Overexpressions of *RHOA, CSNK1A1, DVL2, FZD8*, and *LRP5* Genes Enhance Gastric Cancer Development in the Presence of *Helicobacter Pylori*

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Abstract

Introduction: Intestinal metaplasia (IM), and Helicobacter pylori (HP) infection can be shown as risk factors in the development of gastric cancer (GC). WNT signaling pathway plays a critical role in carcinogenesis. However, the literature studies are limited on the significance of this pathway for the transition from IM to GC.

Methods: We aimed to investigate the importance of the genes of WNT signaling pathways diagnostic and prognostic markers in the presence and absence of HP in conversion from IM to GC. 104 patients, (GC group n=35, IM group n=45, control group n=25) were included in this case-control study. Expression of genes in WNT signalling were searched in study groups with qRT-PCR array and qRT-PCR method. Data were analysed using PCR array data analysis software.

Results: Statistically significant overexpression of RHOA, CSNK1A1, DVL2, FZD8 and LRP5 genes was detected in the GC and IM groups compared to the control group ($p < 0.05$). Statistically significant overexpression of RHOA, CSNK1A1, DVL2, FZD8 and LRP5 genes was observed in patients with metastatic GC compared to patients with GC without metastasis ($p < 0.05$). It was found that the RHOA, CSNK1A1, DVL2, FZD8 and LRP5 genes were statistically significantly over-expressed in diffuse GC patients compared to non-diffuse GC patients ($p < 0.05$). Statistically significant overexpression of RHOA, CSNK1A1, DVL2, FZD8 and LRP5 genes was detected in HP positive IM patients compared to HP negative IM patients ($p < 0.05$).

Conclusion: Overexpression of RHOA, CSNK1A1, DVL2, FZD8 and LRP5 genes in IM may suggest that these genes are important markers in the development of IM and inflammation with HP. In addition, these genes are linked to tumor burden in the GC group. Consequently, we can conclude that these genes are poor prognosis biomarkers for GC and have the potential to be used as markers for future treatment monitoring.

Introduction

Intestinal metaplasia (IM) and *Helicobacter Pylori* (*HP*) are major risk factors for gastric cancer (GC). IM usually develops as a result of atrophic gastritis (AG) infected by *HP* and may cause gastric dysplasia and/or cancer [1, 2]. Throughout this development process, *HP* triggers the response by CD4 Th1 cells due to inflammation. Thus, macrophages and neutrophils soon reach this site and are stimulated by the release of reactive oxygen and reactive nitrogen products. These reactive substances cause DNA damage with superoxide and hydroxyl ions created by the *HP*, as well as oxidative damage [3, 4]. *HP* is considered to trigger cause intestinal type GC with two major direct and indirect mechanisms. The indirect mechanism includes inflammation due to increased cell cycle and accumulation mitotic defects within a few decades. In this process called Correa hypothesis, AG occurs first, followed by IM, colonic metaplasia, dysplasia, and finally GC. The other mechanism is directly associated with DNA damage [3].

WNT signaling pathway which preserved in the evolutionary process have a role in important processes including development, differentiation, cell proliferation, mothology, mortality etc.[5, 6]. Therefore, functional genes on the WNT signaling pathway are important for carcinogenesis and have been intensely studied with respect to the pathophysiology of carcinogenesis. The aberrant activation of the WNT signaling pathway is involved in the development and progression of a significant proportion of GC [7–9]. In a review by Chiurillo (2015), it was suggested that WNT signallingdue to the *HP* infection and suppression of WNT inhibitors play crucial role in GC. Also, *CTNNB1* and *APC* were listed as pivotal genes in the development of GC [8]. GC aggressiveness is dependent on *WNT5A* expression level [10]. *WNT5A* may help building favorable conditions for GC progression (Kazawa 2013) [11]. The *RHOA* gene in the WNT signaling pathway was described as both a potential therapeutic target and a biomarker for GC (Chang et al. 2016) [12]. However, the literature studies are limited on the significance of this pathway for GC development in the presence of *HP*. In this context, this case-control study investigated the activity of WNT signaling pathway genes as diagnostic and prognostic markers in the presence and absence of *HP* in development process from IM to GC.

Materials And Methods

Ethics

Permission for the study was granted by the Local Council of Ethics in 2013 (#147). A signed informed consent form was obtained from each subject prior to any study related procedures.

Study Design and Patients

Between September 2014 and September 2015, 34 GC cases with upper gastrointestinal endoscopy and 45 IM cases who applied to Manisa Celal Bayar University Gastroenterology Department were included in the study. In addition, 25 patients who with normal biopsy-endoscopy underwent endoscopic biopsy due to upper gastrointestinal system/functional dyspepsia complaints, with normal biopsy-endoscopy were selected as the control group. Age, gender, and family history of all subjects were questioned and recorded.

Inclusion Criteria

(1) Adult patients older than 18 years who have not received chemotherapy or radiotherapy treatment with new GC diagnosis, (2) Adult patients over 18 years of age who were diagnosed as IM and underwent control endoscopy for dysplasia, (3) Adult healthy volunteers over 18 years of age who had endoscopic biopsy of the cause of functional dyspepsia and whose endoscopy was normal and biopsy pathology was normal.

Exclusion Criteria

(1) Taking chemotherapy or radiotherapy at any time with the diagnosis of GC or another malignancy, (2)Having a malignancy other than GC,(3)Patients who did not accept biopsy during endoscopy or did not

receive biopsy because they were using antibiotics (4)Cases who underwent subtotal or total gastrectomies for any reasonPET or PET/CT images were taken for staging purposes in all GC patients. CT or PET/CT evaluation of opere and non-opere patients and whose postoperative pathological evaluation were performed according to TNM classification of AJCC.

Endoscopy and Tissue Sampling

Endoscopic examinations of all cases were carried out at Manisa Celal Bayar University, Department of Gastroenterology. Endoscopy was performed with Olympus Lucera CLV260SL device. At least 2 biopsy specimens of antrum and corpustum were taken from tumor tissue in GC patient group, IM, and control patient group using olympus biopsy forceps. Pathology evaluations of biopsy specimens were performed and the data in the results were recorded.

Mucosal biopsy specimens taken for analysis of WNT signaling gene expression from all cases were immediately stored in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) solution.

RNA Isolation

RNA from tissue was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to Kasap et al. (2012 and 2015) and Gerceker et al. (2015)[13–15].

Amount and Purity of RNA.

The amount of RNA was measured at absorbance at 260 nm (A260) and RNA purity was determined by the ratio A260 / A280 using spectrophotometer.

cDNA Synthesis

Conversion of the isolated RNA to cDNA was done with the RT First Strand Kit (SABioscience, Frederick, MD, USA) according to Orenay-Boyacıoğlu et al. (2018) [16]. Eight µl of RNA samples were incubated with 2 µl of GE (5X gDNA Elimination Buffer) at 42 ° C for 5 minutes. A PCR mixture consisting of 4 µl BC3 (5X RT Buffer 3, 1 µl P2 (Primer and External Control mixture), 2 µl RE3 (RT Enzyme Mix 3) and 3 µl H2O was prepared in a different tube and added onto the RNA. It was incubated at 42° C for 15 minutes and then at 95 ° C for 5 minutes.

Human WNT Signaling Pathway Plus RT Profiler PCR Array

Human WNT Signaling Pathway Plus RT² Profiler ™ PCR Array (PAHS-043Y) (SABiosciences, Frederick, MD, USA) was used to measure the expression levels of 84 WNT signaling genes (Table 1).

Table 1
Human WNT Signaling Pathway Plus RT Profiler PCR Array

WNT Signaling Pathways	Canonical	*APC, AXIN1, AXIN2, CSNK1A1, CTBP1, CTNNB1, CTNNBIP1 (ICAT), DKK1, DKK3, DVL1, DVL2, EP300, FRAT1, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, GSK3B, LEF1, LRP5, LRP6, NKD1, PORCN, RUVBL1, SFRP1, SFRP4, SKP2, SOX17, TCF7, TCF7L1, WIF1, WNT1, WNT10A, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT6, WNT7A, WNT7B, WNT8A.*
	Planar Cell Polarity (PCP)	*DAAM1, DVL1, DVL2, MAPK8 (JNK1), NKD1, PRICKLE1, RHOA, VANGL2, WNT9A.*
	WNT/Ca + 2	*FZD2, NFATC1, WNT1, WNT10A, WNT11, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT9A.*
	WNT Signaling Negative Regulation	*APC, AXIN1, AXIN2, BTRC (bTrCP), CCND1, CTBP1, CTNNBIP1 (ICAT), DKK1, DKK3, FBXW11, FRZB (FRP-3), KREMEN1, LRP6, NLK, NKD1, SFRP1, SFRP4, SOX17, WIF1.*
	WNT Signaling Target Genes	*AXIN2, BTRC (bTrCP), CCND1, CCND2, DAB2, FOSL1 (FRA-1), JUN, MMP7, MYC, PITX2, PPARD, WISP1.*
Developmental Processes	**Cell Fate**	*CTNNB1, DKK1, WNT1, WNT3, WNT3A.*
	Tissue Polarity	*AXIN2, FZD2, FZD3, FZD5, FZD6, VANGL2.*
	Cell Growth & Proliferation	*APC, CCND1, CCND2, CTBP1, CTNNB1, CTNNBIP1 (ICAT), DAB2, EP300, FGF4, FOSL1, FZD3, JUN, LRP5, MMP7, MYC, PPARD, WISP1, WNT3A.*
	Cell Migration	*APC, DKK1, LRP5, LRP6, RHOA, WNT1.*
	Cell Cycle	*APC, BTRC (bTrCP), CCND1, CCND2, CTNNB1, EP300, FOSL1, JUN, MYC, RHOA, RUVBL1, TCF7L1.*
	Cellular Homeostasis	*APC, FZD2, JUN, MYC.*
	Pathway Activity Signature Genes	*BOD1, CALM1, CCND1, CCND2, CHSY1, CXADR, CYP4V2, HSPA12A, LEF1, MT1A, MTFP1, MTSS1, MYC, NAV2, PRMT6, SKP2.*

2300 µl RT-PCR array mix containing 102 µl diluted cDNA, 1150 µl 2X RT2 SYBR Green ROX FAST Master mix and 1048 µl H_2O was loaded into 96-well Human WNT Signaling Pathway Plus RT² Profiler ™ PCR Array at 20 µl per well. PCR arrays were placed in the Rotor-Gene RG-3000 (Corbett Research, Qiagen, Hilden, Germany) and a program of 10 minutes at 95 ° C, 40 cycles at 95 ° C for 15 seconds and 30 seconds at 60 ° C were incubated. Array results were analyzed. The 8 highest and lowest expressed genes

(*RHOA, CXADR, CSNK1A1, CCND2, DVL2, FZD8, NFACT1, LRP5)* were selected among the study groups and validated by RT-PCR primer assay.

RT qPCR Primer Assay

Eight genes and one reference gene (*HPRT1*) selected as a result of PCR array analysis were tested by RT qPCR. 8 gene-specific Primer Assays and SYBR Green Master Mix (SABiosciences, Frederick, MD, USA) were purchased and each PCR reaction mix was prepared by adding 12.5 µl SYBR Green Master Mix, 1 µl Primer and 2.2 µl cDNA sample and the total volume was set to 25µl.

PCR mixes were loaded into the Rotor-Gene RG-3000 instrument for amplification. Amplification was carried out at 95 ° C for 5 minutes with the first denaturation step followed by 40 cycles of 94 ° C for 1 minute, 61 ° C for 40 seconds and 72 ° C for 1 minute. The cycling threshold (Ct) values obtained as a result of the RT qPCR primary assay were normalized with the *HPRT1* gene and evaluated using REST 2009 (Relative Expression Software Tool V.2.0.13).

Data analysis

PCR array and qPCR primary assay data were analyzed in the online system (http://www.sabiosciences.com/pcrarraydataanalysis.php). Fold change and fold regulation values above 2, indicative of overexpressed genes; fold change values below 0.5 and fold regulation values below − 2 were considered as indicative of underexpressed genes.

Statistics

In the results, mean values are shown as ± standard deviation, and the 2 - ΔCt values obtained for each gene in the study group were analyzed based on Student's t-test. A p value less than or equal to 0.05 was considered statistically significant.

Results

Patient Groups

When the three groups in the study were compared, there was no difference between the age groups and gender distribution (Table 2).

Table 2
Patient Groups and General Parameters

		GC (n = 34)	IM (n = 45)	Control (n = 25)	*P*
Age		58.85 ± 7.5	54.71 ± 12.5	54.84 ± 7.4	*0.152*
Gender	**Male**	68% (n = 23)	64% (n = 29)	60% (n = 14)	*0.583*
	Female	32% (n = 11)	36 % (n = 16)	40% (n = 11)	

In patients with GC, the tumor was largely localized in the proximal stomach. Localization-based distribution was as follows: Proximal Stomach: 47% (n = 16), Distal Stomach: 35% (n = 12), and Diffuse Stomach Involvement: 18% (n = 16). Thirty of 34 GC cases had full-body computed tomography and distant metastasis was detected in 52% (n = 16) of these cases. Fifty-six percent (n = 9) of those patients had a peritoneal metastasis, 32% (n = 5) had a liver metastasis, 6% (n = 1) had a pancreatic metastasis, and 6% (n = 1) had a lung metastasis.

Regarding survival, 29% (n = 10) of the patients with GC survived less than 3 months, and 24% (n = 8) survived for a period between 3 months and 1 year.

WNT Signaling Pathway Gene Expression Analysis

A comparison between the patients with GC and the control group (n = 34) were showed significantly overexpressed *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes (respectively, p = 0.006, 0.005, 0.003, 0.004, and 0.006) (Fig. 1).

When IM cases(n = 45) were compared with control group, *RHOA, CCND2, DVL2, FZD8* and *LRP5* genes were showed significantly overexpressed (respectively, p = 0.005, 0.003, 0.007, 0.02 and 0.009) (Fig. 2).

When GC cases were compared with IM cases, the GC group displayed no significantly overexpressed gene with regard to the latter ($p > 0.05$).

Gene Expression Analysis based on site of Gastric Involvement

In patients with GC, a comparison between those with a diffuse gastric involvement (n = 6, 18%) and the control group showed a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes ($p < 0.05$).

Comparing GC cases without a diffuse gastric involvement (n = 28, 82%) with the control group, we detected a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes ($p < 0.05$). Another comparison between patients with diffuse GC and GC cases without a diffuse involvement revealed overexpressed *RHOA, CCND2, DVL2, CXADR, NFACT1, FZD8, CSNK1A1*, and *LRP5* genes in the former group. These findings suggest that overexpression of *CXADR, DVL2, NFACT1, FZD8*, and *CCND2* were significant ($p < 0.05$).

Gene Expression Analysis Based on Metastasis

Based on metastasis (31 patients had systemic imaging), the distant metastasis group (52%, n = 16) presented a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes than the control group ($p < 0.05$).

Similar to the metastatic cases, the GC cases without metastasis (48%, n = 15) also had a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes ($p < 0.05$).

A comparison of metastatic GC cases with non-metastatic ones showed that *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes were overexpressed on a higher level, which was significant ($p < 0.05$).

Gene Expression Analysis Based on Survival of Patients with GC

Regarding survival, 53% (n = 16) of the patients with gastric cancer became exitus. Fifty percent of them (n = 8) survived for 3 months, 19% (n = 3) 6 months, and 31% (n = 5) 12 months. The patients who became exitus had a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes than the control group ($p < 0.05$). There was no significant difference between exitus and surviving patients in terms of gene overexpression ($p > 0.05$).

Sub-Group Gene Expression Analysis for Patients with IM

Gene Expression Analysis Based on HP Presence

Thirty-one percent (n = 45) of patients with IM were *HP-positive*. Compared with the control group, *HP-positive* patients had a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes ($p = 0.000$) (Fig. 3). A comparison between *HP-negative* patients (69%, n = 31) and the control group showed a significantly higher overexpression of *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes in the former, which was similar to that of *HP-positive* patients (respectively, $p = 0.006$, 0.000, 0.014, 0.004, and 0.010) (Fig. 4).

A gene analysis comparison between *HP-positive* and *HP-negative* IM cases revealed that overexpressed *RHOA, CCND2, DVL2, FZD8*, and *LRP5* genes were significantly higher in *HP-positive* patients ($p < 0.05$).

Discussion

The present study focuses on the overexpression of *RHOA, CXADR, CSNK1A1, CCND2, DVL2, FZD8*, and *NFACT1* genes belonging to the WNT signaling pathway. Expression rates were compared with GC, IM patients and control group. In addition, effects of *HP* on the expression of these genes in IM patients have been evaluated.

RHOA (Ras homolog gene family member A), a key step along the WNT signaling pathway, is a member of RHO family, which is considered to play a role in the development of various cancers and active in cellular signal transmission over GTPase (Maeda et al. 2015) [17]. It plays a role in events such as cell cycle, polarization, and migration. *RHOA* GTPase activation may be altered in the tumor tissue due to mutation. Consequently, GTPase-influenced G1-S phase of the cell cycle may gain pace or may slow down. A *RHOA* mutation is obtained in GC by cell culture assays. In these mutations, RhoA-specific siRNA, which is active in the G1-S domain of cell division, is detected (Zhang et al. 2009)[18].This represents

another significant evidence for the importance of *RHOA* in cell proliferation (Karlsson et al. 2009) [19].Pan et al (2004), in a study in which they evaluated RHO family expressions in a cell culture assay, demonstrated that high *RHOA* expression was associated with poor-differentiated GC and TNM classification increased with higher expression rates[20]. Ushiku et al (2015) studied 87 patients with GC and found signet-ring cell appearance in 18 out of 22 *RHOA*-mutant patients. The patients frequently presented a mixed and diffuse involvement. Intestinal type GC was a rare clinical picture in RHOA-mutant patients. *RHOA* mutation was not considered a significant prognostic parameter[21]. In another analysis by Kakiuchi et al (2014) on 87 patients with diffuse GC, *RHOA* mutation was detected in 25.3% (22/87) of the patients[22]. A study by Wang et al (2014) presented similar findings as well. They found no *RHOA* mutation in the patients with intestinal type GC, whereas 14.3% (14/98) of the diffuse type GC cases had *RHOA* mutation[23]. Lastly, Röcken et al (2016) retrospectively examined the pathological preparations of 415 patients to evaluate the clinical and pathological situation of *RHOA*-mutant cases. Cases present poor-differentiated and largely distal GC. Interestingly, mutant cases had a lower T grade and did not present distant metastasis[24]. In another analysis by Chang et al (2016), the role of *RHOA* pathway dysregulation in GC and the feasibility of clinically targeting *RHOA* for GC therapy were systematically assessed. As a result, they demonstrated that *RHOA* gene in the WNT signaling pathway was described as both a potential therapeutic target and a biomarker for the Asian GC[12].

The WNT signaling pathway gets activated via canonical and non-canonical ways. In the non-canonical way, the signal is transmitted to *RHOA/RHOU/RAC/CDC42, JNK, PKC, NFAT*, and NLK cascade via *Frizzled (FZD)* receptors and *PTK7/ROR2/RYK* co-receptors. *LRPs (low-density lipoprotein-related receptor)* play a role in the canonical way, whereby *FZD* receptors upregulate *MYC, CCND1, FGF20, JAG1, WISP1*, and *DKK1* genes with *LRP5/LRP6* co-receptors (Katoh et al. 2007; Katoh et al. 2009; Joiner et al. 2013) [25–27]. *LRP5* and *LRP6* are currently being researched for other diseases as well as carcinogenesis. Their overexpression or mutation has been demonstrated as a factor in many diseases (Joiner et al. 2013; Ettenberg et al. 2010) [27, 28]. The literature on adenocarcinomas is limited. A study by Liu et al. on advanced-stage GC cases found that the presence of mutation was inversely proportionated with disease-free and median survival in patients with *LRP5* mutation (Liu et al. 2014) [29].

FZD is a part of *WNT-FZD-LRP5-LRP6* component and functions as a receptor along the WNT signaling pathway. It activates the Beta-catenin complex. The triggering of this signaling pathway results in the activation of disheveled (DVL) proteins, inhibition of GSK-3 kinase, nuclear accumulation of beta-catenin, and activation of WNT target genes (Ueno et al. 2013) [30].. There is limited knowledge on the relation between GC and *FZD* gene, which presents an increased expression in different types of GC. *FZD* receptors were evaluated for GC in an analysis conducted by Kirikoshi et al (2001). They cloned *FZD1-10*of the FZD family and checked expression profiles of patients with GC. The analysis showed increased *FZD2* and *FZD8* in 4 out of 10 patients. The study emphasized that *FZD2* and *FZD8* might serve as a potential biomarker for GC. No association was detected between the expression of *FZD8* and *HP* (Kirikoshi et al. 2001) [31].

Disheveled Segment Polarity Protein 2 (DVL2) is a major step along the WNT signaling pathway. It binds to the cytoplasmic C-terminal of the *FZD* receptor and ensures the downward transmission of the signal. It plays a role in both canonical and non-canonical WNT signaling pathways. *DVL2* binds to the *Axin-APC* complex that downregulates the Beta-catenin signaling pathway and activates the pathway (Gnad et al. 2010; Katoh 2005; Schwarz-Romond et al. 2007) [32–34]. A study by Metcalfe et al (2010) found overexpressed *DVL2* in colorectal adenocarcinoma (CRC) cells. Based on the idea that the inhibition of *DVL2* by siRNA reduces the activity of Beta-catenin signaling pathway, it was thought that *DVL2* activation in patients with CRC led to a hyper-activated Beta-catenin signaling pathway. An analysis conducted on 24 patients with CRC detected that the *DVL2* level in 1/3 of CRC cells was higher than that of the normal tissue on the tumor surgical border. The study proceeded by increasing the number of subjects to 393. Patients were divided into the groups of normal mucosa, hyperplastic, adenomatous polyp, and CRC. When Beta-catenin-positive nuclei were ordered from normal tissue to the carcinomatous tissue, the expression rate was observed to increase within the cell groups. This increased expression level was also the case for *Axin* and *DVL2*, as well. The preservation of this rate across different tumor stages suggests the upregulation of *DVL2* and *Axin* genes in patients with CRC in response to *APC* loss. With a WNT simulation, the same study also demonstrated that the upregulation of *DVL2* might also occur post-transcriptionally. This led the authors to evaluate *DVL2* in *APC*-mutant rats. A low level of *DVL2* was found proportionated to reduced tumor burden; and shortened small intestine, as well as reduced number and diameter of crypts were observed in *DVL2* reduction [35]. A study by Chong et al (2014) found that DVL2 and ETS1 proteins were more specific to diffuse and mixed type GC than the intestinal type. Both genes display a methylation similar to the normal tissue and intestinal type GC, whereas the methylation rate was high in the mixed and diffuse type GC. These findings suggest that abnormal methylation starts from the non-carcinomatous normal tissue in the mixed and diffuse type GC [36].

CyclinD2 (CCND2) is a member of the cyclin family. *CCND2* regulates CDK kinase and forms a complex with *CDK4* and *CDK6*. *CCND2* is the regulator of this complex. It enables cell cycle to proceed from G1 to S phase. *CCND2* also participates in the phosphorylation of RB (Retinoblastoma-associated protein), which is a tumor suppressor protein (Truong et al. 2015) [37]. Therefore, the activation of CDK/Cyclin complex may gain importance in carcinogenesis (Myant et al. 2011) [38]. In a study conducted on rats by Cole et al (2010), authors suggested that the inhibition of WNT signaling pathway could prove to be a treatment alternative for patients with CRC. The study demonstrated that Cyclin D/CDK4-6 complex increased in the intestinal tissues of *APC*-negative rats. In *APC*-positive rats, however, CyclinD2 level was low in the intestinal tissue. The most important findings were as follows: CyclinD2 did not affect normal enterocyte development and CDK4/6 inhibition suppressed the adenomatous tissue in knockout rats[39]. A retrospective study conducted by Shi et al (2015) on patients with GC demonstrated significantly increased *CyclinD2 (CCND2)* in the tumorous tissue compared to the normal tissue ($P < 0.001$). The same analysis also detected that increased *CCND2* expression was associated with the depth of invasion, lymph node positivity and distant metastasis, and advanced TNM staging. Authors suggested that increased *CCND2* expression could be a prognostic factor[40].

Two casein proteins were purified: Casein kinase (CK) proteins CD1 and CD2. Casein kinase 1 (CK1) is a monomeric protein of a serine and threonine structure. It takes part in tasks such as DNA repair, cell division, nuclear settlement, membrane transport, etc. CK1-alpha is thought to phosphorylate beta-catenin and played a role in the degradation process when it is not bound to the WNT receptor (*FZD*). When CK1 binds to the WNT receptor, it phosphorylates Ck1-epsilon intracellular signaling protein, while DVL and CK1-gamma phosphorylate LRP5/6 co-receptors (Wang et al. 2010) [41]. As to its impact on tumorigenesis, studies on *CSNK1A1* present divergent and contradictory values.

CXADR (Coxsackie virus and adeno virus receptor) gene codes type 1 membrane receptor protein for the adeno virus C sub-group and Coxsackie B virus. Bowles et al. mapped the functional *CXADR* gene at chromosome 21 and the pseudo-*CXADR* gene at chromosomes 15, 18, and 21. It was found at 21q11 location by a FISH analysis (Bowles et al. 1999) [42]. Gamma-delta T-cells in the epithelial tissue play a critical role for defense against environmental factors such as infection, trauma and malignancy. The *CXADR* gene, which is included in the present study, has been previously isolated in pancreatic and small intestine tissues and demonstrated to be active in cell proliferation through cytokines, which suggests that this gene could be active in GIS adenocarcinomas.

Studies on *NFACT1* gene, which is a part of this study, are largely conducted osteocytes. There is no study available on its relationship with adenocarcinomas; however, it has been demonstrated that *HP* causes the inhibition of *NFACT*, increases VacA secretion, and consequently leads to the vacuolization of epithelial cells. This intensifies the process whereby *HP* becomes chronic (Gebert et al. 2003) [43]. Although there is no relevant study in the literature and our findings contradict it, there are some data available to claim that *HP* becomes chronic by inhibiting *NFACT*, which suggests that this gene may be associated with adenocarcinoma.

A review of our findings

We classified the findings of genes in two groups. The first group included the genes *RHOA, LRP5, FZD8, DVL2*, and *CCND2.* Similar to the reports in the literature, *RHOA, LRP5, FZD8, DVL2*, and *CCND2* were found significantly overexpressed compared to the patients in the control group. Again, similar to the findings reported in the literature, we detected that these genes were significantly overexpressed in diffuse type GC patients compared with both the control group and patients without a diffuse involvement. Patients with a distant metastasis had a significantly higher overexpression of *RHOA, LRP5, FZD8, DVL2*, and CCND2 than non-metastatic patients. This finding contradicted previous reports for *RHOA*. Compared with survival, exitus patients presented an overexpression of *RHOA, LRP5, FZD8, DVL2*, and *CCND2*, although the difference was not significant. As part of the present study, we checked *RHOA, LRP5, FZD8, DVL2*, and *CCND2* in patients with IM and found that they were significantly overexpressed with respect to the control group. *HP-positive* and/or *HP-negative* patients had a significantly higher overexpression of *RHOA, LRP5, FZD8, DVL2*, and *CCND2*. Patients with *HP-positive* IM showed significantly overexpressed *RHOA, LRP5, FZD8, DVL2*, and *CCND2* than the *HP-negative* ones. A comparison with GC cases revealed no significant difference from the patients with IM in terms of gene expression ($p > 0.05$). *RHOA, LRP5,*

FZD8, DVL2, and *CCND2* were overexpressed in *HP-positive* and *HP-negative* patients. The overexpression was higher in the former group than the latter.

The second group included the genes *CSNK1A1, CXADR*, and *NFACT. CSNK1A1* showed no significant overexpression in GC or IM patient groups compared with the control group. Contrary to previous reports on patients with adenocarcinoma, the present study did not detect a significantly overexpressed *CXADR* in GC or IM patient groups than the control group. Although our findings do not support it, previous reports claiming that *HP* becomes chronic by causing *NFACT* inhibition suggest that this gene could be associated with adenocarcinoma.

Conclusion

1. We are of the opinion that the significantly higher overexpression of *RHOA, LRP5, FZD8, DVL2*, and *CCND2* in the patient group than the control group and in the metastatic group than the non-metastatic group as well as the fact that all of them were overexpressed in patients with diffuse involvement and metastatic patients are associated with tumor burden. The absence of a significant difference between exitus and surviving patients in the GC group suggest that it may not be related to prognosis. Analyses with a larger sample size and to include other factors may provide us with deeper knowledge about its impact on survival.
2. Given that *RHOA, LRP5, FZD8, DVL2*, and *CCND2* were significantly overexpressed in IM cases compared with the control group, one may think that carcinogenesis starts at the IM phase.
3. The significant overexpression of *RHOA, LRP5, FZD8, DVL2*, and *CCND2* in *HP*-positive patients than the *HP-negative* ones suggest that *HP* might influence gene expression by intensifying inflammation. These findings need to be supported by further studies.
4. In accordance with the *HP*-B/catenin relationship reported in the literature, the present study also found that *HP-positive* and/or *HP-negative* IM cases presented a significant overexpression of *RHOA, LRP5, FZD8, DVL2*, and *CCND2* compared with the control group. *HP-positive* IM cases had a significantly higher overexpression of *RHOA, LRP5, FZD8, DVL2* and *CCND2* than the *HP-negative* cases.
5. It is likely that *RHOA, LRP5, FZD8, DVL2*, and *CCND2*, which play a key role in the gastric carcinogenesis, are triggered by inflammation and influenced by environmental factors such as *HP*.
6. In the light of the data we obtained and the literature, we are of the opinion that *CSNK1A1* does not have an impact on the pathogenesis of adenocarcinoma.
7. Contrary to the data in the literature, we did not detect a significant overexpression of *CXADR* in GC or IM cases compared with the control group. Therefore, further studies are required to shed light on its role in the pathogenesis of GC.
8. Although it is not supported by our data, previous studies reporting that *HP* becomes chronic by causing the inhibition of *NFACT* suggest that this gene might be associated with adenocarcinoma.

More studies with larger study groups are needed to elucidate the role of the detected genes in the pathogenesis of GC.

Limitations

The sample size in our study is limited. A total of 11 cases were excluded from the study due to RNA degradation. In this respect, this study was categorized as a case-control study. Further and comprehensive studies are needed for these genes about this topic.

Declarations

Funding: This study was supported by Manisa Celal Bayar University Scientific Research Projects (#2014-094).

Conflicts of Interest: The authors have no conflict of interest to declare.

Ethics approval: Local Council of Ethics in 2013 by Manisa Celal Bayar University (#147)

Data will be available upon request, not in repository since it includes genetical data.

All authors have given consent to publication.

UD, SB, EK, MK Constructing an idea / hypothesis for research

UD, SB, EK, MK Planing methodology

EK, HY, ARB, EG Collect samples from patients

SB, HY Samples worked

UD, EK Writing the article

EK, HY, EBE, MK Reviewing the article

All authors read and approved the manuscript

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Figures

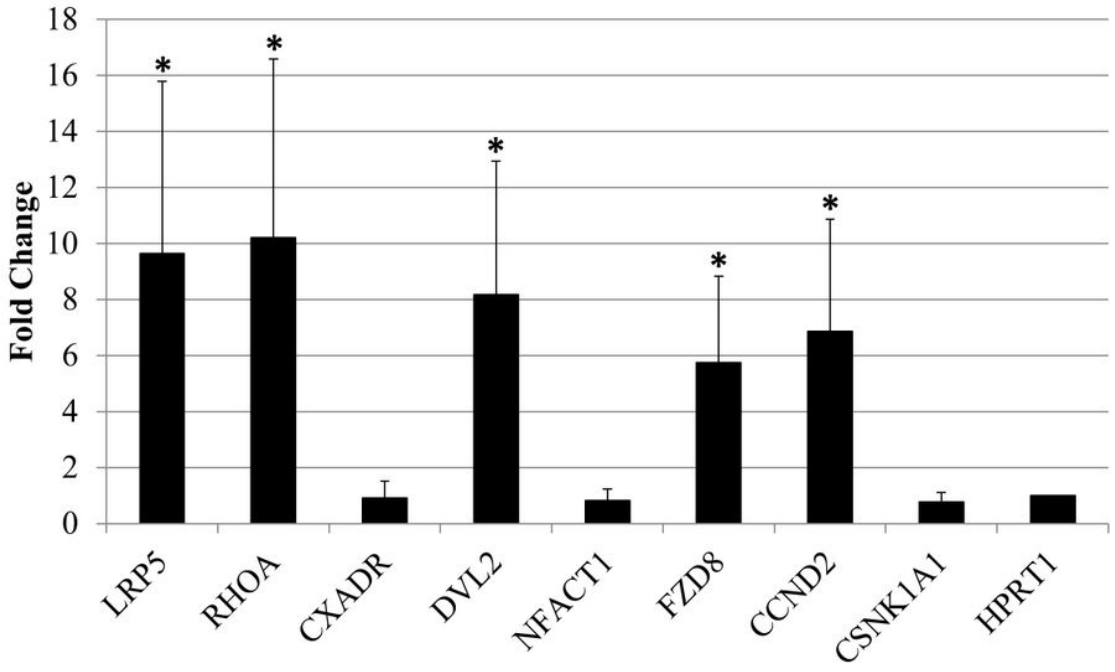

Figure 1

WNT Signaling Pathway Gene Expression in GC

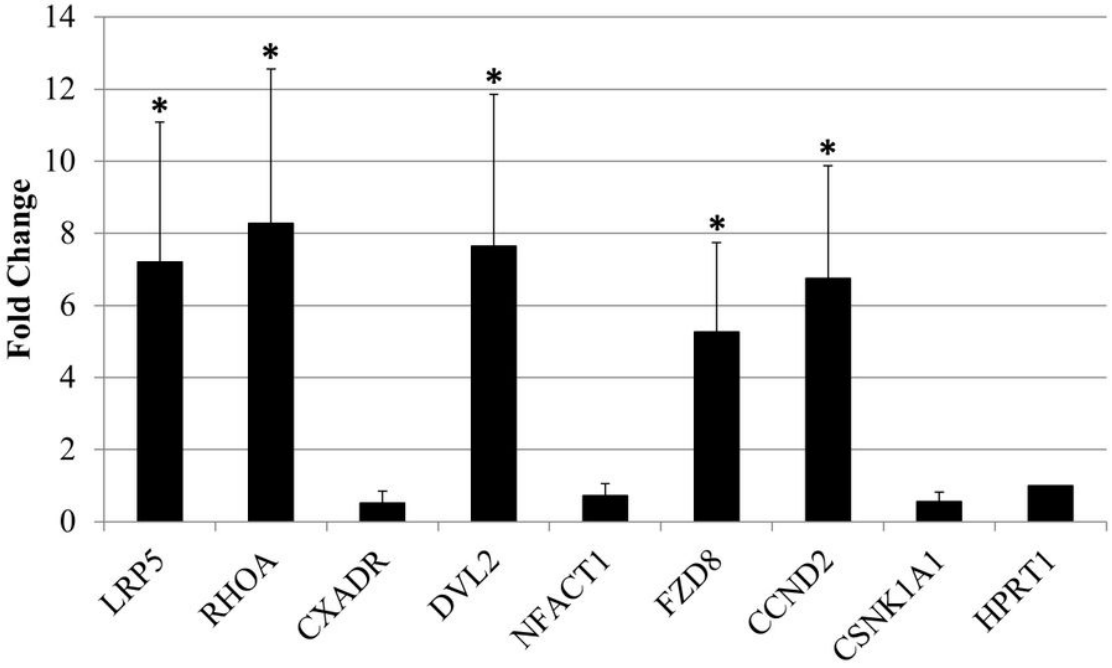

Figure 2

WNT Signaling Pathway Gene Expression in IM

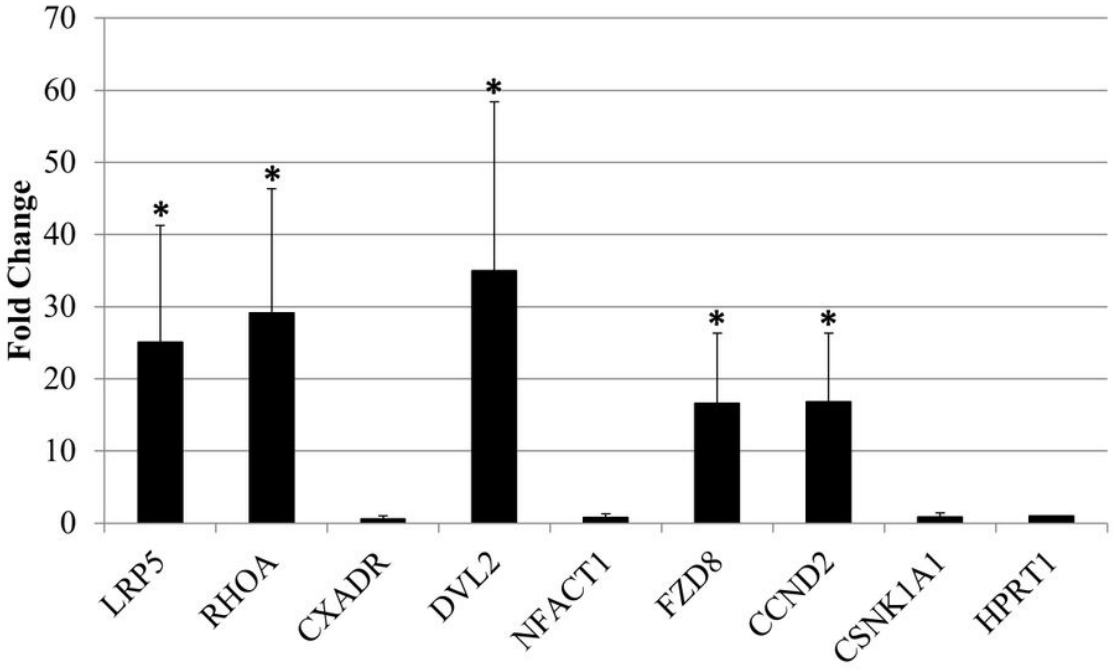

Figure 3

WNT Gene Expressions in HP Positive IM

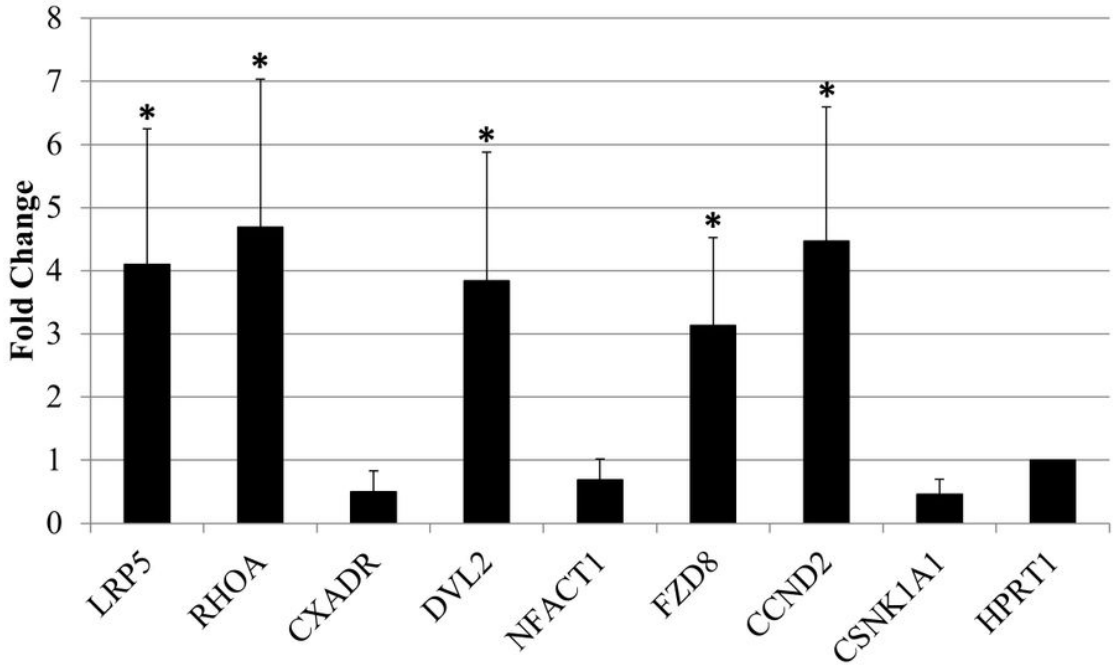

Figure 4

WNT Gene Expressions in HP Negative IM