Replication of *LCE3C–LCE3B* CNV as a Risk Factor for Psoriasis and Analysis of Interaction with Other Genetic Risk Factors

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Recently, a deletion of two late cornified envelope (*LCE*) genes within the epidermal differentiation complex on chromosome 1 was shown to be overrepresented in 1,426 psoriasis vulgaris (PsV) patients of European ancestry. In this study, we report a confirmation of this finding in 1,354 PsV patients and 937 control individuals of German origin. We found an allele frequency of the deletion of 70.9% in PsV patients and of 64.9% in control individuals ($\chi^2 = 17.44$, $P = 2.97 \times 10^{-5}$, odds ratio (95% confidence interval) = 1.31 (1.15–1.48)). The overall copy number of the two *LCE* genes had no influence on the age of onset, but we observed a dosage effect at the genotype level. There was no evidence of statistically significant interaction with copy number of the β-defensin cluster on 8p23.1 or with an IL-23R pathway variant in a combined data set of German and Dutch individuals, whereas evidence for interaction with the PSORS1 risk allele in German individuals was marginal and did not remain significant after correction for multiple testing. Our study confirms the recently published finding that the deletion of the two *LCE* genes is a susceptibility factor for PsV with dosage effect, while, because of power limitation, no final conclusion regarding interaction with other PsV risk factors can be made at this stage.

INTRODUCTION

Psoriasis vulgaris (PsV) is a common inflammatory skin disorder characterized by epidermal hyperproliferation, altered keratinocyte differentiation, and inflammation. An *HLA-C* allele or a variant in strong linkage disequilibrium (LD) with it is the major risk allele, especially in PsV patients manifesting before the age of 40 years. This finding probably corresponds to the most evidentiary psoriasis susceptibility locus (PSORS1). Besides this risk factor and PSORS4 (see below), replicated associations with candidate genes (*RAPTOR* and *SLC12A8*) have been reported so far at PSORS2 and PSORS5 (Hewett *et al.*, 2002; Helms *et al.*, 2003; Capon *et al.*, 2004; Huffmeier *et al.*, 2005). In addition, variants in the IL-23R pathway have been identified by two genome-wide association studies for PsV (Capon *et al.*, 2007; Cargill *et al.*, 2007) and could be confirmed by further independent studies (Chang *et al.*, 2007; Nair *et al.*, 2008, 2009; Huffmeier *et al.*, 2009b). Finally, copy number variation (CNV) of a genomic segment on chromosome 8p23.1 harboring a cluster of genes encoding β-defensins (DEFB) small antimicrobial peptides, was identified to be associated with psoriasis in a Dutch and a German case–control cohort (Hollox *et al.*, 2008) and variants of the NF-κB-pathway were recognized to be risk factors for PsV (Nair *et al.*, 2009).

PSORS4 is a susceptibility locus on chromosome 1 initially identified in an Italian genome-wide linkage analysis of Italian families (Capon *et al.*, 1999). This locus is of special interest for PsV, because it comprises the epidermal differentiation complex, a group of genes expressed in the upper strata of the epidermis. Although several genes at PSORS4—e.g., *LOR*, *LCE1C*, *PGLYRP*, *SPRR* genes, *PRR9* genes, and *IVL*—have been suggested to account for psoriasis susceptibility (Giardina *et al.*, 2006; Chen *et al.*, 2009; Liu *et al.*, 2008; Kainu *et al.*, 2009), very recently, a CNV

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within the late cornified envelope (*LCE*) gene cluster was identified by a genome-wide scan using pooled DNAs. The deletion of two *LCE* genes (*LCE3C* and *LCE3B*) was shown to be at higher frequency in 1,426 psoriasis patients from several European countries than in controls and to be associated with psoriasis in a large family-based cohort (de Cid *et al.*, 2009). Expression data in the same study suggested that carriers of the deletion may have a compromised repair response after barrier disruption of the skin (de Cid *et al.*, 2009). In addition, in an independent genome-wide association study of a Chinese cohort, single-nucleotide polymorphisms (SNPs) in strong LD with the deletion were identified as risk factors for psoriasis (Zhang *et al.*, 2009).

This study investigates the contribution of *LCE3C–LCE3B*-del to PsV susceptibility in German patients and also whether this contribution to disease is independent of three other known genetic risk factors. We, therefore, analyzed a large case–control cohort comprising 1,354 PsV patients and 937 control individuals for the presence of *LCE3C–LCE3B*-del and also genotyped three SNPs known to be in strong LD with it (rs10888502, rs4112788, and rs4845456). In addition, we analyzed a possible interaction between *LCE3C–LCE3B*-del and other known risk factors, PSORS1 risk allele, the copy number of the β-defensin cluster on chromosome 8p23.1 and an SNP (rs6887695) near the *IL12B* gene, which represents the strongest genetic variant of the IL-23R pathway in German cohorts (Huffmeier *et al.*, 2009b).

RESULTS

Genotyping of the *LCE* CNV as well as all SNPs fulfilled Hardy–Weinberg equilibrium both in groups of German patients and in control individuals. Genotyping rates were between 94.7 and 95.6% for the three SNPs and 99.4% for the *LCE* CNV. All variants except rs10888502 were in near-perfect LD with each other, while LD was strongest between *LCE* CNV and variant rs4112788, with an R^2 value of 0.94.

We observed strong evidence for association of psoriasis with *LCE3C–LCE3B*-del, with a significant allele frequency difference of 6.0%, a *P*-value of 2.97×10^{-5} and a corresponding odds ratio (OR) of 1.31 (95% confidence interval: 1.15–1.48) (Table 1). Association with single SNPs was strongest for rs4112788, but also significant for the two other SNPs at allele level (Table 2). Similar results were obtained at haplotype level: the haplotype ''GGA deletion'' (order of variants: rs10888502—rs4112788—rs4845456—*LCE* CNV) was the most common one in cases and controls (39.4 vs 35.7%), with a corresponding *P*-value of 0.011 (Table 3). Even stronger was the effect of a two-marker haplotype consisting of rs4112788 and *LCE* CNV: risk haplotype ''G-deletion'' was significantly associated with a *P*-value of 0.0010 (Table 3). The *P*-value indicating the distribution of *P*-values was one order of magnitude higher in the two-marker haplotypes analysis compared with four-marker analysis. To determine whether the risk alleles of the three SNPs have independent effects from the *LCE* CNV, we performed a conditional analysis. Thereby, we identified marginal evidence for the independent effects of rs4112788 (*P* = 0.044), but not for the other two SNPs (*P*-values not significant). The *P*-value for rs4112788 remained not significant after correction for multiple testing.

No significant differences in age of disease onset were observed between the three *LCE* CNV genotypes. This was also true when the analysis was restricted to patients with an age of onset before 40 years (also known as type I psoriasis), which represented the majority of our cases. The OR for psoriasis patients homozygous for *LCE3C–LCE3B*-del was 1.61 (1.21–2.14), which was higher than the OR for heterozygous patients (1.16 (0.87–1.55)), indicating a dosage effect.

As only part of the German cohort had been previously genotyped for copy number of β-defensin cluster, we performed a joint analysis with the Dutch study group to

Table 1. Allele and genotype frequencies (absolute number (percentage)) in 1,354 PsV patients and 937 control probands, results of χ^2-statistics, and odds ratios (95% confidence intervals) for the *LCE* CNV

(A)

Allele	CON	PsV	χ^2	*P*	OR (95% CI)
LCE3C–LCE3B-del	1,215 (64.9)	1,899 (70.9)	17.44	2.97×10^{-5}	1.31 (1.15–1.48)
Non-deletion	657 (35.1)	785 (29.2)			

(B)

Genotype	CON	PsV	χ^2	*P*	OR (95% CI)[1]
LCE3C–LCE3B-del/*LCE3C–LCE3B*-del	391 (41.8)	678 (50.5)			
LCE3C–LCE3B-del/non-deletion	433 (46.3)	543 (40.5)	18.010	1.228×10^{-4}	1.37 (1.05–1.80)
Non-deletion/non-deletion	112 (12.0)	121 (9.0)			

Abbreviations: CI, confidence interval; CNV, copy number variation; CON, control probands; *LCE,* late cornified envelope; *LCE3C–LCE3B*-del, deletion of the two *LCE* genes (*LCE3C* and *LCE3B*); OR, odds ratio; PsV, psoriasis vulgaris.
[1]Carriers—homozygous or heterozygous—of LCE3C-LCE3B-del compared to non-carriers.

Table 2. Allele and genotype frequencies (absolute number (percentage)) in 1,354 PsV patients and 937 control probands, results of χ^2-statistics, and odds ratios (95% confidence intervals) for the three SNPs

(A)

SNP	Allele	CON	PsV	χ^2	P	OR (95% CI)
rs10888502	G	685 (38.6)	1,080 (42.2)	5.585	0.018	1.16 (1.03–1.31)
	C	1,091 (61.4)	1,482 (57.8)			
rs4112788	G	1,151 (64.7)	1,799 (69.1)	9.314	0.002	1.22 (1.07–1.39)
	A	627 (35.3)	803 (30.9)			
rs4845456	A	675 (37.7)	1,053 (40.8)	4.295	0.038	1.14 (1.01–1.29)
	G	1,117 (62.3)	1,529 (59.2)			

(B)

SNP	Genotype	CON	PsV	χ^2	P	OR (95% CI)[1]
rs10888502	C/C	332 (37.4)	432 (33.7)			
	C/G	427 (48.1)	618 (48.2)	5.885	0.053	1.17 (0.98–1.4)
	G/G	129 (14.5)	231 (18.0)			
rs4112788	A/A	106 (11.9)	118 (9.1)			
	A/G	415 (46.7)	567 (43.6)	9.502	0.009	1.36 (1.03–1.79)
	G/G	368 (41.4)	616 (47.3)			
rs4845456	A/A	120 (13.4)	221 (17.1)			
	A/G	435 (48.5)	611 (47.3)	5.780	0.056	1.11 (0.93–1.33)
	G/G	341 (38.1)	459 (35.6)			

Abbreviations: CI, confidence interval; CON, control probands; OR, odds ratio; PsV, psoriasis vulgaris; SNP, single-nucleotide polymorphism.
[1]Carriers—homozygous or heterozygous—of the risk allele compared with non-carriers.

Table 3. Frequencies of four- and two-marker haplotypes, results of association statistics, and odds ratios (95% confidence intervals) in 1,354 PsV patients and 937 control probands

Haplotype	CON	PsV	P	OR (95% CI)
(A) Haplotypes consist of four variants in the order rs10888502, rs4112788, rs4845456, LCE CN. P-value indicating distribution of haplotypes: 9.31×10^{-5}				
G G A Del	35.7	39.4	0.011	1.17 (1.04–1.32)
C A G Non-Del	32.4	27.1	1.1×10^{-4}	0.78 (0.68–0.88)
C A G Del	0.4	1.0	0.021	2.69 (1.17–6.18)
C G A Del	1.8	1.2	NS	NA
C G G Del	26.2	27.7	NS	NA
G A G Non-Del	2.6	2.1	NS	NA
(B) Haplotypes consist of two variants in the order rs4112788, LCE CN. P-value indicating distribution of haplotypes: 1.80×10^{-6}				
G Del	64.5	69.3	0.0010	1.24 (1.10–1.41)
A Non-Del	35.0	29.2	3.4×10^{-5}	0.77 (0.68–0.87)
A Del	0.5	1.5	2.7×10^{-4}	3.19 (1.54–6.57)

Abbreviations: CI, confidence interval; CON, control probands; *LCE*, late cornified envelope; NS, not significant; NA, not applicable; OR, odds ratio; PsV, psoriasis vulgaris.

increase the statistical power for interaction analyses. Logistic regression analysis on data from 1,073 individuals did not reveal any evidence for interaction ($P = 0.22$, Table 4). In a combined German and Dutch case–control study consisting of 1,567 patients and 1,272 individuals, no evidence for interaction of the *LCE3C–LCE3B*–CN with rs6887695 (SNP

Table 4. Uncorrected results (*P*-values) of interaction analyses in case–control cohorts

(A) Dutch or German individuals

	LCE3C_3B-CN	IL12B	DEFB-CN
LCE3C_3B-CN	—	0.55	0.22
IL12B	0.55	—	0.80
DEFB-CN	0.22	0.80	—

(B) German individuals

	LCE3C_3B-CN	PSORS1	IL12B	DEFB-CN
LCE3C_3B-CN	—	0.02[1]	0.49	0.32
PSORS1	0.02[1]	—	0.98	0.66
IL12B	0.49	0.98	—	0.56
DEFB-CN	0.32	0.66	0.56	—

Abbreviations: *LCE,* late cornified envelope; PSORS, psoriasis susceptibility locus.
[1]*P*-value did not remain significant when corrected for number of interaction analyses performed.
P-values for interaction of two risk factors can be found in a square, with the corresponding risk factors above and on the left-hand side.

near the *IL12B* gene) was observed ($P = 0.55$). As we observed significant differences in homogeneity of ORs for the PSORS1 risk allele between both cohorts, we analyzed the interaction of PSORS1 with other risk factors only in the subset of 2,291 German individuals. We observed a tendency toward interaction with *LCE3C–LCE3B*–CN, while the *P*-value did not remain significant after correction for the number of interaction analyses performed. Further calculations on possible interactions revealed no significant *P*-values (Table 4).

DISCUSSION

We present the first replication of *LCE3C–LCE3B*—del's association with psoriasis, which was previously reported by de Cid *et al.* (2009). The frequency of the *LCE3C–LCE3B*-del allele in our German case–control cohort was very similar to that in the Dutch and US case–control samples (de Cid *et al.*, 2009). The single SNPs were all associated at allele level, while effects were strongest for rs4112788. This variant also shows strongest LD to *LCE* CNV, and haplotype analyses revealed the stronger effects of a haplotype consisting of these two variants than those of all four variants. Testing for independence of *LCE* CNV and rs4112788 was inconclusive. Currently, we cannot exclude that rs4112788 might modify the risk at this locus, while the *LCE* CNV seems to be the more plausible risk factor. Further functional studies and maybe the upcoming meta-analysis (see below) will hopefully elucidate the role of the two variants in the pathogenesis of PsV.

Several independent studies (see, e.g., Gudjonsson *et al.*, 2002) have observed that the PSORS1 risk factor is strongly associated with early age of disease onset. We performed a similar analysis for *LCE3C–LCE3B*-del, which indicated that it

is not associated with early disease onset. But confirming the previous results from the European study (de Cid *et al.*, 2009), we observed a dosage effect of the *LCE3C–LCE3B*-del at genotype level.

The major risk allele for psoriasis has been found to be epistatic to *LCE3C–LCE3B*-del in the subgroup of Dutch individuals (de Cid *et al.*, 2009), but not in the other subgroups. We observed only marginal evidence for interaction in the German cohorts, which remained not significant after correcting for testing of multiple interactions by Bonferroni. A large multicenter meta-analysis on the possible interaction between PSORS1 risk allele and *LCE3C–LCE3B*-del is underway (Riveira *et al.*, in preparation). The meta-analysis will provide more generalizable data regarding this possible interaction and might show differences in various European populations. We did not observe evidence for interaction of *LCE3C–LCE3B*-del with the β-defensin CNV nor with a variant of the *IL12B* locus, whereas we did not test the interaction with newly identified risk alleles in the genes *TNIP1* and *TNFAIP3* (Nair *et al.*, 2009). Regarding possible interaction(s), we have to consider limited power because of study size, and this limit does not allow a final conclusion. In general, no evidence for interaction is not a wholly unexpected finding, as interaction in Crohn's disease—a disease in which many more loci/risk factors have been identified in total—has been observed for only single risk factors (Hampe *et al.*, 2007; Barrett *et al.*, 2008) and evidence for the replication of these findings is scarce.

MATERIALS AND METHODS
Study groups
The German case–control study consisted mainly of 1,114 PsV patients and the 937 control probands, previously described in Huffmeier *et al.* (2009b). A minority of the 1,354 cases were index patients of 240 trios of a family-based cohort (Huffmeier *et al.*, 2009a). The studies were approved by the ethical committees of the University of Erlangen-Nuremberg and of the University of Münster. The Dutch case–control group consisted of 213 patients and 335 control individuals and has been described earlier (Hollox *et al.*, 2008). Permission for these studies was obtained from the local medical ethics committee (Comissie Mensgebonden Onderzoek). Written informed consent was obtained from each patient and control proband before enrollment. The investigations were conducted according to the Declaration of Helsinki principles.

Genotyping
Late cornified envelope variants. We genotyped the CNV in German samples with a protocol modified from that used by de Cid *et al.* (2009): a multiplex assay of two fluorescently marked PCR products detected on an ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA). Briefly, we used 40 ng DNA and the Amplitaq Gold polymerase (Applied Biosystems) to amplify a breakpoint-spanning PCR product of 351 bp (F: 5′-GGATACTAAG AAGTTCTCAC-3′, R: 5′-GTGGTGAGAGAGGGCATCTC-3′) for deletion alleles and a second amplicon for wild-type alleles (primers within the deleted region, product size 561 bp (F: 5′-CATTAGCCTG GAGCTTTTGC-3′, R: 5′-ACAAGTGATAACATTGTCAGGAGG-3′). The multiplex reaction was diluted 1:20; a volume of 5 μl was

analyzed using size standard LIZ600 (Applied Biosystems) on the capillary sequencer. Genotypes passing quality control showed peak intensities >2,000 fluorescent units, and in putative heterozygote individuals, ratios of peak heights of *LCE3C–LCE3B*-del to those of the non-deletion alleles were >0.5 and <3. To estimate the error rate of genotyping, we performed duplicate genotyping of six 96-well-microtitre plates. In all, 515 DNAs yielded an amplication in both runs and were used to compare between genotypes. Within these, 449 (87.2%) passed both quality criteria (see above) and were concordant in both experiments. Seven DNAs (1.4%) passed quality control in both runs, but showed divergent genotypes. We therefore have to assume a genotyping error rate of approximately 1.4%. For genotyping failures of the *LCE* CNV, we used the three primers LCE3CF (5′-TCACCCTGGAACTAGACCTCA-3′), LCE3CR (5′-CTCCA ACCACTTGTTCTTCTCA-3′), and LCE3CR2D (5′-CATCCCAGGGA TGCTGCATG-3′) in a multiplex reaction as previously described (de Cid *et al.*, 2009) and performed agarose gel electrophoresis to separate the deletion allele (199 bp) and non-deletion allele (240 bp). Using this method, we also confirmed the genotypes of 37 individuals who had been successfully genotyped by the mainly used method.

Single-nucleotide polymorphisms were genotyped with TaqMan assays (Applied Biosystems) in the German study groups. In addition, we sequenced 24 randomly selected individuals for the three variants as described earlier (Huffmeier *et al.*, 2009b) and could verify their genotypes.

Genotyping of the *LCE* CNV in Dutch patients and controls was performed as described in de Cid *et al.* (2009). rs6887695, the variant of the IL12B gene, was successfully genotyped in Dutch cases and controls with a genotyping rate of 96.1%.

DEFB CNV and available genotypes for interaction analyses

Copy numbers of the β-defensin cluster were determined in subgroups of 317 German PsV patients and 305 control individuals, as well as in 179 Dutch psoriasis patients and 272 control probands as described by Hollox *et al.* (2008). The copy number of β-defensin cluster was not available for the rest of the cohort.

For interaction analyses, we had data for the *LCE* copy number variant and the *IL12B* variant res6887695 in all German and Dutch individuals. For the main risk factor for psoriasis (PSORS1), we had information on carriers/non-carriers of the HLA-Cw0602 allele in Dutch individuals and used an estimation in German individuals as described earlier (Huffmeier *et al.*, 2009b).

Statistical and interaction analyses

To determine allele frequency differences between cases and controls, we used chi-square statistics, with one degree of freedom for comparisons of allele frequencies and two degrees of freedom for comparisons of genotypes. For comparisons resulting in significant statistical values, an OR with 95% confidence interval was calculated (OR (confidence interval)). The LD between the four variants was calculated with the software Haploview vs 4.0, Broad Institute, Harvard, Massachusetts (Barrett *et al.*, 2005). Haplotypes were calculated with FAMHAP (Herold and Becker, 2009).

An unpaired *t*-test statistic was calculated to test whether PsV patients carrying one or two *LCE3C–LCE3B*-del alleles develop disease earlier than non-carriers/carriers of one *LCE3C–LCE3B*-del allele.

To test for a dosage effect of *LCE3C–LCE3B*-del, logistic regression analysis was performed using as reference homozygotes for the *LCE3C_LCE3B* non-deletion allele.

To adjust for the effect of *LCE3C_3B*-del, we followed the logistic regression framework described in Cordell and Clayton (2002). Significant *P*-values were corrected by Monte–Carlo simulation for three markers.

For stratification for other psoriasis risk factors, we used genotypes of the strongest associated variant of the IL-23R pathway (rs6887695 of *IL12B* gene) in the German cohort (Huffmeier *et al.*, 2009b). To stratify for single risk factors, carriers of the common risk allele ''G'' of variant rs6886795 and carriers of more than four copies of the β-defensin cluster were regarded as risk groups versus non-risk groups.

To test whether the German and Dutch cohorts could be combined in one logistic regression model, the Cochran–Mantel–Haenszel statistical analyses were performed. For *LCE* and *IL12B* data, the test of homogeneity of the OR showed no significant difference ($P = 0.526$ for a recessive model and $P = 0.186$ for a dominant model), whereas it showed significant differences regarding the PSORS1 risk allele.

To test for interaction, we followed the logistic regression framework described in Cordell and Clayton (2002). Our model contained one variable (risk/non-risk) for each potential interaction partner and an interaction term to test for deviation from a multiplicative model. The significance of the interaction term was evaluated using a chi-square distribution with one degree of freedom. Significant *P*-values were corrected using the Bonferroni method, i.e., *P*-values were multiplied by 6 (for the number of interaction analyses performed).

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