ZBTB6 is hyper-methylated and differentially expressed in the blood of patients with Crohn's disease.

Shahan Mamoor¹

¹Thomas Jefferson School of Law

San Diego, CA 92101

mamoorsk@tjssl.edu

Crohn's disease, an inflammatory bowel disease of the gastrointestinal tract (1), causes significant morbidity and nearly 3.5 billion dollars in lost economic productivity in the United States (2) due to complications of the disease. We mined transcriptome and methylome datasets (3, 4) to understand, in an unbiased manner, the most significant changes in gene expression and DNA methylation in the hematopoietic system of patients with Crohn's disease (CD). We identified the zinc finger and BTB (broad complex, tramtrack, bric-à-brac) domain-containing gene ZBTB6 (5, 6) as one of the most differentially expressed genes in the whole blood of patients with Crohn's disease. Analysis of a separate data revealed that the ZBTB6 locus was one of the most differentially methylated sites globally in the blood of patients with Crohn's disease when compared to the blood of healthy patients. ZBTB6 is differentially methylated and differentially expressed in the blood of patients with Crohn's disease, and more significantly so than the vast majority of the human genome. These data point to inhibition of ZBTB6 gene expression by hyper-methylation of the ZBTB6 locus and suggest that titration of some function or transcriptional target of ZBTB6 may be an important event in the pathogenesis of Crohn's disease.

Keywords: inflammatory bowel disease, IBD, Crohn's disease, ZBTB6, targeted therapeutics in Crohn's disease.

1 The inflammatory bowel diseases include Crohn's disease (CD) and ulcerative colitis (1).
2 Major clinical differences between these diseases include depth of inflammation and ulceration,
3 and location of the gastrointestinal (GI) tract affected. The disease process in Crohn's disease
4 is transmural (7), invading the mucosa that lines the bowel lumen deep into the muscular layer,
5 while in ulcerative colitis inflammation is mucosal and submucosal (8). The affected portion of
6 the GI tract in ulcerative colitis is strictly limited to the large intestine while in Crohn's disease
7 any portion of the GI tract from the mouth to the anus can be affected (7). Fistulizing disease,
8 an aggressive behavior in which the disease penetrates the bowel tissues and generates
9 aberrant connections to the skin, other loops of bowel, the bladder or the vagina is restricted to
10 Crohn's disease (8). Surgery is only considered curative for patients with ulcerative colitis as
11 the disease will generally recur in patients with Crohn's disease (9). Treatments include use of
12 aminosalicylates derived from sulfasalazine, "immunomodulators" including mercaptopurine and
13 azathioprine, and a newer generation of biologic therapeutics such as infliximab and
14 natalizumab that target the inflammatory cytokine tumor necrosis factor alpha (TNF- α) and the
15 integrin-mediated cell adhesion system that facilitates leukocyte migration to the bowels,
16 respectively (1, 10). There is a significant portion of patients with Crohn's disease that fail to
17 respond or lose response to the most advanced treatment options and in the most severe cases
18 stem cell transplantation has been offered an option of last resort (11). There is a real need for
19 a basic understanding of the fundamental biology of Crohn's disease and for identification of
20 novel therapeutic targets in Crohn's disease.

23 Systems-level analyses make no assumptions and instead query all of the events or
24 elements in question to understand changes in DNA, RNA or epigenetic processes. Comparing
25 systems-level data between a disease state and in health can reveal the most significant
26 biological differences that accompany that disease state. Here, we mined two datasets (3, 4) to
27 understand the most significant changes in the gene expression and DNA methylation of
28 patients with Crohn's disease. We found that the BTB (broad complex, tramtrack, bric-à-brac)

domain-containing gene ZBTB6 (5, 6) was among the genes whose expression was most significantly different when comparing the blood of patients with CD to that of healthy patients (3). Analysis of a separate dataset (4) revealed that the ZBTB6 locus was among the genomic sites that was most differentially methylated when comparing the blood of patients with CD to that of healthy patients. These data indicate that quantitatively, ZBTB6 is one of the genes that is transcribed and epigenetically modified most differently in the blood of patients with Crohn's disease and suggest that ZBTB6-related transcriptional events are of importance to understand in Crohn's disease.

Methods

We used datasets GSE94648 and GSE32149 for this analysis in conjunction with GEO2R. GSE94648, a global transcriptional profiling dataset (3), was generated using Affymetrix Human Genome U133 Plus 2.0 array technology. GSE32149, a whole genome methylation profiling dataset (4), was generated using Illumina HumanMethylation450 BeadChip technology.

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 and DNA methylation levels of ZBTB6 in 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

To identify the most significant changes in the gene expression and DNA methylation in the hematopoietic system of patients with Crohn's disease, we mined published and public datasets (3, 4) to compare mRNA expression and DNA methylation, globally, in the blood of

1 patients with Crohn's disease with that of non-affected patient blood. We identified changes in
2 gene expression and DNA methylation of ZBTB6 as among the most quantitatively significant
3 changes in the blood of patients with Crohn's disease.

4
5 ZBTB6 is one of the most differentially expressed genes in the blood of patients with Crohn's
6 disease.

7 Differential expression of ZBTB6, the change in mRNA expression between CD patient
8 blood and healthy, non-affected blood, was ranked 136 out of 19674 transcripts (Table 1).
9 Global differential expression of ZBTB6 was statistically significant (Table 1; $p=3.33E-05$).

10 ZBTB6 is expressed at significantly lower levels in the blood of patients with Crohn's disease
11 compared to that of healthy patients.

12 To compare the levels of ZBTB6 expression in the blood, between patients with Crohn's
13 disease and healthy, non-affected patients, we obtained the exact mRNA expression levels of
14 ZBTB6 from both groups. ZBTB6 was expressed at significantly lower levels in the blood of
15 patients with Crohn's disease when compared to the blood of healthy patients (Figure 1). This
16 difference was statistically significant (Figure 1; $p<0.0001$).

17
18 ZBTB6 is one of the most differentially methylated sites in the blood of patients with Crohn's
19 Disease.

20 Next, we mined a public dataset that generated global DNA methylation profiles using
21 blood from non-affected patients and patients with Crohn's disease. A site at the ZBTB6 locus
22 was one of the most differentially methylated sites in the blood of patients with Crohn's disease.
23 The change in DNA methylation at the ZBTB6 locus was ranked 200 out of 65534 loci probed
24 (4). This was statistically significant (Table 1; $p=0.00026176$).

25
26 The ZBTB6 locus undergoes a significant gain in methyl marks in the blood of patients with
27 Crohn's Disease as compared to the same site in the blood of healthy patients.

28 To quantitatively compare DNA methylation at the differentially expressed ZBTB6 locus
between the blood of patients with Crohn's disease and healthy, non-affected patients, we

1 obtained the exact levels of ZBTB6 methylation from both groups. DNA methylation at the
2 identified ZBTB6 locus was significantly higher in the blood of patients with Crohn's disease
3 when compared to the blood of healthy patients (Figure 2). Hyper-methylation of the ZBTB6
4 locus was statistically significant (Figure 2; $p=0.0003$).

5 Thus, we identified ZBTB6 as one of the most differentially expressed genes in the blood
6 of patients with Crohn's disease, genome-wide, and found that a site at ZBTB6 locus possessed
7 significantly higher quantities of methyl marks in Crohn's disease patient blood compared to that
8 of non-affected patients.

10 Discussion

11 We mined transcriptome and epigenome datasets (3, 4) to understand the most
12 significant biological differences at the systems-level in the blood of patients with Crohn's
13 disease. We found that ZBTB6 was differentially expressed and differentially methylated in the
14 blood of patients with Crohn's disease. Differences in gene expression and methylation of
15 ZBTB6 were quantitatively among the most significant changes in the gene expression and DNA
16 methylation of the whole blood of patients with Crohn's disease, globally. ZBTB6 was
17 expressed at significantly lower levels and methylated at significantly higher levels in the blood
18 of patients with Crohn's disease when compared to the blood of healthy, non-affected patients.

19 ZBTB6, also known as ZID (6) contains four zinc fingers (5) and a 120 amino acid BTB
20 domain at its amino terminus (6). The BTB domain, also known as a POZ (poxvirus zinc finger
21 domain) (6), functions as a silencer of transcription, preventing binding of its zinc finger to
22 specific DNA sequences (5) by serving as a protein-protein interaction domain (6). Studies
23 describing the functions of ZBTB6 are otherwise limited. ZBTB6 is expressed throughout the
24 hematopoietic system (5).

25 We identified changes in gene expression and DNA methylation at ZBTB6 as among the
26 quantitatively significant changes, globally, in the blood of patients with Crohn's disease. Future
27 studies should be aimed at validating these findings in larger cohorts, assessing ZBTB6
28

expression in specific blood cell types in patients with Crohn's disease, and determining the functional consequence of decreased ZBTB6 expression in the blood in Crohn's disease.

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Rank	ID	p-value	t	B	Gene
GSE94648					
136	10773_at	3.33E-05	-4.4240491	2.202178	ZBTB6
GSE32149					
200	cg14582970	0.00026176	4.13	-0.78309	ZBTB6

Table 1: ZBTB6 is differentially expressed and differentially methylated in the blood of patients with Crohn's disease when compared to blood from healthy, non-affected patients.

The rank of differential expression out of 19674 (GSE94648) or 65534 (GSE32149) transcripts, probe ID, p-value (of global differential expression), t, a moderated t-statistic, and B, the log-odds of differential expression between the two groups compared are represented in this chart (obtained from GEO2R).

Figure 1: ZBTB6 is expressed at significantly lower levels in the blood of patients with Crohn's disease.

The mRNA expression values of ZBTB6 in the blood of patients non-affected patients (left; n=22) and in the blood of patients with Crohn's disease (right; n=50) is displayed with the mean mRNA expression value in each group shown with mean mRNA expression values marked and p-value listed.

Figure 2: In the blood of patients with Crohn's disease, a site at the ZBTB6 locus is hyper-methylated when compared to the blood of healthy patients.

The level of DNA methylation at the differentially methylated site at the ZBTB6 locus in the blood of healthy patients (left; n=15) and the blood of patients with Crohn's disease (right; n=16) is displayed with mean mRNA expression values marked and p-value listed.