CARD16 is differentially expressed in the blood of patients with Crohn's Disease.

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Crohn's Disease (CD) is an inflammatory bowel disease that causes significant morbidity, declines in quality of life and loss of economic productivity (1, 2). Twin studies show concordance rates at 58.3% and 63.3% in monozygotic twins demonstrating that genetic factors contribute to the disease etiology in a significant manner (3, 4). We mined a published microarray dataset to perform global differential gene expression profiling using the blood of patients with Crohn's Disease (5). We identified the caspase recruitment domain family member 16, CARD16, also known as COP and Pseudo-ICE as among the most differentially expressed genes in the blood of patients with Crohn's Disease. CARD16 expression in the whole blood of patients with Crohn's Disease was significantly higher than in the blood of healthy, non-affected subjects. Analysis of a separate dataset (6) revealed that CARD16 is a transcriptionally activated in peripheral blood mononuclear cells following stimulation with muramyl dipeptide. CARD16 has been described as both a negative and positive regulator of interleukin-1 β (IL-1 β) secretion (7, 8) and an activator of NF-kB signaling (9). These data suggest that differential and significantly increased expression of CARD16 in the blood of patients with Crohn's Disease, in conjunction with the contrasting roles of CARD16 in inflammasome versus NF-kB signaling might contribute to the dysregulated cytokine profile seen in the hematopoietic tissues of patients with Crohn's Disease (10-15).

Keywords: Crohn's Disease, muramyl dipeptide, CARD16, cytokines in Crohn's Disease.

Crohn's Disease (CD) is an inflammatory bowel disease that can affect any part of the digestive tract from the mouth to the anus but most commonly affects the distal portion of the small intestine, the ileum (1). CD has been shown to be a disease of polygenic nature with concordance rates in monozygotic twins reported at 58.3% and 63.3% (3, 4). This demonstrates that there is a significant genetic contribution to the development of Crohn's Disease. Genome-wide association studies have demonstrated that the most significant genetic risk factor for the development of Crohn's Disease resides in the nucleotide oligomerization domain 2 (NOD2) locus (16).

We used published transcriptome data to identify the most significant changes in the transcriptional behavior of the blood cells of patients with Crohn's Disease by comparing total transcription in the blood of patients with Crohn's Disease versus that of non-affected subjects (5). We identified CARD16 (caspase recruitment domain family member 16) as a differentially expressed gene in the blood of patients of Crohn's Disease. CARD16 was expressed at significantly higher levels in the blood of patients with Crohn's Disease in the absence of any stimulus. Moreover, using a separate published dataset we identified CARD16 as a transcriptional target of the gene expression program activated following exposure of peripheral blood mononuclear cells to muramyl dipeptide (6), the ligand for the gene product of the sentinel CD susceptibility locus NOD2, also known as CARD15 (12, 17, 18). CARD16 is composed almost exclusively of a CARD domain and has been described as both an activator and inhibitor of inflammasome signaling leading to IL-1b secretion (7, 8), as well as an activator of NF-kB signaling by binding to RIP2 (9). Variant alleles of NOD2 found more frequently in patients with Crohn's Disease lead to abnormalities in cytokine production that remain incompletely understood due to differences in phenotypes based on species-divergent biology and differences in knock-out versus mutant allele modeling (19, 20). CARD16 may be a contributing factor to the cytokine imbalance found in patients with Crohn's Disease (21).

Methods

We used datasets GSE94648 (5) and GSE68765 (6) for this analysis in conjunction with GEO2R. GSE94648 (5) was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology with n=22 whole blood from controls and n=50 whole blood from patients with Crohn's Disease. GSE68765 (6) was generated using Illumina HumanHT-12 V4.0 expression beadchip technology with n=6 untreated PBMC and n=5 PBMC stimulated with MDP.

The Benjamin and Hochberg method of p-value adjustment was used for ranking of differential expression but raw p-values were used for assessment of statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI generated category of platform annotation was used.

A statistical test was performed to evaluate the significance of difference between mRNA expression levels of CARD16 in the blood of patients with Crohn's disease as compared to healthy controls, using a two-tailed, unpaired t-test with Welch's correction. Only p-values less than 0.05 were considered statistically significant.

Results

We mined published datasets (5, 6) to identify the most significant transcriptional changes in the blood of patients with Crohn's Disease by comparison to blood from normal subjects.

CARD16 is a differentially expressed gene in the blood of patients with Crohn's Disease.

We identified CARD16 as among the most differentially expressed genes when comparing the global transcriptional profiles of blood from patients with CD when compared to blood from non-affected, healthy patients. CARD16 was the 22nd ranked gene out of 19,674 transcripts when ranking all of the transcripts produced in the blood based on the change in expression between CD patient blood and blood from non-affected subjects (Table 1). Differential expression of CARD16 was statistically significant (Table 1; $p=2.61E-06$).

CARD16 is expressed at significantly lower levels in the blood of patients with Crohn's Disease when compared to the blood of non-affected, healthy subjects.

Next, we obtained exact mRNA expression values for CARD16 from CD patient blood and from the blood of non-affected subjects. We also performed a statistical test to determine if the difference in expression of CARD16 between patients with Crohn's Disease and non-affected control subjects was statistically significant. CARD16 was expressed at significantly higher levels in the blood of patients with Crohn's Disease (Figure 1; $p < 0.0001$).

CARD16 is a differentially expressed gene in peripheral blood mononuclear cells following muramyl dipeptide exposure.

We identified CARD16 as among the most differentially expressed genes when comparing the global transcriptional profiles of peripheral blood mononuclear cells (PBMC) from control donors. CARD16 was the 249th most differentially expressed gene when ranking all of the transcripts produced in PBMC based on the change in expression between PBMC and PBMC treated with MDP (Table 1). Differential expression of CARD16 in PBMC following MDP exposure was statistically significant (Table 1; $p = 1E-04$).

CARD16 is transcriptionally activated in PBMC following exposure to muramyl dipeptide (MDP).

Next, we obtained exact mRNA expression values for CARD16 from PBMC and from the PBMC treated with MDP. We also performed a statistical test to determine if the difference in expression of CARD16 between PBMC and PBMC stimulated with MDP was statistically significant. CARD16 was expressed at significantly higher levels PBMC treated with MDP than in PBMC in resting state (Figure 2; $p = 0.0006$).

Thus, the caspase recruitment domain family member 16, CARD16 is differentially expressed in the blood of patients with Crohn's Disease, at significantly higher levels than in non-affected subjects, and is transcriptionally activated in normal peripheral blood mononuclear cells following exposure of PBMC to the ligand for the gene product of most significant genetic risk factor for the development of Crohn's Disease, NOD2.

Discussion

Nearly 20 years after the identification of NOD2/CARD15 as the most significant genetic risk factor for the development of Crohn's Disease, over 140 genetic susceptibility loci that confer risk of development of Crohn's Disease have been discovered but there has been little therapeutic translation of these findings. To facilitate target discovery in Crohn's Disease at the systems level but from a different approach, we mined publicly available transcriptome data to identify the genes whose expression was most quantitatively different between the blood of patients with Crohn's Disease and the blood of non-affected patients (5). This approach differs from GWAS in two major ways. First, GWAS utilizes genetic information, DNA, that is identical to all somatic cells. Second, we targeted our discovery on the hematopoietic compartment of patients with Crohn's Disease. Many studies that have investigated the mechanism of action of GWAS-identified alleles have focused on the effect of these variant alleles on the gut tissues and on intestinal epithelial cells (20, 22, 23) despite the fact that bone marrow transplantation has successfully treated patients with advanced treatment refractory Crohn's Disease, indicating that the hematopoietic compartment is relevant to this disease (24). In one study, of five patients with Crohn's Disease that survived allogeneic hematopoietic stem cell transplantation for leukemia (out of six patients total), four remained free of disease 4.5 to 15.3 years after transplantation (24). There was only one patient that possessed mixed donor-host chimerism, and this patient relapsed 1.5 years after transplantation (24). This is experimental evidence that manipulation of the hematopoietic compartment alone can provide remission from treatment-refractory Crohn's Disease and emphasizes that the hematopoietic compartment should not be neglected in deciphering the function of GWAS-identified CD susceptibility loci.

CARD16, also known as Pseudo-ICE and COP, is composed almost exclusively of a CARD domain (7), and as such is known as a CARD-only protein, only one of three CARD-only proteins including CARD17 and CARD18 (25). CARD domains (26) function as recruitment platforms for caspases, enzymes that have diverse immunological functions such as activation

1 of functionally competent interleukin-1 β by caspase 1 cleavage of pro-interleukin-1 β (27). We
2 found that CARD16 was transcriptionally induced following exposure of peripheral blood
3 mononuclear cells to muramyl dipeptide, the ligand for NOD2. NOD2 is also known as CARD15
4 and variants in NOD2/CARD15 are the single strongest risk factors for the development of
5 Crohn's Disease (16). Interestingly, a short isoform of NOD2, NOD2-S, acts as an inhibitor of
6 NOD2 signaling through RIP2 kinase (28). Similarly, CARD16 was initially described as an
7 inhibitor of interleukin-1 β signaling by acting as a dominant negative caspase recruitment
8 platform (7, 8). However, CARD16 has more recently been reported as an activator of
9 caspase-1 signaling as well as an activator of NF-kB signaling (9).

12 Though it remains unclear exactly what the function of CARD16 is, what is clear is that
13 CARD16 in certain contexts has the ability to function as a positive regulator of IL-1 β signaling,
14 the canonical cytokine produced following activation of the inflammasome, while also acting as
15 an activator of NF-kB signaling (7, 8). We found here that CD patient blood expresses
16 significantly higher levels of CARD16 than the blood of non-affected patients. It has been
17 reported that both the gut mucosa and peripheral blood mononuclear cells express significantly
18 more IL-1 β than in homeostasis (10-11), while another study found that NOD2 Leu1007fsinsC
19 homozygotes, while expressing significantly increased expression of IL-1 β mRNA, expressed
20 significantly lower levels of the mature IL-1 β cytokine (12). Increased expression of CARD16, in
21 conjunction with CARD16 transcriptional induction by muramyl dipeptide and moreover in the
22 presence of NOD2 variant alleles that perturb the MDP-induced transcriptional network may
23 contribute to the cytokine imbalance found in patients with Crohn's Disease (21). Increased
24 expression of CARD16 should be evaluated in larger and separate CD patient cohorts and the
25 effect of CARD16 on inflammasome signaling leading to IL-1 β processing and secretion as well
26 as NF-kB activation downstream of MDP stimulation of NOD2 should be evaluated *in vitro* and

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1 in CARD16 deficient mice. Understanding of the genetic contributions to Crohn's Disease
2 pathogenesis will remain incomplete without a broader understanding of the role the
3 hematopoietic compartment plays in induction, maintenance and resolution of the disease
4 process.

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Rank	ID	p-value	t	B	Gene	Gene name
GSE94648						
22	114769_at	2.61E-06	5.0983805	4.521191	CARD16	caspase recruitment domain family member 16
GSE68765						
249	ILMN_1726591	1E-04	5.684572	1.64449	CARD16	caspase recruitment domain family member 16

Table 1: CARD16 is among the most differentially expressed genes, in the blood of patients with Crohn's Disease, and in peripheral blood mononuclear cells treated with muramyl dipeptide, the ligand for the gene product of the sentinel CD susceptibility locus NOD2.

The rank of differential expression, probe ID, p-value with respect to differential expression, t, a moderated t-statistic, B, the log-odds of differential expression between the two groups, the gene symbol and gene name are represented in this chart.

Figure 1: CARD16 is expressed at significantly higher levels in the blood of patients with Crohn's Disease when compared to non-affected controls.

The expression of CARD16 in the blood of non-affected subjects and in the blood of patients with Crohn's Disease is represented with mean value marked and p-value listed.

Figure 2: CARD16 is transcriptionally activated in peripheral blood mononuclear cells following stimulation with muramyl dipeptide.

The expression of CARD16 in peripheral blood mononuclear cells from healthy donors and in the PBMC stimulated with muramyl dipeptide (MDP) is represented with mean values marked and p-value listed.