ORIGINAL ARTICLE

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Relationship between villous atrophy and Wnt pathway gene expressions in pediatric celiac patients

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SUMMARY

OBJECTIVE: Celiac disease is an autoimmune disease characterized by an abnormal immune response occurring in the small intestine linked to consumption of food containing gluten in individuals with a genetic predisposition. Dysregulation of Wnt signal transduction plays a role in the pathogenesis of many diseases including autoimmune diseases like celiac disease. In this study, the correlation of Wnt pathway gene expressions with each other and the correlation with clinical data were researched in pediatric celiac disease cases grouped according to the Marsh classification.

METHODS: Gene expression levels of *FZD8*, *DVL2*, *LRP5*, *RHOA*, *CCND2*, *CXADR*, and *NFATC1*, which are involved in the Wnt pathway, were determined using quantitative real-time polymerase chain reaction in 40 celiac disease and 30 healthy individuals.

RESULTS: All cases with the short height symptom were observed to be in Marsh 3b\3c groups (p=0.03). The gene expressions of *DVL2*, *CCND2*, and *NFATC1* were high in the Marsh 3b group, and these genes showed positive correlation with each other (p=0.002). *LRP5* and *CXADR* gene expressions were lower in the Marsh 3b group compared to other Marsh groups, and these genes showed a positive correlation with each other (p=0.003). *CCND2* gene expression was associated with Marsh 3b group, diarrhea, and vomiting symptoms. *DVL2* gene expression was correlated with Marsh 2 group and constipation symptom (p<0.05).

CONCLUSION: Wnt signaling in the early stages of the disease of Marsh 1–2 involves high expression of *LRP5* and *CXADR* genes, while expression of these two genes reduces, and *DVL2*, *CCND2*, and *NFATC1* gene expressions clearly increase with a transduction variation observed from Marsh 3a stage when villous atrophy begins to form. It appears that the Wnt pathway may contribute to disease progression through expression changes.

KEYWORDS: Celiac disease. Wnt signaling pathway. Gene expression. Autoimmune diseases.

INTRODUCTION

Celiac disease (CD) is an autoimmune disease occurring with infection and chronic atrophy of the small intestine linked to intake of foods containing gluten and some prolamins by individuals with genetic predisposition. Generally, symptoms in children are growth and development retardation, chronic diarrhea, loss of appetite, abdominal bloating, malabsorption, and gastrointestinal irregularities. Disease symptoms may begin at 6 months of age. CD diagnosis is made by observing human leukocyte antigen *(HLA)-DQ2 HLA-DQ8* haplotypes of HLAs, antibodies specific to CD, and the presence of enteric atrophy[1,2]. Playing an important role in many autoimmune diseases including CD, the Wnt signaling pathway participates and regulates many biological processes like cell proliferation, differentiation, regulation of transcription of a variety of target genes, and cell adhesion in both embryonic and adult periods[3,4]. Wnt signal transduction begins with frizzled (FZD) transmembrane receptors and low-density lipoprotein receptor-related protein (LRP) coreceptors triggering canonic and noncanonic signal transductions[5,6]. With FZD mediation, noncanonic Wnt signals activate disheveled segment polarity protein (DVL)-dependent Ras homolog family member A (RHOA)-ROCK, G-protein-dependent calcineurin-nuclear factor of activated T cells (NFAT), and RTK-dependent P13K-AKT[7]. The *Cyclin D2* (*CCND2)* gene on the Wnt signaling pathway interacts with cyclin-dependent kinases in the cell cycle, playing an important role especially in G1/S transition[8]. CXADR Ig-like cell adhesion molecule (CXADR) is effective in cell adhesion via β-catenin inactivation[9]. Abnormalities in Wnt signal transduction play roles in pathogenesis of many diseases. Therefore, in this case-control study, it was aimed to determine the correlations of *FZD8*, *DVL2*, *LRP5*, *RHOA*, *CCND2*, *CXADR*, and *NFATC1* gene expressions with disease symptoms and Marsh classification, as well as with *HLA-DQ2/8* haplotypes and other clinical data.

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METHODS

Study subjects and ethics

The study included a total of 70 children attending the Haseki Education Research Hospital Pediatric Gastroenterology Clinic. Of these children, 40 received diagnosis of CD and 30 had normal gastrointestinal endoscopy results. The diagnosis of CD was made using the criteria of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)[1] according to the results of the histopathological examination of the endoscopic tissues of the cases and the Marsh classification. The age, gender, clinical findings, hematological, and biochemical parameters at diagnosis along with symptoms and signs were recorded. Patients with other chronic gastrointestinal system diseases such as inflammatory bowel disease and autoimmune diseases other than chronic gastrointestinal system disease were excluded from the study. Ethics permission was received from Aydın Adnan Menderes University Non-Interventional Clinical Research Ethics Committee. Volunteers were informed about the study and were included after providing consent forms before the study. This study was completed in accordance with the standards determined by the ethics committee and the Declaration of Helsinki.

DNA isolation

Peripheral blood samples were used to isolate genomic DNA according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA concentration and purity were determined by the absorbance value at 260 nm (A260) and the ratio of A260/A280, respectively, using a spectrophotometer (NanoDrop, Thermo Scientific, USA).

Detecting *HLA-DQ* genotypes

The primary susceptibility genotype for CD is *HLA-DQ2* consisting of *HLA-DQA1*05* and *DQB1*02*. The remainder of the cases were associated with *HLA-DQ8* consisting of *HLA-DQA1*03* and *DQB1*03:02*. Case DNA samples were genotyped according to the sequence-specific primers-polymerase chain reaction (SSP-PCR) method for *HLA-DQA1* and *DQB1* according to the manufacturer's instructions (Olerup SSP® DQ low-resolution AB, Sweden). The commercial diagnostic kit includes 22 primer mixes and one negative control for the *DQA1* and *DQB1* alleles. The typing was interpreted with the lot-specific interpretation and specificity tables from kit.

RNA isolation and cDNA synthesis

Sections taken from FFPE blocks belonging to volunteers had RNA isolation completed using a RNeasy FFPE Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) synthesis was completed using an RT2 First Strand Kit (SA Bioscience, Frederick, MD, USA) in line with the manufacturer's instructions.

Q-PCR primer assay

Expression levels for seven genes acting on the Wnt signaling pathway of *FZD8*, *DVL2*, *LRP5*, *RHOA*, *CCND2*, *CXADR*, *NFATC1*, and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*) as a housekeeping gene were determined using a Rotor-Gene 3000 (Corbett Research, Qiagen, Germany) device in accordance with the manufacturer's directions.

Data analysis

Normalization of expression data and data analysis were completed using an online data analysis robot offered by the manufacturer (https://geneglobe.qiagen.com/us/analyze). ΔΔCt method was used for the quantification of gene expression.

Statistical analysis

Demographic characteristics and clinical data were analyzed using IBM SPSS Statistics Version 25 (IBM Company, New York, USA) using the χ^2 test or Fisher-exact χ^2 and correlation tests. ΔCt values for each gene were calculated based on a Student's t-test. $p<0.05$ was considered statistically significant.

RESULTS

There were no statistical differences in terms of age distribution between the CD group (10.71±5.63) and control group (11.03±5.49) ($p>0.05$). In the CD group, the proportion of boys was 37.5% and that of girls was 62.5%, while in the control group the proportion of boys was 40% and that of girls was 60% (Table 1).

In CD cases, 75% had *HLA-DQ2* and 15% had *HLA-DQ8*, with 10% having both *HLA-DQ2* and *HLA-DQ8*. Among cases, 90% had abdominal pain, 65% had an inability to gain weight, 55% had anemia, 30% had short height, 25% had constipation, 20% had diarrhea, and 10% had vomiting symptoms (Table 1). All cases with the short height symptom were in the Marsh 3b and 3c groups ($p=0.031$). Although not statistically significant, it was observed that the inability to gain weight and anemia symptoms intensified in the Marsh 3b and 3c groups (Table 2).

LRP5 and *CXADR* gene expressions were higher in Marsh 1-2 and 3a groups compared to controls, and displayed a clear reduction in the Marsh 3b group. Expression of these genes

showed a significant positive correlation in terms of Marsh classification (p=0.002). Expressions of *DVL2*, *CCND2*, and *NFATC1* genes were close to or below controls in the Marsh 1-2 and 3a groups, with a pronounced elevation in the Marsh 3b group. These gene expressions showed significant positive correlation in terms of Marsh classification (*DVL2-CCND2* p=0.004), (*DVL2-NFATC1* p<0.001), and (*CCND2-NFATC1* p<0.001). *CCND2* and *DVL2* genes displayed statistically significant expression in terms of Marsh classification (p=0.04 and p=0.04, respectively). *CXADR* gene expression showed statistically significant negative correlation with the expression of *DVL2*, *CCND2*, and *NFATC1* genes (p=0.026, p=0.006, and p=0.038, respectively). *LRP5* gene expression displayed a statistically significant negative correlation with *DVL2* and *CCND2* gene expressions (p=0.011 and p=0.008, respectively); however, there was no statistically significant correlation with *NFATC1* gene expression (p=0.068) (Figure 1).

Table 1. Demographic features.

Characteristics	Group	Number of patients	Percentage
Gender	Female	25	62.5
	Male	15	37.5%
Age	2–12	19	47.5
	13–20	21	52.5
HLA	DQ2/-	30	75
	DQ8/-	6	15
	DQ2/DQ8	4	10
Marsh classes	Marsh 1–2	8	20
	Marsh 3a	8	20
	Marsh 3b	10	25
	Marsh 3c	14	35
Symptom	Abdominal pain	34	85
	Inability to gain weight	26	65
	Anemia	22	55
	Short height	12	30
	Constipation	10	25
	Diarrhea	8	20
	Vomiting	4	10

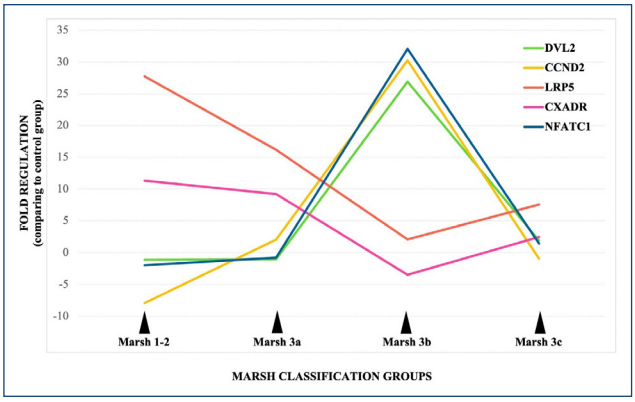

Figure 1. Fold regulations of gene expressions comparing to the control group. The changes in gene expressions in Marsh groups compared to the control group and their correlations with each other are shown. It is observed that some genes act together in the formation of mucosal damage. *DVL2*, *CCND2*, and *NFATC1* gene expressions act together in the formation of mucosal damage and are expressed at the highest level in the Marsh 3b group. *LRP5* and *CXADR* genes also act together and are expressed high level in Marsh 1 and 2 groups, which are the initial stages of mucosal damage, and decrease to their lowest levels in Marsh 3b stage.

Table 2. Comparison of Marsh classification by symptoms and HLA-DQ haplotype.

Total n=40	Marsh 1–2 (n=8)	Marsh 3a (n=8)	Marsh 3b (n=10)	Marsh 3c (n=14)	p-value
Abdominal pain (n=34)	6	7	9	12	0.668
İnability to gain weight (n=26)	4	4	8	10	0.176
Anemia (n=22)	2	4	8	8	0.106
Short height (n=12)	0	0	5	7	0.031*
Constipation (n=10)	2	1	3	4	0.711
Diarrhea (n=8)	1	2	3	2	1.000
Vomiting (n=4)	0	1	2	1	0.638
HLA-DQ2 (n=30)	6	6	7	11	0.998
HLA-DQ8 (n=6)	1	1	2	2	0.998
HLA-DQ2/DQ8 (n=4)	1	1	1	1	0.998

*Significant p<0.05.

In terms of disease symptoms, *CCND2* gene expression was associated with vomiting and diarrhea symptoms at statistically significant levels (p=0.001 and p=0.028, respectively). *DVL2* gene expression was found to be associated with the constipation symptom at a statistically significant level (p=0.003).

DISCUSSION

In our study, there were higher rates for the female sex, similar to the literature[10-12]. Similar to the literature, the *HLA-DQ2* rate was identified to be dominantly higher[10,12,13]. Symptoms like chronic diarrhea, abdominal pain, and growth retardation in addition to vomiting, constipation, and anemia were observed in our cases, similar to the literature[2,14]. The finding of short height was identified in all cases in the Marsh 3b and 3c groups, and similarly, cases with lack of weight gain and anemia were observed more intensely in these two groups compared to the other groups. We think these symptoms may be caused by disruption of small intestine tissue function in these two Marsh groups, where the highest levels of villous atrophy and crypt hyperplasia are observed.

When our study is examined in terms of the detected gene expressions, expression levels among cases in the Marsh 3c class were identified at levels close to those of the control group. We think this may be due to Wnt signaling returning to normal levels after completing the task of total atrophy of villous and intense hyperplasia in crypts. In our study, three genes had increased expression (*DVL2*, *CCND2*, and *NFATC1*), and two genes had reduced expression (*LRP5* and *CXADR*) from Marsh 3a class on, when villous atrophy and crypt hyperplasia began to occur. These data create the idea that the Wnt signaling pathway responds to increased lymphocyte infiltration at the onset of disease by displaying an expression pattern where *LRP5* and *CXADR* genes are effective. With the continuation of pathological status, cells adapt to the situation with *DVL2*, *CCND2*, and *NFATC1* gene expressions on the Wnt signal gaining efficacy and the Wnt signal transduction pattern causing villous atrophy and crypt hyperplasia being adopted. When we examine the literature, we think small intestinal cells may show expression on the LRP5/β-catenin/CCND1 axis for renewal against stress caused by lymphocyte infiltration induced by the immune response[15], while *CXADR* gene expression activates CDC42, supporting cell adhesion[9]. Increased cell renewal and adhesion may have begun to disrupt the villous architecture by slowing the migration of cells toward the villous tip. In the Marsh 3a class where villus atrophy formation starts, it is seen that the expression of these two genes started to decrease, and the expression of *DVL2*, *CCND2*, and *NFATC1* genes started to increase. There is a strong correlation between these genes because they are similarly negatively correlated in Marsh 3b class. This suggests that they may be involved in the regulation of each other and that there may be a Wnt signal transduction pattern specific to villus atrophy. *NFATC1* gene expression has an important role in the non-canonical Wnt signal pathway of Wnt/Ca^{+2} signal transduction and especially comes to the agenda during embryogenesis[16] and additionally undertakes important duties in the immune response induced by T cells and the activation of B cells[17]. We think the high *NFATC1* gene expression we identified in Marsh 3b cases is due to T cells and causes induction of the immune response at high levels. *CCND2* gene expression is known to suppress the G1/S stage of the cell cycle, stopping proliferation and allowing the opportunity for differentiation[18]. *CCND2* gene expression at high levels in our Marsh 3b cases brings to mind the formation of villous atrophy as a result of suppression of proliferation. On the contrary, *DVL2* expression increases cell proliferation in crypts and is known to regulate tight junctions directly[19]. Increased tight junctions and dysregulated proliferation may be effective in the formation of crypt hyperplasia.

Limitations

Studies with higher case numbers including gene expression will support our findings and contribute to understanding molecular mechanisms of disease and creation of treatment targets.

CONCLUSION

We think that all these gene expressions act in accordance with a certain order to create pathogenesis in small intestinal tissue. Our findings suggest that villus atrophy and crypt hyperplasia occur as a result of increased activity of the non-canonical Wnt pathway, which plays a role in cytoskeleton and cell adhesion. Suppression of the noncanonical Wnt pathway can be considered a treatment strategy to prevent small intestine tissue damage.

AUTHORS' CONTRIBUTIONS

MC: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **GD:** Resources, Writing – review & editing. **SOB:** Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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