A genome-wide association study identifies 6p21 as novel risk locus for dilated cardiomyopathy

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Aims

Dilated cardiomyopathy (DCM) is one of the leading causes for cardiac transplantations and accounts for up to one-third of all heart failure cases. Since extrinsic and monogenic causes explain only a fraction of all cases, common genetic variants are suspected to contribute to the pathogenesis of DCM, its age of onset, and clinical progression. By a large-scale case-control genome-wide association study we aimed here to identify novel genetic risk loci for DCM.

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Introduction

Dilated cardiomyopathy (DCM) is a severe cardiovascular disorder with an estimated prevalence of 37 in 100,000 people. It is the most frequent cause of heart failure and cardiac transplantation in young adults and accounts for up to 30–40% of all heart failure cases as found in large randomized trials. About one-third of all patients have a suspected familial disease indicating a genetic basis of DCM. Linkage analyses and consecutive candidate gene sequencing or recently next-generation sequencing have facilitated the identification of monogenic causes of DCM, making genetic testing for the early identification of disease carriers a clinical option. However, the genes identified so far still explain only a small fraction of all cases. Furthermore, the genotype–phenotype relationship in DCM is highly variable and even in a single family carrying the very same mutation the clinical findings and disease progression may vary markedly. Hence, the search for novel susceptibility mechanisms is a major challenge in DCM research.

So far, only a few common variants associated with DCM have been identified by candidate approaches. Recently, we identified a 600 kilobase (kb) large region in linkage disequilibrium on chromosome 5q31.2–3.1 that shows associations with dilated and ischaemic cardiomyopathy. Cappola et al. described a candidate gene association study based on single nucleotide polymorphism (SNP) genotyping in genes coding for proteins with a known cardiovascular function. The authors identified an association between rs9262636 located in the Heat shock protein beta-7/CLNCNKA locus and DCM, which was consequently supported by another candidate gene association study as well as a pooled screening approach for genome-wide associations (GWA). The latter study furthermore identified a genetic susceptibility locus on chromosome 10q26 within the BCL2-associated ataxanogene 3 (BAG3) gene. BAG3 was subsequently also found as monogenic cause of DCM.

Here we present the results from a three-stage case–control GWA study conducted within the German National Genome Research Network (NGFN), the German Center for Cardiovascular Research (DZHK), the Competence Network Heart Failure (CNHF), the German–French network INSIGHT DCM, and the European DCM network INHERITANCE to further elucidate the complex genetic basis of DCM. We found a close association of genetic variants on chromosome 6p21 with DCM and show the association of HLA-C gene expression with this locus. These findings indicate a link between genetic variants, the susceptibility to idiopathic DCM, and inflammatory disease mechanisms.

Materials and Methods

Ethics and study design

The study was conducted in accordance with the principles of the Declaration of Helsinki. All participants of the study have given written informed consent and the study was approved by the ethic committees of the participating study centres.

The present study relied on a three-staged case–control design. Stage 1 (screening phase) included 909 genome-wide genotyped individuals of European descent with DCM recruited between 2005–08 and 2120 controls from the PopGen and KORA population-based cohorts. In a first replication stage, SNPs on locus 6p21 were genotyped in 2597 DCM cases from Germany and Italy recruited between 2007 and 2011 as well as in 4867 controls from the population-based SHIP study (SHIP-0 and SHIP-TREND) and from Italy. In a second replication stage, the lead SNP was replicated in a cohort of 637 DCM cases and 723 healthy controls representing European Caucasians of French decent. Supplementary material online, Table S1 gives the origin of cases and controls.

Patients and controls

Dilated cardiomyopathy was diagnosed according to the guidelines of the World Health Organization. The inclusion criteria for DCM cases in Stages 1 and 2 were at least moderately [left ventricular ejection fraction (LVEF) < 45%] reduced left ventricular systolic function (assessed by echocardiography or left ventricular angiography) in the absence of a relevant coronary artery disease (CAD). In replication 2, we genotyped a cohort of DCM patients from France, which had an at least moderately to severely reduced LVEF (< 35%). Patients with valvular, hypertensive, or congenital heart disease, history of myocarditis, or cardio-toxic chemotherapy were excluded. Controls derived from KORA, PopGen, SHIP, Italy, or France had no history of heart disease, such as valvular, hypertensive, or congenital heart disease, myocarditis or cardio-toxic chemotherapy, CAD, myocardial infarction, heart failure, or cardiomyopathies.

Genotyping

Please refer to Supplementary material online, Methods for details.

Statistical analysis

Case–control association tests were conducted using the PLINK software package version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink). Associations were tested using logistic regression assuming an underlying additive genetic model with 1 degree of freedom (df). For detailed information on the statistical analysis please refer to the Supplementary material online, Methods.


Results

Screening for dilated cardiomyopathy-associated loci by a genome-wide association study

In the screening analysis (Stage 1), we investigated a German cohort comprising 909 patients with idiopathic DCM and a population-based control group with 2120 individuals from the KORA and PopGen consortia. The characteristics and origin of study samples can be found in Table 1 and Supplementary material online, Table S1.

When assuming an additive model of inheritance adjusted for age and sex, we identified six signals with \( P \)-values surpassing genome-wide significance of \( P = 1.7 \times 10^{-7} \); rs9262636, rs9262635, rs9262615, rs4947296, rs3130000 on chromosome 6, and rs10859313 on chromosome 12) (Figure 1; Table 2; refer to Supplementary material online, Table S2 for unadjusted \( P \)-values). On chromosome 6, we find multiple, closely located SNPs in a 31 kb large region with \( P \)-values ranging from \( 10^{-5} \) down to \( 7.09 \times 10^{-9} \) (Figure 2A), underlining a robust disease association for this locus. Since the estimated inflation factor of the screening study was 1.18, we additionally adjusted for potential population stratification using genomic control (GC) (Supplementary material online, Figure S1A and B). After correction, rs9262636 and rs9262635 on chromosome 6 still surpassed the Bonferroni corrected level of genome-wide significance, showing odds ratios (ORs) of 1.48 (95% confidence interval (CI): 1.29–1.68) after correction for GC or 1.41 (1.23–1.62) after correction for the first 10 principal components, respectively.

In addition to the novel candidate loci for DCM, we confirmed weaker associations with SNPs in the HSPB7 locus (rs1763610: \( P = 0.002 \) and rs4661346: \( P = 0.024 \)) and the CD14 locus (rs2569193: \( P = 0.049 \)), which were previously identified as susceptibility loci for heart failure due to DCM.

Replication of dilated cardiomyopathy-associated SNPs on chromosome 6p21

To further substantiate our findings from the screening stage, we subsequently carried out an independent replication study by genotyping a large cohort of 2597 DCM patients and 4867 controls to validate the observed association signals. We selected 12 SNPs based on stringent quality criteria and significance of association for follow-up genotyping (see the section Materials and methods). When applying an additive genetic model of inheritance adjusted for age and sex, 2 out of 12 selected SNPs replicate the association observed in the screening cohort (\( P < 0.05 \); see Table 2). In the combined analysis of Stages 1 and 2, we find an association signal on the 6p21 locus with a \( P \)-value of \( P = 4.90 \times 10^{-9} \) for rs9262636 under an additive penetrance model adjusted for age and sex (\( P = 7.25 \times 10^{-9} \) adjusted for age, sex, and GC). When combining the screening and replication stages using inverse variance weighting, we observe a combined OR and a corresponding 95% CI of 1.195 (1.113–1.283) for rs9262636.

Since the successfully replicated SNPs reside all on chromosome 6, we tested for an underlying DCM risk haplotype. As depicted in Figure 2B, we observe that the haplotype GCGGG is significantly associated with DCM in the screening stage (\( P = 3.23 \times 10^{-7} \)) and shows a trend towards significance in the replication stage (\( P = 0.055 \)). The estimated attributable risk for this haplotype is 3.3% based on the haplotype frequencies from pooled samples of both stages of the study (attributable risk separated by stages: screening: 7.0%, replication: 1.7%).

Next, we conducted an independent second replication in a cohort of 637 cases and 723 controls from France by direct genotyping of the lead-SNP rs9262636. In an additive model adjusted for gender and age, we find an OR of 1.22 (1.020–1.459; \( P = 0.029 \)). Since one of the phenotypic criteria (LVEF) of this cohort was slightly more stringent (\( \leq 35\% \)) than for the screening and first replication cohort (\( < 45\% \)), we did not include them in the combined analysis.

Associated single nucleotide polymorphisms on chromosome 6p21 indicate the contribution of inflammatory mechanisms in the pathogenesis of dilated cardiomyopathy

The replicated SNPs on chromosome 6 (Figure 2A) are located within the major histocompatibility complex (MHC) region 6p21.3, \( \sim 300 \) kb telomeric of the HLA-B locus. The genes MUC21 and MUC22 are located upstream of our lead-SNP rs9262636. The two

Table 1  Study sample characteristics of the screening and replication cohorts

<table>
<thead>
<tr>
<th>Cohort Description</th>
<th>n</th>
<th>Women (%)</th>
<th>Age (years)</th>
<th>LVEF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Screening (Stage 1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM: Germany (NGFN)</td>
<td>909</td>
<td>25.2</td>
<td>56.6 ± 12.9</td>
<td>28.5 ± 10.9</td>
</tr>
<tr>
<td>Controls: Germany (KORA and PopGen)</td>
<td>2120</td>
<td>49.7</td>
<td>57.4 ± 14.1</td>
<td>n.a.</td>
</tr>
<tr>
<td>Replication (Stage 2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM: Germany and Italy</td>
<td>2597</td>
<td>24</td>
<td>51.4 ± 12.5</td>
<td>30.5 ± 10.1</td>
</tr>
<tr>
<td>Controls: Germany (SHIP and SHIP-TREND) and Italy</td>
<td>4867</td>
<td>50</td>
<td>47.7 ± 16.4</td>
<td>n.a.</td>
</tr>
<tr>
<td>Replication (Stage 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCM: France</td>
<td>637</td>
<td>19.6</td>
<td>47.3 ± 11.7</td>
<td>23.3 ± 6.8</td>
</tr>
<tr>
<td>Controls: France</td>
<td>723</td>
<td>11.1</td>
<td>48.8 ± 10.5</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

LVEF, left ventricular ejection fraction.
SNPs with the lowest P-values (rs9262635 and rs9262636) are located directly within intron 2 of the predictably non-coding gene ‘HLA complex group 22’ (HCG22), while two additional SNPs (rs4713429 and rs9262615) are located ~2 kb upstream (Supplementary material online, Figure S2).

To further elucidate the most likely candidates for DCM susceptibility, we next performed expression quantitative trait locus (eQTL) analyses in 986 samples to investigate the gene expression levels as molecular or intermediate phenotypes. The transcriptomic data from this cohort (SHIP-TREND) were generated using RNA prepared from whole blood samples. Based on this cohort, we find a highly significant association between our lead-SNP rs9262636 and HLA-C mRNA levels ($P = 4.05 \times 10^{-17}$) (Figure 3) as well as associations with additional transcripts (Table 3). Three out of the five most significant associations ($P < 10^{-5}$) were found for genes besides HLA-C that also encode heavy chain paralogues of the major histocompatibility antigen complex, namely HLA-DRB5 ($P = 5.96 \times 10^{-15}$), HLA-DRB1 ($P = 1.22 \times 10^{-10}$), and HLA-DQB1 ($P = 1.52 \times 10^{-06}$). For HLA-C, HLA-DRB5, and HLA-DQB1, mRNA levels decreased with each additional minor G allele of rs9262636 (estimated $\beta = -0.47$, $-0.29$, and $-0.12$, respectively). In contrast, HLA-DRB1 transcript levels increased per G allele ($\beta = 0.21$) (Figure 3). The fifth gene, VARS2, encodes a putative mitochondrial valyl-tRNA synthetase of unclear physiological relevance within the context of heart disease. Among the five most significant associations, the effect of rs9262636 on the VARS2 transcript level was smallest ($\beta = 0.08$).

**Discussion**

Genetic variants affect disease penetrance and modulate phenotypic expression of many complex diseases. In the cardiovascular field, for example, the prominent role of common genetic variants was repeatedly demonstrated for CAD and its associated risk factors, such as hypercholesterolaemia, arterial hypertension, or diabetes mellitus. However, only few studies were reported so far on genetic modifiers of DCM or other causes of systolic heart failure. Here we report on a novel susceptibility locus identified by a case-control GWA study for DCM relying on individual genotyping of study samples.

We identified and replicated SNPs surpassing genome-wide significance that are located within the MHC region on chromosome 6. Early studies have linked this region harbouring several candidate genes to psoriasis, which is an inflammatory skin disease. Since common variants that affect the coding regions of proteins account for only a minority of observed disease associations, the elucidation of the genetic architecture of human disorders has recently focused on variants residing in non-coding regions. Since the identified DCM risk SNP rs9262636 also resides within a non-coding gene, we have performed here eQTL analyses to further prioritize the most likely candidates for DCM susceptibility. eQTL studies are a powerful tool to define regulatory elements that affect the levels of gene expression, providing important insight into affected biological pathways that might best explain the observed phenotypic variation and susceptibility to complex diseases. As demonstrated above, we identified a strong association between our lead-SNP rs9262636 and gene-specific mRNA levels including HLA-C and -D genes. These genes were in distances of 210 kb (HLA-C), 1459 kb (HLA-DRB5), 131 kb (VARS2), 1520 (HLA-DRB1), and 1600 kb (HLA-DQB1) to rs9262636. Therefore, it still remains unclear whether the observed regulation involves only cis or also trans regulatory effects, both potentially involving the non-protein coding gene HCG22.
### Table 2  Dilated cardiomyopathy-associated single nucleotide polymorphisms

<table>
<thead>
<tr>
<th>SNP</th>
<th>CHR</th>
<th>Screening (Stage 1)</th>
<th>Replication (Stage 2)</th>
<th>Combined P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A1 F (aff)</td>
<td>F (unaff) OR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>rs13428663*</td>
<td>2</td>
<td>G 0.1908 0.1382</td>
<td>1.45 (1.24–1.7)</td>
<td>3.40E–06</td>
</tr>
<tr>
<td>rs933199*</td>
<td>6</td>
<td>G 0.09167 0.05737</td>
<td>1.75 (1.41–2.17)</td>
<td>3.22E–07</td>
</tr>
<tr>
<td>rs3130000*</td>
<td>6</td>
<td>T 0.05639 0.09793</td>
<td>0.52 (0.41–0.66)</td>
<td>8.23E–08</td>
</tr>
<tr>
<td>rs4713429</td>
<td>6</td>
<td>G 0.2475 0.19</td>
<td>1.43 (1.25–1.64)</td>
<td>2.79E–07</td>
</tr>
<tr>
<td>rs9262615</td>
<td>6</td>
<td>C 0.2629 0.1983</td>
<td>1.46 (1.28–1.67)</td>
<td>2.21E–08</td>
</tr>
<tr>
<td>rs9262635</td>
<td>6</td>
<td>G 0.2704 0.2014</td>
<td>1.48 (1.29–1.68)</td>
<td>7.85E–09</td>
</tr>
<tr>
<td>rs9262636</td>
<td>6</td>
<td>G 0.2704 0.2014</td>
<td>1.48 (1.29–1.68)</td>
<td>7.09E–09</td>
</tr>
<tr>
<td>rs2523883/rs2517471</td>
<td>6</td>
<td>A 0.4481 0.3794</td>
<td>1.34 (1.19–1.51)</td>
<td>7.64E–07</td>
</tr>
<tr>
<td>rs4947296</td>
<td>6</td>
<td>C 0.1134 0.07197</td>
<td>1.7 (1.4–2.06)</td>
<td>9.09E–08</td>
</tr>
<tr>
<td>rs12552255*</td>
<td>9</td>
<td>G 0.176 0.1278</td>
<td>1.5 (1.28–1.76)</td>
<td>7.10E–07</td>
</tr>
<tr>
<td>rs10904002*</td>
<td>10</td>
<td>A 0.08773 0.04793</td>
<td>1.81 (1.44–2.28)</td>
<td>4.11E–07</td>
</tr>
<tr>
<td>rs10859313*</td>
<td>12</td>
<td>A 0.06107 0.1153</td>
<td>0.53 (0.43–0.67)</td>
<td>2.81E–08</td>
</tr>
<tr>
<td>rs7192626*</td>
<td>16</td>
<td>T 0.07531 0.04107</td>
<td>1.84 (1.44–2.34)</td>
<td>9.13E–07</td>
</tr>
</tbody>
</table>

Given are P-values and ORs with 95% confidence intervals for SNPs found to be significantly associated with DCM applying an additive model adjusted for sex and age. Replication analysis was additionally adjusted for place of origin (Germany/Italy). SNPs outside the locus 6p21 are marked by an asterisk and have been genotyped in a subset of 5700 samples within the replication stage. In the combined analysis, rs2523883 of screening stage was combined with rs2517471 of replication stage, because no Taqman assay for rs2523833 was available (see the section Materials and methods). For rs10904002, P-values were not combined because of differing minor alleles.

Chr, chromosome; A1, minor allele; F (aff), allele frequency in affected samples; F (unaff), allele frequency in unaffected samples; OR, odds ratio; CI, confidence interval; P, P-value of association analysis; GC-adj, P-values adjusted by genomic control; Bonf, P-values adjusted for multiple testing using the Bonferroni correction.
**Figure 2** A regional plot of associations on the 6p21 locus and haplotype analysis. (A) A regional association plot showing the association results between genotyped single nucleotide polymorphisms (black dots), imputed single nucleotide polymorphisms (grey dots) and dilated cardiomyopathy based on the screening cohort. The plot displays minus log10 P-values from an additive logistic regression model adjusted for age and sex. Multiple imputation relied on the CEU (Utah residents with ancestry from northern and western Europe) population in HapMap and on genotyped single nucleotide polymorphisms around the strongest signal of association. Linkage disequilibrium blocks are calculated from the genotype data of the screening cohort and open reading frames are given. (B) A linkage disequilibrium plot of seven single nucleotide polymorphisms located on chromosome 6p21 based on data from the screening stage showing a block of five single nucleotide polymorphisms in close linkage disequilibrium.
The molecular pathways by which genetic variants in MHC heavy chains may affect DCM and its progression remain elusive. The cell membrane-bound MHC consists of the subclasses I–III. Class I molecules, such as HLA-D and -C, play a central role in the immune surveillance by presenting peptides to immune-competent cells. In contrast to other class I genes, polymorphisms within

**Figure 3** Boxplots of the expression quantitative trait loci probes. Boxplots of the five genes with expression quantitative trait loci P-values below $1 \times 10^{-3}$ via association of single nucleotide polymorphism rs9262636. The y-axis shows the residual log2 expression values per genotype (x-axis) adjusted for sex, age, and the first 50 principal components obtained from principal component analysis over the expression values. The band in the box denotes the median and the bottom and top of the box are the 25th and 75th percentiles, respectively, whereas the whiskers extend them by the 1.5 interquartile range. The genotypes were estimated using the best guess genotype from allele dosage probabilities.
eQTL associations of rs9262636 with P-values < 1 × 10⁻³. The beta references to the increase or decrease of the expression value per minor G allele (forward strand) adjusted for sex, age, and the first 50 principal components obtained from principal component analysis over the expression values. Associations showing a P-value below 1 × 10⁻⁴, which corresponds to a Bonferroni correction of all 48,802 tested expression probes, are significant. SE is the standard error of the beta. Mean and SD are the mean and the standard deviation of the normalized log₂ expression values of all individuals, respectively.

As shown, we have successfully identified a novel risk locus for idiopathic DCM on chromosome 6p21. By further increasing the number of patients, one might identify additional loci in future studies. Since DCM can be the endpoint of various cardiac disorders including hypertensive or ischaemic heart disease, myocarditis, or cardio-toxicity, it is pivotal to carefully phenotype patients recruited for such studies, which may also explain that the overlap with previous studies relating polymorphisms in genes encoding HLA-C, in the pathogenesis of idiopathic DCM and support the hypothesis of genetically driven, inflammatory mechanisms in DCM. This may involve alterations of autoimmunity as well as immune competency against viruses, eventually promoting viral persistence in the myocardium.

One potential limitation of the current study is the estimated inflation factor in the screening stage of 1.18, for which we corrected by using GC. For polygenic diseases such as DCM substantial genomic inflation is expected independently from the presence of population stratification, potentially interfering with the identification of associations in these diseases. Additionally, although the control subjects from the KORA and PopGen cohorts are well established and widely used within the scientific community and show little genetic differentiation along a north-south gradient within Germany, apparently part of the observed genomic inflation in our study is driven by population differences between these control cohorts. For instance, when PopGen samples are removed from the screening stage, genomic inflation is reduced to 1.13. Importantly no significant association of our lead-SNP rs9262636 is present when calculating associations between KORA controls and PopGen controls, which together with two independent replication stages shows that the here identified signals are indeed due to true associations.

In the past decades larger evidence for inflammatory mechanisms as important pathophysiological pathways in heart failure progression has emerged. However, a profound clinical benefit of anti-inflammatory therapies for DCM, such as anti-TNFα, immunoglobulin, or interferon application, could not be proved in larger randomized trials. Hence, besides identification of novel inflammatory targets a better understanding of underlying mechanisms and improved patient selection is thought to be key for successful future developments.

Our study reveals a novel susceptibility region for DCM and thus expands our knowledge of the genetic variance contributing to this complex disease. For the associated locus on chromosome 6, we identified novel candidate genes that support the involvement of autoimmunity and inflammatory processes in DCM aetiology. While our findings are in good agreement with previous studies relating polymorphisms in genes encoding HLA-D antigens to DCM susceptibility, HLA-C and the mediation of HLA-D gene transcription by the here identified DCM susceptibility locus represent intriguing novel pathophysiological insights. Since HLA molecules are ubiquitously expressed, it might be speculated that a distinct profile of these proteins on the leucocyte or cardiomyocyte surface, defined by specific amounts of class I and class II heavy chain paralogues of the MHC antigen complex, may be mediated by the here identified genetic variants and thereby modify individual susceptibility to DCM and response to anti-inflammatory therapies. Similar to almost all GWA studies, additional functional investigations are needed to fully understand the functional roles of the here identified genetic associations.

### Supplementary material

Supplementary material is available at European Heart Journal online.
Authors’ contributions


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