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The role of CXORF21 in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) has a strong genetic contribution and, to date, more than 50 SLE risk loci have been mapped in Europeans. In a recent SLE GWAS (genome-wide association studies), CXORF21 was found to be associated with the disease. After re-sequencing in 117 SLE cases of European origin, a cluster of rare coding variants (3 missense and 1 nonsense) were detected in exon 3 of CXORF21. A follow-up cohort of 519 SLE patients were Sanger sequenced to detect rare coding variants in CXORF21 exon 3. The protein CXorf21 is understudied and its function is unknown. Therefore an expression analysis (Flow cytometry and Western blot) of B cells and monocytes in SLE patients and healthy controls was conducted. It was not possible in this study to show evidence of association of rare coding variants in CXORF21 with SLE, supporting the emerging hypothesis that rare coding variants might play a negligible role in complex disease. The expression analysis enabled new insights into the expression of CXorf21 in various cell types. Interestingly, cell-type specific reactions to stimulants were detected. Although no difference is seen in unstimulated cells, the comparison between SLE patients and controls shows disease-dependent differences in CXorf21 expression following cell stimulation. The Western blot analysis suggests a genotypic difference in expression of CXorf21, which we hope to replicate by acquiring the genotype data of the individuals analysed by flow cytometry.

Keywords:

CXORF21, SLE

Introduction

Systemic lupus erythematosus (SLE) is a severe rheumatic disease with a strong genetic contribution, caused by an abnormal autoimmune response. The presence of autoantibodies against a variety of autoantigens can induce symptoms and tissue damage in any organ system. It often affects skin, joints or kidney with various degrees of severity (Tsokos 2011). Both innate and adaptive immune cells play a role in SLE, e.g. autoantibody-producing B cells or monocytes. Influences on the SLE pathogenesis might be hormones, epigenetic mechanisms or environmental factors and certainly a genetic influence.
To date, more than 50 SLE risk loci have been mapped in Europeans. In a recent SLE genome-wide association study (GWAS), **CXORF21** was found to be associated with the disease. The topSNP rs887369 (hg19) is a synonymous variant and is located in exon 3 of **CXORF21** (Bentham et al. 2015). Additionally, re-sequencing **CXORF21** in 117 SLE cases of European origin by Next Generation Sequencing (NGS), identified a cluster of rare (minor allele frequency (MAF) <1%) coding variants (3 missense and 1 nonsense) in exon 3. The sex imbalance of SLE, shown by a 9:1 female to male ratio (Rahman / Isenberg 2008) and the evidence that **CXORF21** escapes X-inactivation (Zhang et al 2013) makes the X-linked gene very interesting as well. To date, the gene product of **CXORF21**, the protein CXorf21, is understudied and its function in the cell is unknown. It has been reported to be associated with mental impairment (Zhang et al. 2013) and might play a role in Klinefelter's syndrome (47, XXY) (Vawter et al. 2007).

**Aims of the study**

The aims of this study were two-fold. Firstly, to screen for an association between rare coding variants of **CXORF21** exon 3 and SLE and secondly to analyse the expression of CXorf21 protein. Expression levels were measured in B cells and monocytes of SLE patients and healthy controls in both resting and interferon α (IFNα) and lipopolysaccharide (LPS) stimulated cells. Also, the expression differences of CXorf21 in EBV immortalised B cell lines with various genotype (rs887369: CC risk and AA non-risk) were investigated.

**Methods**

PCR and Sanger sequencing were used to detect rare coding variants in **CXORF21** exon 3 of 519 SLE patients and GeneScreen software (Carr et al. 2011) was used to analyse the data. For the statistical analysis, the collapsing method was used (Li / Leal 2008). The Exome Aggregation Consortium (ExAC) database was chosen as control dataset (ExAC database, http://exac.broadinstitute.org/, May 2016).

For the expression analysis of the protein CXorf21, an intracellular, indirect flow cytometry and a Western blot analyses were conducted. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of 17 female SLE patients and 13 female, healthy individuals. Additionally, the PBMCs were stimulated with interferon α or LPS for 20 h. After the surface staining (CD19 for B cells and CD14 for monocytes), the fixed and permeabilised cells were stained with the primary (anti-CXorf21 or isotype control [IC]) and subsequently the labelled secondary antibody. The flow cytometry analysis was conducted on a FACS Canto II machine. The median fluorescence intensities (MFI) of CXorf21 were obtained by the software FlowJo (FlowJo, LLC, Data Analysis Software, version 10.1, for Windows, http://www.flowjo.com, March 2016).

For the Western blot analysis, 8 EBV immortalised B cell lines of female, healthy individuals were used to investigate the genotypic expression of CXorf21. They were selected for their genotype (SNP rs887369, located in exon 3 of **CXORF21**): 4 homozygous risk (CC) and 4 homozygous non-risk (AA) cell lines. Additionally, the cells were analysed after stimulation for 24 h with interferon α. The Western blot bands were quantified by the software ImageJ (Schneider et al. 2012) and normalised to the “housekeeping gene” β actin.

All statistical analyses and graph creations were conducted with GraphPad Prism software (version 5.02, for Windows, www.graphpad.com, April 2016). The SLE Patients from the Louise Coote Lupus unit (4th floor, Tower Wing, Guy's Hospital) satisfy the revised criteria for the classification of systemic lupus erythematosus (Tan et al. 1982 & Hochberg 1997). Peripheral blood of healthy controls for the flow cytometry analysis was obtained from the Department of Twin Research & Genetic Epidemiology,
King’s College London (Spector et al. 2006). The eight human EBV growth-transformed lymphoblastoid cell lines for the Western blot analysis were obtained from Coriell Biorepository (Coriell institute for medical research, https://catalog.coriell.org/, February 2016).

Results

Three out of four NGS-detected rare coding variants were confirmed by Sanger sequencing, the nonsense mutation was an NGS false positive. However, in the 519 additional Sanger sequenced SLE patients only two variants were found, one was a synonymous variant. Although the preliminary work showed a significantly higher frequency of variants in SLE patients compared to the unaffected controls, the Sanger sequencing analysis failed to confirm this finding. Since all previously detected variants were found in patients with early disease onset, the analysis was conducted again, focusing on patients with an age of diagnosis ≤25. Again, this could not confirm the prior findings.

The expression analysis suggested strong evidence that CXorf21 is not expressed on the cell surface but intracellular. It also indicated higher expression in monocytes than in B cells (figure 1).

![Box whiskers plot of MFI to compare CXorf21 expression between monocytes and B cells in SLE patients (n=17, left figure) and healthy controls (n=13, right figure). The x-axes show the stimulation (unstimulated, IFNα or LPS-stimulated), the y-axes the median fluorescence intensity (MFI) of CXorf21 (flow cytometry analysis); the whiskers display the minimum and maximum MFI. * indicates significance (p-value < 0.028), ** displays p values < 0.005, *** labels p values < 0.0005, ns stands for ‘not significant’.](image)

In general, it appears that in monocytes 20 h LPS stimulation has a lowering effect on the expression of CXorf21, whereas in B cells it tends to increase the protein level (although not significant in the control dataset), suggesting a cell type-specific effect. Interferon α stimulation results in higher CXorf21 expression in B cells (again not significant in the controls), but it seems not to have an impact on monocytes (figure 2).
Figure 2: Box whiskers plot of MFI to compare CXorf21 expression between different stimulation conditions in monocytes and B cells of SLE patients (n=17, left figure) and healthy controls (n=13, right figure). The x-axes show the stimulation (unstimulated, IFNα or LPS stimulated), the y-axes the median fluorescence intensity (MFI) of CXorf21 (flow cytometry analysis); the whiskers display the minimum and maximum MFI. * indicates significance (p-value < 0.028), ** displays p values < 0.005, ns stands for ‘not significant’

Furthermore, between SLE patients and healthy individuals no difference in the CXorf21 level in unstimulated monocytes, as well as in unstimulated B cells was detectable. When stimulated with IFNα, monocytes of patients showed higher CXorf21 levels than monocytes of healthy individuals, whereas when stimulated with LPS, B cells of patients showed higher expression (figure 3).

Figure 3: Box whiskers plot of MFI to compare the expression of CXorf21 between SLE patients (n=17, left figure) and healthy controls (n=13, right figure) in monocytes and B cells. The x-axes show the stimulation (unstimulated, IFNα or LPS stimulated), the y-axes the median fluorescence intensity (MFI) of CXorf21 (flow cytometry analysis); the whiskers display the minimum and maximum MFI. * indicates significance (p-value < 0.028), ns stands for ‘not significant’

The Western blot showed a trend, although not significant, that CXorf21 is higher expressed in the risk group (rs887369, CC). When stimulated with interferon α, the tendency reverses, visible by a strong decline of CXorf21 in the risk group and the slight increase in the non-risk group (figure 4).
**Discussion**

It was not possible in this study to show evidence of association of rare coding variants in **CXORF21** with SLE. However, as described before by others, rare coding variants might contribute negligibly to the heritability of autoimmune diseases (Hunt et al. 2013). Common variants, which were associated with disease by a GWAS (as rs887369) might tend to influence the gene expression. This is suggested by the Western blot analysis for this project as well. When these common variants of the gene are predicted to influence the gene expression, rare coding variants, which change the protein coding sequence, might not influence the disease risk.

The expression analysis enabled new insight into the expression of CXorf21 by e.g. revealing the higher expression in monocytes, compared to B cells. Also, different reactions to stimulants, depending on the cell type, were detected: LPS seems to decline CXorf21 expression in monocytes, whereas it elevates the protein level in B cells. The impact of interferon α was only seen in B cells, by decreasing CXorf21 expression. The comparison between SLE patients and controls did not show unambiguous results, although it seems that only stimulation leads to disease dependent different CXorf21 expression. However, we note the relatively small sample size (13 controls and 17 SLE cases). Ongoing work to increase the cohort size may provide sufficient power to detect additional significant differences.

The Western blot analysis of EBV immortalised cell lines suggests a genotypic difference in expression of CXorf21. We hope to replicate this finding in the ex vivo data by acquiring the genotype data of the individuals analysed by flow cytometry.

**Relevance for work**

SLE is a severe autoimmune disease; however, there is still a huge lack of knowledge about the cause. Therefore research about the genetic background and examination of the proteins associated with the disease, might bring new insights in the disease etiology and might induce new concepts for diagnostic tests or therapies.
Short summary

Systemic lupus erythematosus (SLE) has a strong genetic contribution and, to date, more than 50 SLE risk loci have been mapped in Europeans. In a recent SLE GWAS (genome-wide association studies), CXORF21 was found to be associated with the disease. After re-sequencing in 117 SLE cases of European origin, a cluster of rare coding variants (3 missense and 1 nonsense) were detected in exon 3 of CXORF21. A follow-up cohort of 519 SLE patients were Sanger sequenced to detect rare coding variants in CXORF21 exon 3. The protein CXorf21 is understudied and its function is unknown. Therefore an expression analysis (Flow cytometry and Western blot) of B cells and monocytes in SLE patients and healthy controls was conducted.

It was not possible in this study to show evidence of association of rare coding variants in CXORF21 with SLE, supporting the emerging hypothesis that rare coding variants might play a negligible role in complex disease.

The expression analysis enabled new insights into the expression of CXorf21 in various cell types. Interestingly, cell-type specific reactions to stimulants were detected. Although no difference is seen in unstimulated cells, the comparison between SLE patients and controls shows disease-dependent differences in CXorf21 expression following cell stimulation. The Western blot analysis suggests a genotypic difference in expression of CXorf21, which we hope to replicate by acquiring the genotype data of the individuals analysed by flow cytometry.

References

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