity underlying the calcium-activated chloride conductance CaCC.

We have based our basic defect assessment and the phenotype analyzed in the association study on rectal suction biopsies, and thus our finding shows that *CLCA4* modifies the manifestation of the basic defect in intestinal cells. In this context, it is interesting to note that *CLCA4* has been described by global transcriptome analysis as a region-selective gene for colon in a tissue scan of the gastrointestinal tract while also being a marker for Crohn's disease and ulcerative colitis,<sup>14</sup> suggesting that *CLCA4* is a key player of epithelial integrity beyond the disease condition CF.

Taken together, our data argue strongly for the involvement of *CLCA4* in determining which type of residual chloride secretion, if any, is expressed in colonic tissue. The latter finding is in consistency with the now favored role of the CLCA proteins in signal transduction in epithelial cells. Correspondingly, as it was previously shown that

**Table 2 Association of CLCA4 haplotypes with residual chloride secretion in ICM**

		Reference ICM – no Res.	Case ICM – DIDS Res.	OR <sup>a</sup>	Case ICM – Res.	OR <sup>a</sup>
rs11807298—rs6684219	1–1	0.276	0.595	3.844	0.688	5.720
	1–2	0.613	0.055	0.037	0.250	0.212
	2–1	0.001	0.055		0.000	
	2–2	0.110	0.295		0.063	
	$P_{\text{raw}}^a$			0.0013		0.0338
rs113894048—rs6684219	1–1	0.222	0.350	1.885	0.688	7.700
	1–2	0.722	0.350	0.207	0.313	0.175
	2–1	0.056	0.300		0.000	
	2–2	0.000	0.000		0.000	
	$P_{\text{raw}}^a$			0.0750		0.0049
rs11807298—rs113894048—rs6684219	1-1-1	0.222	0.300		0.688	7.700
	1-1-2	0.611	0.050	0.033	0.250	0.212
	1-2-1	0.056	0.300		0.000	
	2-1-1	0.000	0.050		0.000	
	2-1-2	0.111	0.300	3.429	0.063	
	$P_{\text{raw}}^a$			0.0021		0.0200

<sup>a</sup> $P$  and  $OR$  calculated vs 'ICM no Res.' as reference.

$P_{\text{raw}}$ : uncorrected  $P$ -value for marker combination;  $P$ -values corrected for 9 SNPs within block 4 and 5 SNPs are:  $P_{\text{ICM DIDS Res.}} = 0.0174$ ;  $P_{\text{ICM Res.}} = 0.059$ ;  $P$ -values corrected for the entire data set of 17 SNPs are:  $P_{\text{ICM DIDS Res.}} = 0.0157$ ;  $P_{\text{ICM Res.}} = 0.181$ . Abbreviations: ICM, intestinal current measurement; ICM – no Res., absence of chloride secretion upon stimulation with carbachol and histamine; ICM – DIDS Res., presence of chloride secretion upon stimulation with carbachol but absence of chloride secretion upon stimulation with histamine after inhibition with DIDS (chloride secretion mediated by alternative ion channels); ICM – Res., presence of chloride secretion upon stimulation with carbachol and presence of chloride secretion upon stimulation with histamine after inhibition with DIDS (chloride secretion mediated by CFTR). Sample sizes are: ICM – no Res.:  $n = 17$ ; ICM – DIDS Res.:  $n = 13$ ; ICM – Res.:  $n = 22$ .

patients who exhibit DIDS-sensitive residual chloride conductance display a more severe phenotype,<sup>4</sup> our findings suggest that mechanisms that govern epithelial cell differentiation deserve further attention to clarify the role of modifying genes in CF.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## WEB RESOURCES

dbSNP at [www.ncbi.com](http://www.ncbi.com) GEO database at [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/) MatInspector at [www.genomatix.de](http://www.genomatix.de)

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