**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY**

# EXAMINATION OF THE CORRELATION BETWEEN *HELICOBACTER PYLORI* AND T CELL RESPONSE WITH THE DETERMINATION OF PD-1 EXPRESSION LEVEL IN GASTRIC PATHOLOGIES

**M.Sc. THESIS**

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**JULY 2018**

**ISTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# MİDE PATOLOJİLERİNDE PD-1 EKSPRESYON SEVİYESİNİN TAYİNİ İLE *HELİKOBAKTER PYLORİ* VE T HÜCRE CEVABI ARASINDAKİ KORELASYONUN İNCELENMESİ

**YÜKSEK LİSANS TEZİ**

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**TEMMUZ 2018**

Tevriz Dilan Demir, a M.Sc. student of İTU Graduate School of Science Engineering and Technology student ID 521151120, successfully defended the thesis/dissertation entitled "EXAMINATION OF THE CORRELATION BETWEEN *HELICOBACTER PYLORI* AND T CELL RESPONSE WITH THE DETERMINATION OF PD-1 EXPRESSION LEVEL IN GASTRIC PATHOLOGIES", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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**Date of Submission : 4 May 2018**
**Date of Defense : 23 July 2018**

***To my family,***

## FOREWORD

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**ABBREVIATIONS**

**Mg** : Microgram
**Ml** : Microliter
**Mm** : Micromolar
**Mm** : Micrometer
**ACG** : Active Chronic Gastritis
***Baba*** : Blood Group Antigen-Binding Adhesion Protein A
**Bp** : Base Pair
***Caga*** : Cytotoxin-Associated Gene A
**Cag-T4SS** : Cag Type IV Secretion System
**Cdna** : Complementary DNA
**CO2** : Carbon Dioxide
**DNA** : Deoxyribonucleic Acid
**Dntp** : Deoxyribonucleotide
***Dupa*** : Duodenal Ulcer Promoting Gene A
**EPIYA** : *Glu-Pro-Ile-Tyr-Ala* Motif
**Fla A/B** : Flagella A/B
**FOXP-3** : Forkhead Box Protein 3
**H2** : Hydrogen
***Hpaa*** : Putative Neuraminyllactose-Binding Hemagglutinin Homolog A
***H. Pylori*** : *Helicobacter Pylori*
**ICG** : Inactive Chronic Gastritis
**IFN-Γ** : Interferon Gamma
**Ig** : Immunoglobulin
**IHC** : Immunohistochemistry
**K** : Cohen's Kappa Coefficient
**IL** : Interleukin
**IM** : Intestinal Metaplasia
**$Le^b$** : Lewis B Antigen
**M** : Molar
**MALT** : Mucosa-Associated Lymphoid Tissue
**Mg** : Milligram
**MHC** : Major Histocompatibility Complex
**Min** : Minute
**Ml** : Milliliter
**Mm** : Millimolar
**Mm** : Millimeter
**Mrna** : Messenger Ribonucleic Acid
***Napa*** : Neutrophil - Activating Protein A
**NH3** : Ammonia
**NKT** : Natural Killer T Cell
**Oipa** : Outer Membrane Protein A
**PAI** : Pathogenicity Island
**PCR** : Polymerase Chain Reaction
**PD-1** : Programmed Cell Death Protein 1

**PD-L1** **:** Programmed Death Ligand 1
**Ph** **:** Power Of Hydrogen
**PMN** **:** Polymorphonuclear Leukocytes
**PPI** **:** Proton-Pump İnhibitor
**RNA** **:** Ribonucleic Acid
**RUT** **:** Rapid Urease Test
**Rorγt** **:** RAR-Related Orphan Receptor Gamma T
**Rrna** **:** Ribosomal RNA
**RT-PCR** **:** Real-Time Polymerase Chain Reaction
**T-Bet** **:** T-Box Transcription Factor
**T4SS** **:** Type IV Secretion System
**TCR** **:** T Cell Receptor
**TGF-B** **:** Transforming Growth Factor-Beta
**Th1** **:** T Helper 1
**Th2** **:** T Helper 2
**Th17** **:** T Helper 17
**Tm** **:** Melting Temperature
**TLR** **:** Toll-Like Receptor
**TNF** **:** Tumor Necrosis Factor
**Treg** **:** Regulatory T Cell
**SAT** **:** Stool Antigen Test
**STAT** **:** *Helicobacter Pylori*
**Urea** **:** Urease A
**Ureb** **:** Urease B
***Vaca*** **:** Vacuolating Toxin Gene A

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**EXAMINATION OF THE CORRELATION BETWEEN *HELICOBACTER PYLORI* AND T CELL RESPONSE WITH THE DETERMINATION OF PD-1 EXPRESSION LEVEL IN GASTRIC PATHOLOGIES**

## SUMMARY

*Helicobacter pylori (H. pylori)* is a gram negative microaerobic bacterium that coccoid or sphiral shaped bacterium that colonizes in stomach. Bacteria is around 2.5-3 μm length. Each *H. pylori* has around 6 flagella. This flagellas enables the bacterium movement and survival under harsh environmental conditions by escaping.

More than 50% of people all around the world are infected by this bacterium and world health organization accepts *H. pylori* as primary cancerous. Most of the infected people by *H. pylori* do not show any clinical disease evidence, it maintains its existence sub clinically. Generally, infection starts at childhood and maintain lifelong. However, if bacterium could not detect early and treated properly, it caused several gastric pathologies. These pathologies are ulcer, gastritis, gastric carcinoma and MALT. Gastric Cancer has two main subtypes; intestinal type gastric cancer and diffuse type gastric cancer. Intestinal type gastric cancer is more frequently diagnosed than diffuse type. The prevalence of intestinal type is around 60% and there is a stepwise progression of disease. When the healthy gastric epithelium infected with *H. pylori*, host immune system responds it. Inflammation starts and if bacterium is not treated or eliminated by immune system, inflammation becomes a chronic phase; chronic gastritis. If bacterium does not eradicated atrophic changes in gastric epithelium takes place. One of the most important atrophic changes is degeneracy of glandular structure of gastric epithelium. After the degradation of gastric epithelium structure, disease could be progressed to intestinal metaplasia stage. Intestinal metaplasia is a pathological discrimination of gastric disease, which is mainly the differentiation of gastric epithelial cells to small intestine or colon epithelial cells. After the formation of these epithelial changes, treatments against *H. pylori* could not efficient to preventation of gastric cancer. Therefore, bacterium detection must be take place in early stages and this detection must be accurate. Detection of *H. pylori* could be happened by invasive or non-invasive methods. Non-invasive and invasive methods could be used at the same time for more accurate diagnosis.

All those *H. pylori* related diseases are directly related with the host immune responses, genetic susceptibility and virulence factors of bacterium. Especially the resistance of bacterium to the treatment and increasing the survival period in host stomach is directly associated with the virulence factors. Even the genetic strain of *H. pylori* shows geographical differences in between countries, most important and prevalent virulence factors are urease subunit A (*ureA*), urease subunit B (*ureB*) Cytotoxin-associated gene A (*cagA*), vacuolating cytotoxin gene A (*vacA*), outer inflammatory protein A (oipA), blood group antigen-binding adhesion (BabA), putative neuraminyllactose-binding hemagglutinin homolog A (*hpaA*), neutrophil

activating protein A (NapA), duodenal ulcer promoting gene A (*dupA*). Not every *Helicobacter pylori* strain has to carry all of those virulence factors but they have to carry one of the urease subunit; *ureA* or *ureB*. These virulence factors are importants for bacterial infection, adhesion, colonization and survival periods.

When the *H. pylori* infected, host immune system starts an immediate response against it by innate or adaptive immunity. Innate immunity gives immediate but non-specific responds. However, adaptive immune response is highly specific to pathogenic antigens. Especially, as an adaptive immune response, T cells have an importance on determining the clinical outcome of the infection. One of the recently discovered subtypes of T helper cell is Th17. T helper 17 (Th17) cells are functional in defending the host against the extracellular microorganism infections and has role in induction and propagation of autoimmunity. The master regulatory transcription factor of Th17 is Retineic-acid-receptor-related orphan nuclear receptor gamma t (RORγT). RORγT mainly expresses in thymus and controls the lineage of Th17. Th17 has been secreted 8 cytokines known. IL-17 is main cytokine groups of Th17.

PD-1 is programmed death protein that act as immune checkpoint protein. It is expressing by the effector T cells as a surface receptor. Normally, absent on resting T cells. This surface receptor protein is responsible in controlling the T cell response of the body. When it binds its ligand PD-L1, it turns active T cells to inactive T cells. This feature of receptor is important in progression of many diseases. The effect of *H. pylori* on T cells and gastric epithelium is known but, effect on PD-1 is not clearly explained yet.

In this thesis, we worked with the gastric biopsy and resection samples. In total of 60 patients were selected from 5 different pathological conditions. With in this 5 pathological groups; there is 15 normal, 20 active chronic gastritis, 3 inactive chronic gastritis, 9 intestinal metaplasia and 14 gastric cancer patient exists. Normal group was used as uninfected and unaffected control group for gastric malignecies. Patients were selected from patients who admitted to Acıbadem Hospital Gastroenterology Department who undergo endoscopies and Acıbadem Hospital General Surgery Department who undergo resection in between 18 to 80 years old admitted in this study. Each patient informed about the study, and his or her permissions taken. Endoscopic biopsy samples were collected from antrum of patients and resection samples were from tumor sites. Tumor site was detected by macroscopic examination and verified by hematoxylin and eosin staining.

First step of this study, we detected the each virulence factors by PCR assay. *H. pylori* G27 strain used as positive control. G27 strain expresses all of those 9 virulence factors. We correlated the virulence factors expression profiles in between active chronic gastritis and intestinal metaplasia patients. Then verification of datas were confirmed with multiplex PCR assay. Then, we compared our PCR results with pathology reports of patients, which were prepared, based on histological appearance of *H. pylori*. According to this result, our PCR assay shows almost perfect agreement. Secondly, we examined the gene expression profiles of IL-17, RORγT and PD-1 by RT-PCR. We tried to correlate expression of these three genes with *H. pylori* infection. We observed that only PD-1 showed significant increase expression profile in infected patiens. The expressions of IL-17 and RORγT did not show significant varience between infected and uninfected patients. Then, we examined and tried to correlate expression profiles of those genes in between normal

(uninfected and unaffected), precancerous lesions (inactive chronic gastritis, active chronic gastritis and intestinal metaplasia) and gastric cancer. For, IL-17 and RORγT, highest expression found in normal (uninfected and unaffected) patients. PD-1 had highest expression in active chronic gastritis. Consequently, there was not any stepwise increase or decrease of IL-17 and RORγT expression in between progression steps of gastric cancer but PD-1 showed increasing expression profile.

# MİDE PATOLOJİLERİNDE PD-1 EKSPRESYON SEVİYESİNİN TAYİNİ İLE HELİKOBAKTER PYLORİ VE T HÜCRE CEVABI ARASINDAKİ KORELASYONUN İNCELENMESİ

## ÖZET


*Helikobakter pylori,* midede kolonize olan kokoid veya spiral şekilli gram negatif mikroaerobik bir bakteridir. Bakteri yaklaşık olarak 2.5-3 mikron uzunluğundadır. Her bakteri yaklaşık 6 flagellaya sahiptir. Bu flagellalar sert çevre koşullarında bakteri hareketini ve hayatta kalmasını sağlar.

Dünyanın dört bir yanındaki insanların % 50'sinden fazlası bu bakteri tarafından enfekte edilmiştir. Dünya Sağlığı Örgütü (World Health Organization*) H. pylori'*yi birincil kanserojen olarak kabul etmektedir. *Helikobakter pylori* tarafından enfekte olmuş kişilerin çoğu, herhangi bir klinik hastalık kanıtı göstermez ve varlığını subklinik olarak korur. Genellikle bakteri enfeksiyonu çocuklukta başlar ve yaşam boyu devam eder. Bununla birlikte, bakteri erken teşhis edilmediyse ve uygun şekilde tedavi edilmediyse, çeşitli mide patolojilerine neden oldu. Bu patolojiler ülser, gastrit, mide karsinomu ve MALT'dır. Gastrik kanserde iki ana alt tip vardır; intestinal tipi mide kanseri ve diffüz tip mide kanseri. İntestinal tipi mide kanseri, diffüz tipten daha sık teşhis edilir. İntestinal tipin prevalansı yaklaşık olarak % 60'tır ve hastalığın adım adım ilerlemesi vardır. *H. pylori* ile enfekte olmuş normal (sağlıklı) gastrik epitelyumun konakçı immün sistemi buna cevap verir. Enflamasyon başlar ve eğer bağışıklık sistemi tarafından bakteri tedavi edilmez veya ortadan kaldırılmaz ise, iltihaplanma kronik bir evreye dönüşür; Kronik gastrit. Mide epitelinde bakteri yok edilmezse atrofik değişiklikler yer almaya başlar. En önemli atrofik değişikliklerden biri gastrik epitelyumun glandüler yapısının dejenerasyonudur. Gastrik epitelyum yapısının bozunmasından sonra hastalık intestinal metaplazi safhasına doğru ilerleyebilir. İntestinal metaplazi, gastrik epitelyal hücrelerin ince bağırsak veya kolon epitelyal hücrelerine farklılaşması olan gastrik hastalığın patolojik bir ayrımımdır. Bu epitelyal değişikliklerin oluşmasından sonra, *H. pylori'*ye karşı tedavi, mide kanserinin önlenmesinde olan etkisini kaybetmeye başlar. Bu nedenle, bakteri tespiti erken aşamalarda yapılmalı ve bu tespit hassas bir doğrulukta olmalıdır. *Helikobakter pylori*'nin saptanması, invazif veya invazif olmayan yöntemlerle gerçekleşebilir. Daha doğru bir teşhis için aynı zamanda non-invazif ve invaziv yöntemler kullanılabilir.

Tüm bu *Helikobakter pylori* sebepli hastalıklar doğrudan konakçı immün yanıtları, genetik yatkınlık ve bakterinin virülans faktörleri ile ilişkilidir. Özellikle bakterinin tedaviye karşı direnci ve konak midede sağkalım süresini arttırması doğrudan bakterinin sahip olduğu virülans faktörleri ile ilişkilidir. *Helikobakter pylori*'nin genetik türü, ülkeler arasında coğrafi farklılıkları gösterse de, en önemli ve yaygın virülans faktörleri, üreaz alt birimi A (*UreA*), üreaz alt birimi B (*UreB*), sitotoksin ilişkili gen A (cagA), vakuolasyon sitotoksin gen A'dır (*vacA*) dış enflamatuar protein A (OipA), kan grubu antijen bağlama adezyonu (BabA), putatif

nöraminillaktoz bağlayıcı hemaglutinin homolog A (*hpa*A), nötrofil aktive edici protein A (NapA), duodenal ülseri teşvik eden gen A (dupA). Her *Helikobakter pylori* soyu, bu virülans faktörlerini taşımak zorunda değildir, ancak üreaz alt biriminden birini taşımak zorundadırlar; *ureA* veya *ureB*. Bu virülans faktörleri, bakteriyel enfeksiyon, adhezyon, kolonizasyon ve hayatta kalma süreleri için önemlidir.

*Helikobakter pylori* enfekte olduğunda, konakçı immün sistemi, doğuştan gelen veya adaptif immünite ile buna karşı derhal bir yanıt başlatır. Doğuştan gelen bağışıklık, anında, spesifik olmayan bir yanıt. Bununla birlikte, adaptif immün yanıt patojenik antijenlere oldukça özgüdür. Özellikle adaptif bir immün yanıt olarak T hücreleri, enfeksiyonun klinik sonuçlarını belirlemede büyük bir öneme sahiptir. T yardımcı hücresinin yeni keşfedilen alt tiplerinden biri Th17'dir. T yardımcı 17 (Th17) hücreleri konakçıyı hücre dışı mikroorganizma enfeksiyonlarına karşı savunmada işlevseldir ve otoimmünitenin indüksiyonu ve yayılmasında rol oynar. Th17'nin ana düzenleyici transkripsiyon faktörü ROR'dur. RORγT ağırlıklı olarak timüste eksprese edilir ve Th17 gelişimi ve değişimini kontrol eder. Th17, bilinen 8 ana sitokin salgılar. IL-17, Th17'nin ana sitokin grubudur.

PD-1, immün kontrol noktası proteini olarak görev yapan programlanmış hücre ölüm proteinidir. Bir yüzey reseptörü olarak efektör T hücreleri tarafından ifade edilmektedir. Normal olarak, aktif olmayan T hücreleri üzerinde yoktur. Bu yüzey reseptör proteini, vücudun T hücre yanıtını kontrol etmekten sorumludur. Ligand PD-L1'e bağlandığında aktif T hücrelerini inaktif T hücrelerine çevirir. Reseptörün bu özelliği, birçok hastalığın ilerlemesinde önemlidir. *Helikobakter pylori* 'nin T hücreleri ve gastrik epitelyum üzerindeki etkisi bilinmektedir, ancak PD-1 üzerindeki etki henüz açık bir şekilde açıklanmamıştır.

Bu tez çalışmasında mide biyopsisi ve rezeksiyon örnekleri ile çalıştık. Toplamda 60 hasta 5 farklı patolojik durumdan seçilmiştir. Bu 5 patolojik grupta; 15 normal, 20 aktif kronik gastrit, 3 inaktif kronik gastrit, 9 intestinal metaplazi ve 14 mide kanseri hastası vardır. Endoskopic biyopsi hastaları, Acıbadem Hastanesi Gastroenteroloji Anabilim Dalı'na başvuran ve bu çalışmaya kabul edilen 18-80 yaşları arasındaki kişilerden ve rezeksiyon yapılan mide kanserli hastalar Acıbadem Hastanesi Genel Cerrahi Kliniği'ne başvuran hastalardan seçildi. Normal grup, midede enfeksiyon ve buna bağlı etkileri olmayan kontrol grubu olarak kullanılmıştır. Her hasta çalışma hakkında yazılı ve sözlü olarak bilgilendirildi ve imzalı onamları alındı. Endoskopik biyopsi örnekleri hastaların antrumundan, rezeksiyon örnekleri çıkarılan midenin tümör bölgelerinden alındı. Tümör bölgesi makroskopik inceleme ile tespit edildi ve hematoksilin ve eozin boyaması ile doğrulandı.

Bu çalışmanın ilk adımı olarak her bir virülans faktörünü PZR testi ile tespit ettik. *Helikobakter pylori* G27 türü pozitif kontrol olarak kullanıldı. G27 türü, bu 9 virülans faktörünün hepsine sahiptir. Aktif kronik gastrit ve intestinal metaplazi hastaları arasındaki virülans faktörleri ekspresyon profillerini ilişkilendirdik. Daha sonra verilerin doğrulanması multipleks PZR testi ile yapıldı. Daha sonra, *Helikobakter pylori'*nin histolojik görünümüne dayanarak hazırlanan patoloji raporlarıyla PZR sonuçlarımızı karşılaştırdık. Bu sonuçlara göre, PZR testimiz neredeyse mükemmel bir anlaşma göstermektedir. İkincil olarak, gerçek zamanlı PZR ile IL-17, RORγT ve PD-1'in gen ekspresyon profillerini inceledik. Bu üç genin ekspresyonunu *Helikobakter pylori* enfeksiyonu ile ilişkilendirmeye çalıştık. Enfekte

edilen hastalarda sadece PD-1'in belirgin bir artış ekspresyon profili gösterdiğini gözlemledik. IL-17 ve RORγT ifadeleri enfekte olmuş ve enfekte olmamış hastalar arasında belirgin bir farklılık göstermedi. Daha sonra, normal, prekanseröz (inaktif kronik gastrit, aktif kronik gastrit ve intestinal metaplazi) ve mide kanseri arasında bu genlerin ekspresyon profillerini inceledik ve bunları test etmeye çalıştık. IL-17 ve RORγT için normal hastalarda en yüksek ekspresyon seviyesi bulundu. PD-1, aktif kronik gastritte en yüksek ekspresyona sahipti. Sonuç olarak, mide kanserinin ilerleyen aşamaları arasında IL-17 ve RORγT ekspresyonunun herhangi bir kademeli olarak artması veya azalması olmamıştır ancak PD-1 ekspresyonu artış göstermiştir.

# 1. INTRODUCTION

## 1.1 Human Stomach

Stomach is a thick walled muscular organ which lies on the upper left side of human abdomen and constitutes the gastrointestinal tract (alimentary tract) with mouth, pharynx, esophagus, small and large intestine, rectum and anal canal (Bhise & Yadav, 2008). Human stomach directly connects with esophagus and small intestine which is also named as duodenum.

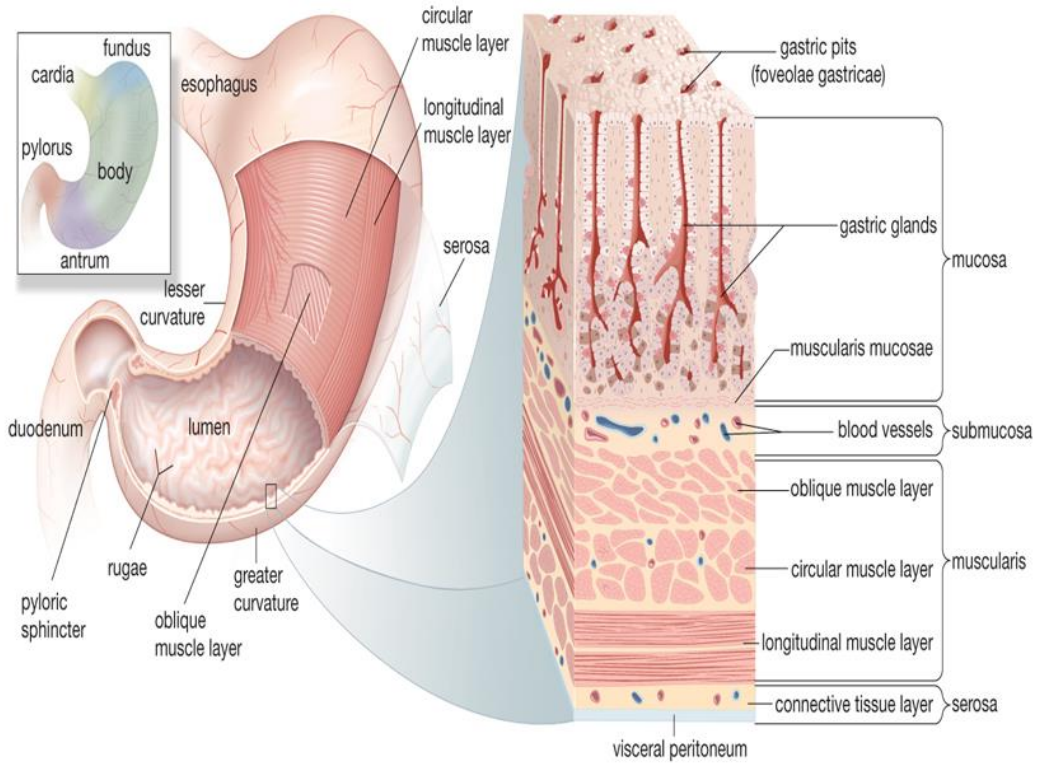


**Figure 1.1 :** Structure of human stomach (adapted from Encyclopædia Britannica Online, 2003).

This first intra-abdominal part of gastrointestinal track mainly serves as temporary reservoir for nutrients (Bhise & Yadav, 2008). By the intake of foods and nutrients, around ten trillion microorganisms can enter the human stomach (Marchesi, 2014). However, thanks to acidic pH of stomach which changes in between 1 to 2, many of those microorganisms are intrinsically eliminated. On the other hand, some species

may tolerate the acidic environment and determine those acidic environments as an ecological niche for instance, *Helicobacter pylori* (*H. pylori*) which is is primary cause of many chronic inflammations and diseases in stomach (Wascher, 2010).

### 1.2 *Helicobacter pylori*

*Helicobacter pylori* is a bacterium found in the human gastric epithelium and infected more than half of the world's human population (Kabamba, Tuan, & Yamaoka, 2018). This infectious agent is primarily discovered by Barry Marshall and Robin Warren in 1983. Previously, even there were no proofs that any bacterium could live in the acidic environment of human stomach, Barry and Marshall observed a spiral or curved shaped bacilli in the biopsy specimens from antral mucosa of gastric patients (Marshall & Warren, 1984). To prove this bacterium could live in acidic environment of human stomach and caused the ulcer, Barry Marshall drank it. As a result of it, he developed gastritis and the relation between *H. pylori* and gastric disease is proved.

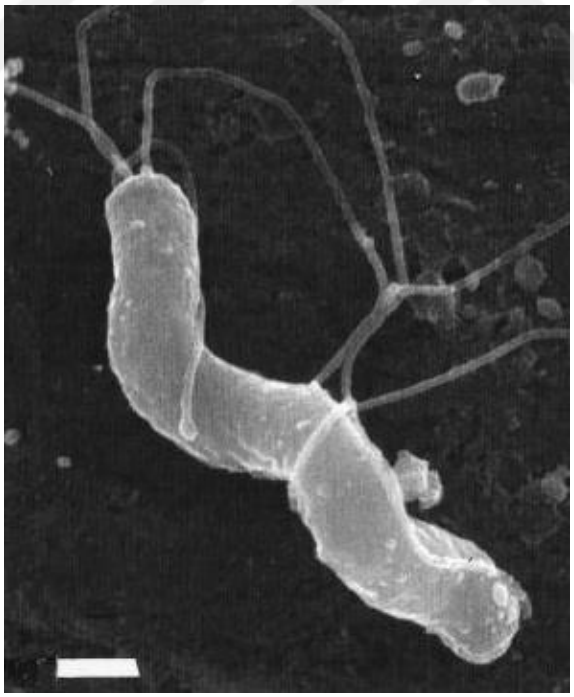

**Figure 1.2 :** Scanning electron microscopy image of *H. pylori* with flagella. The field emission SEM, bar= 0.05 μm (O'Rourke & Bode, 2001)

#### 1.2.1 The Morphology of the *Helicobacter pylori*

*Helicobacter pylori* is a gram negative that can be spiral shaped or curved rods bacteria. In the stomach, they are mostly founded as spiral shaped form. In vitro

experiments show that, they are in curved shape forms in culture. However, when they removed from their natural niche and cultured several times they forms coccoid shape (Chan, et al., 1994). The main reason for this shape change is mainly because of the increase of the pH of environment in culture. This shape change is also observable at the damaged areas of human gastric tissue where the environment becomes more alkali. *H. pylori* shows specific cell wall structure. Even most of gram negative bacteria have structurally homogenous cell wall with thin peptidoglycan, *H. pylori* have thicker peptidoglycan structure. It is because, there is an additional murein fragments which could develop specific interaction with host cell (Linchtman, et al., 1993). Based on the in vitro observations, bacterium is in between 2-4 μm long and 0.5-0.9 μm wide (O'Rourke & Bode, 2001). *H. pylori* prefers to live under microaerophilic conditions (Giudice & Rappuoli, 2003). Therefore, in its niche there has to be oxygen but its level must be less than atmosphere oxygen level.

The harsh condition of human stomach is relatively impossible for living of many other pathogenic species. However, *H. pylori* is a specialized bacterium to resists these environmental conditions. First of all, it is a highly motile bacterium with one to six sheathed flagella which are extended from out of the outer membrane. It allows the movement of bacterium in between the stomach lumen to the epithelium (Loconte, Kekez, Matkovic-Calogovic, & Zanotti, 2017). By this way, *H. pylori* can avoid the acidic milieu of stomach. Also by covering each of its flagellum with unipolar flagellar sheath, this rotatory structure is conserved from the invasion of stomach acid (Hawtin, Stacey, & Newell, 1990). Each of these flagella are around 30 μm length and 2.5 μm thickness.

### 1.2.2 The Genome of the *Helicobacter pylori*

*H. pylori* strains show varieties in their gene clusters into different regional populations. Based on geographic origins, there is classified seven subtype of bacterium exists; hpEurope, hpNEAfrica, hpAfrica1, hpAfrica2, hpAsia2, hpSahul and hpEastAsia (Gutiérrez-Escobar, Trujillo, Acevedo, & Bravo, 2017). In between these populations, the most prevalent strain is hpEurope which is mainly dominated in Europe but also arise in Asia. In 1997, the genome of *H. pylori* strain 266695 from gastritis patient is fully sequenced. They found that; *H. pylori* has circular genome

which is consisting 1667867 base pairs with known 1590 coding sequence (Nejati, et al., 2018). The average G+C content is 39%. However, the cag pathology island has 35% G-C content with 539-579 kb (Censini, 1996). Based on genetic analysis, *H. pylori* have an advanced system to move, obtain ferrous and adjustment of DNA. The most important part of bacterium is the genes that are expressing the virulence factors.

### 1.2.3 *Helicobacter pylori* Virulence Factors

The pathogenicity, cytotoxicity and colonization characterization of *H.pylori* is directly associated with the virulence factors of bacterium. Virulence factors that related with increasing the risk of developing *H. pylori* related gastric are; *VacA*, *CagA*, OipA, *HpaA*, *DupA*, NapA, BabA, *UreA*, and *UreB*. Those virulence genes are located within DNA segment of 35-40 kbp; pathogenicity island (Abu-Lubad, et al., 2018).

#### 1.2.3.1 Vacuolating cytotoxin gene A *(vacA)*

*vacA* is a gene encodes vacuolating cytotoxin A which an exotoxin that increase the risk of peptic ulcer and gastric cancer (Kabamba, Tuan, & Yamaoka, 2018) (Abu-Lubad, et al., 2018). It is a 88 kDa protein. *H. pylori* secreted vacuolating cytotoxin A by type IV auto transport secretion system and protein enters the host cell via endocytosis (Nejati, et al., 2018). Accumulation of *vacA* into the host cell causes the membrane depolarization, autophagy, mitochondrial dysfunction, T cell inhibition and finally apoptosis of cell (Nejati, et al., 2018). Also, it forms "vacuole-like'' membrane vesicles in the cytoplasm of gastric cells. All those functions are necessary for colonization of bacterium into the stomach epithelium.

Based on the *vacA* genes that isolated from *H. pylori* strains; gene has two regions. First region is a signal region (s) and has two alleles; s1 and s2. Second region is a middle region (m) with two alleles m1 and m2. Different combinations of these alleles determine the cytotoxicity level of the *H. pylori*. The most cytotoxic version of *vacA* gene is with s2/m2 allelic combination (Abu-Lubad, et al., 2018). However, vacuolating activity is highest in s1/m1 genotype and absent in s2/m2 (Atherton, et al., 1998).

#### 1.2.3.2 Cytotoxin-associated gene A *(cagA)*

*cagA* is immunogenic protein that highly associated with peptic ulcer and gastric cancer like *vacA. cagA* encoding *H. pylori* strains are directly associated with gastritis. Whole atrophic gastritis patients are *cagA* positive and shows higher grade of mucosal inflammation (Nejati, Karkhah, Darvish, Validi, Ebrahimpour, & Nouri, 2018).

Around 50% of *H. pylori* strains are *cagA* positive. When it is trans-located to host cell by a type IV secretion system, it can be phosphorylated by either Abl kinase or Src kinases on EPIYA motifs at the C-terminal region of the protein. After phosphorylation, *cagA* protein deregulates many signaling pathways and leading morphological changes in the cell (Abu-Lubad, et al., 2018). When the EPIYA motifs number increases on *cagA, H. pylori* becomes more pathogenic. Translocation of *cagA* cause several signal transduction events such as defects in proliferation of B cells. To respond the *cagA* enterences, gastric epithelial cells express interleukin-8 (IL-8). This redundant level of IL-8 expression is not related with *H. pylori* but it is directly increasing when the *H. pylori* strain expresses *cagA* (Pattis, Weiss, Laugks, Haas, & Fischer, 2007).

#### 1.2.3.3 Cag Type IV Secretion System *(*Cag-T4SS*)*

Type IV secretion system is used by many gram negative bacteria to inject their genetic materials, proteins or virulence factors directly into the cytoplasm of target host cells. It's accomplished by syringe like pili or pilus structure.

In *H. pylori*, this syringe like structure used to inject cytotoxic associated proteins; *cagA* into the gastric epithelium cells of human stomach. For this translocation protein, Cag-T4SS express 18 gene products (Pattis, Weiss, Laugks, Haas, & Fischer, 2007). This secretion system is encoded by the *cag* Pathogenicity Island (PAI) which is around 40-kb chromosomal region (Horridge, Begley, Kim, Aravindan, Fan, & Forsyth, 2017). 14 gene products is responsible for synthesis of secretion apparatus and rest of 4 gene product are responsible for translocation of *cagA* and its homologous proteins *cagb* and *cagF* (Pattis, Weiss, Laugks, Haas, & Fischer, 2007).

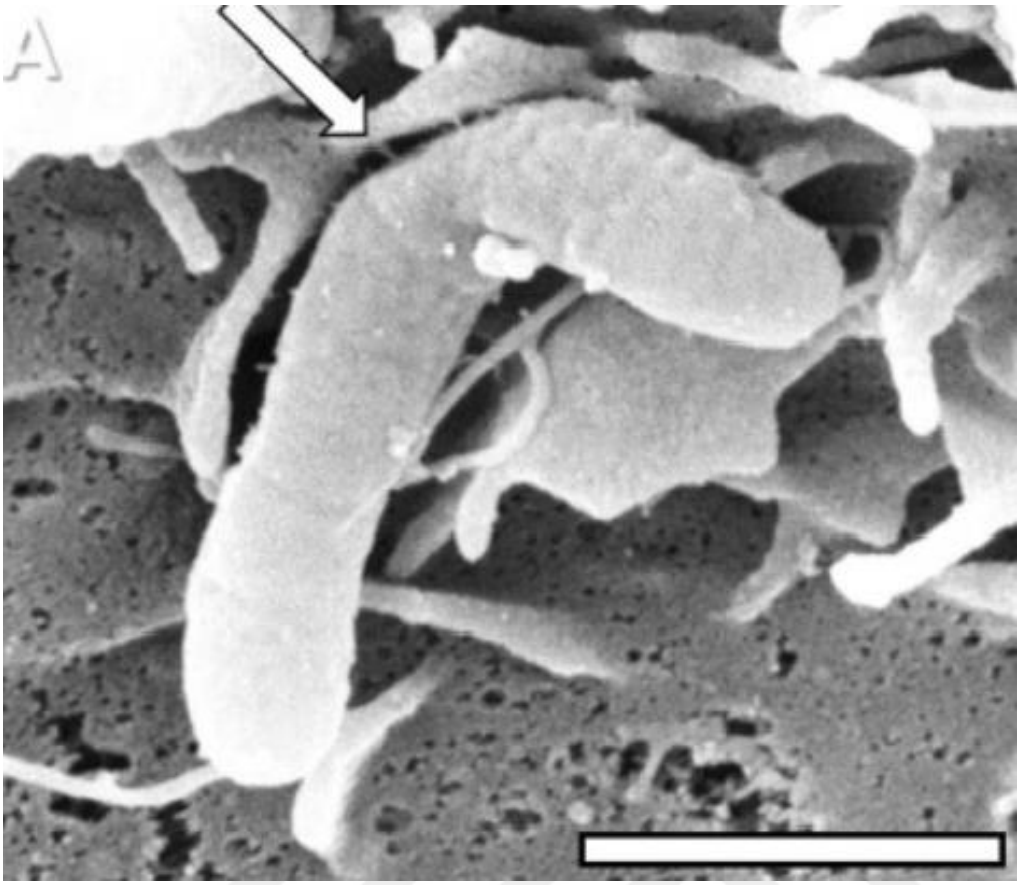


**Figure 1.3 :** High resolution SEM analysis of *H. pylori* Cag-T4SS pilis. (Haley, Blanz, & Gaddy, 2014).

#### 1.2.3.4 Outer Inflammatory Protein A *(oipA)*

*Helicobacter pylori* success among the avoidance from immune response, colonization and persistence against the treatments are mostly depend on the function of the outer membrane proteins (OMP) encoding genes. oipA is the one of the outer membrane protein that specific for the *H. pylori* and encoded by the oipA gene which is located approximately 100 kb from the bacterium cagPAI (Farzi, Yadegar, Aghdaei, Yamaoka, & Zalia, 2018). As like as the other outer membrane proteins, oipA is functional in adherence to host cell and colonization. Especially, it induces the inflammation in the host and increases the expression of IL-8 (Farzi, Yadegar, Aghdaei, Yamaoka, & Zalia, 2018).

Regulation of oipA expression is maintaining by the slipped strand mispairing (SSM) within CT dinucleotide repeat motifs in the 5' terminus of the gene. The number of CT repeats determines whether active protein will be expressed or not. Each *H. pylori* strain shows variety in those numbers of CT repeats (Horridge, Begley, Kim, Aravindan, Fan, & Forsyth, 2017).

#### 1.2.3.5 Blood group antigen-binding adhesion protein A *(*BabA*)*

BabA is the other outer membrane proteins that also functional in adherence to host cell and colonization. It could be named as the adhesion and binds to the fucosylated Lewis b blood group antigens (Farzi, Yadegar, Aghdaei, Yamaoka, & Zalia, 2018). Those fucosylated blood group antigens are highly expressed in the gastric epithelium and refer 0 blood group phenotype with in the ABO blood group system (Aspholm-Hurtig, et al., 2004). Adhesin babA binding to those fucosylated glycoproteins is required for *H. pylori* access to nutrients from host cells.

#### 1.2.3.6 Duodenal ulcer promoting gene A *(dupA)*

*DupA* virulence factor was discovered at 2005 which is located in the plasticity region of the bacterium genome. Till the duodenal ulcer promoting gene discovery, the direct relation with any virulence factor and digestive diseases are not proven. *dupA* is the first *H. pylori* virulence factor that accepted primer marker for increasing the risk for ulcer (Abadi & Perez-Perez, 2016). Therefore, patients that infected with *DupA* positive *H. pylori* strains are more susceptible to develop ulcer than the patients infected with negative bacterium. As like as the OipA, it is highly associate with the IL-8 secretion, especially in the antrum of the gastric.

#### 1.2.3.7 Neutrophil - activating protein A *(*napA*)*

Neutrophil activating protein (napA*)* is one of the main virulence factors that found whole *H. pylori* strains (Liu, et al., 2014). It is functional in promoting endothelial adhesion of neutrophils. On the other hand, napA knock out experiments are proved that, this virulence factor has role in stimulating high production of oxygen radicals from polymorphonuclear leukocytes (PMNs) and adherence to the host cells (Petersson, et al., 2006). Release of the napA is accomplished upon the autolysis of bacterium. After that, napA binds the external surface of the outer membrane and enables the bacterium binding to host cell surfaces (Petersson, et al., 2006).

#### 1.2.3.8 Putative Neuraminyllactose-Binding Hemagglutinin Homologue A (*hpa*A*)*

Putative neuraminyllactose-binding hemagglutinin homologue A (*hpaA*) is one of the adhesion molecules of *Helicobacter pylori* to mediate specific binding to host epithelium and colonization. By the Evans works, *hpaA* structure is defined as

subunit molecular weight of 20,000 and has putative filamentous structure (O'toole, et al., 1995). It is mainly located at the inner membrane of the cytoplasm. It contains lipoprotein cleavages motifs (O'toole, et al., 1995).

#### 1.2.3.9 UreaseA gene *(ureA)* and UreaseB gene *(ureB)*

Urease enzyme activity is the one of the most important feature for *Helicobacter pylori* colonization in stomach. It is because; urease is an enzyme that catalyzes the urea catabolism to ammonia and carbon dioxide (Calam, Gibbons, Healey, Bliss, & Arebi, 1997). The production of ammonia, suppress the acid secretion by acting $H^+$ ion acceptor in stomach. Therefore, the local pH of the stomach increases and it constitutes proper environment for *H. pylori* colonization.

Urease gene cluster is organized into two transcriptional units; *ureAB* and *ureIEFGH*. The firsy two genes of cluster are; *ureA* and *ureB*, and they directly involve to production of an assembled apoenzyme. *UreEFGH* is an accessory gene that required for the assembly of Ni into the urease apoenzyme and *UreI* encodes the inner membrane proton-gated channel involved in importing the urea into the bacterium (Benoit & Maier, 2011). Also, ammonium production gives rise to $NH_3^-$ derived compounds formation which could be cytotoxic for gastric epithelium cells such as; monochloramine (Suzuki, et al., 1992).

### 1.2.4 *Helicobacter pylori* Colonization

*Helicobacter pylori* mainly colonize into the stomach. Stomach has harsh environment that not suitable for living of most bacterium due to the high acidity of stomach lumen where the pH is in between 1 to 5. Normally, survival period of *H. pylori* in these conditions are around 30 minutes. To avoid this harsh condition and increase survival time, bacteria initially colonize into more hospitable localization in stomach; 15 μm from the gastric epithelial cells and deep within gastric glands (Johnson & Ottemann, 2018). After that, to overcome the harsh environment of stomach, bacteria develop some features.

First of all, Chemo-tactical feature of *H. pylori* enables it to live in stomach conditions. Chemotaxis is movement of organism in response to a chemical stimulus (Johnson & Ottemann, 2018). By sensing the environmental signals of stomach, bacteria could escape from immune cells and acidity. For this motility, *H. pylori* use

flagella. After initial colonization, urease enzyme activity of bacterium changes the acidity of the stomach to pH 4.0-8.0. In this pH range bacteria could successfully colonize. Finally, adhesion to gastric epithelium is the other vital factor. By this, *H. pylori* could not eliminated by the stomach mucus turnover and gastric peristalsis.

### 1.2.5 *Helicobacter pylori* Related Diseases

After Barry Marshall demonstrated the relation between gastritis and *Helicobacter pylori*, *H. pylori* has attracted the attention of many scientists. In generally, this common gastric pathogen continues its existence in human stomach as asymptotically (Vakil, 2016). However, because of it can be altered the normal physiology of stomach, it could be histo-pathologically associated with several diseases (Hoffman & Cave, 2001). Nowadays, many important upper gastrointestinal diseases are associated with this infectious bacterium such as; chronic gastritis, ulcer, gastric cancer and MALT (Cremonini, Gasbarrini, Armuzzi, & Gasbarrini, 2001).

At the initial phase of *H. pylori* infection, bacterium colonizes without showing any clinical evidence. Therefore, there is no change in the normal phenotype of the gastric epithelial cells. In this first step of infection and colonization of bacteria, normal cell loss and cell regeneration processes are continuing as like as the normal stomach conditions (Correa, 1988). This initial step is known as acute phase gastritis. In acute phase of gastritis, *H. pylori* colonizes at surface epithelium but at the same time tries to penetrate through mucous layer. Due to *H. pylori* starts to release its chemotactic agent's especially LPS (lipopolysaccharide), epithelial surface of stomach is damaged (Dixon, 2001). As a result of human immune system respond, preliminary inflammation starts (Moller, Heseltine, & Vainio, 1994). If this initial response fails to clear *H.pylori* from stomach, more specific secondary response takes place which involves the recruitment of primed B cells into lymph follicles (Dixon, 2001). This more consistent *H. pylori* colonization and inflammation is known as active chronic gastritis. Another type of gastritis is an erosive gastritis which erodes the stomach lining (Dixon, 2001). Therefore, in general we can define the gastritis as the inflammation of the stomach (Moller, Heseltine, & Vainio, 1994).

The second *H. pylori* related disease is peptic ulcer. There is around 10% to 20% risk for developing peptic ulcer because of *H. pylori* infection (Eicher, Berns, &

Wells, 2018). There are many facts to confirm that acid is the one of the most important factor in peptic ulcer development (Wu P. P., 1935). The relation between *H. pylori* and peptic ulcer is that, colonization of bacteria causes the increasing of gastrin. Elevated level of gastrin, promotes the acid secretion from stomach. As a result of aberrant acid level in epithelial of human stomach, there is damage in intestinal lining which is causing peptic ulcer.

Addition to peptic ulcer and gastritis, there is a 1% to 2% risk for developing gastric cancer in people who infected with *H. pylori* (Eicher, Berns, & Wells, 2018). Even it seeming like a low risk to develop gastritis cancer as a result of infection, in 1994 The World Health Organization's Agency Research Center defined the *H.pylori* as a carcinogen (Moller, Heseltine, & Vainio, 1994). Every year, around 990,000 new gastric cancer patients are diagnosed (Kim & Oh, 2018).

Finally, mucosa associated lymphoid tissue (MALT) lymphoma is the second cancer type that related with *H. pylori* infection. It is actually one of the most common non-Hodgkin lymphoma type which is arising in the gastrointestinal tract (Kobayashi, et al., 2018). To respond the antigenic stimulation based upon the *H. pylori* infection, lymphoid tissue is acquired in the stomach and end with the MALT lymphoma (Wotherspoon, 1996).

### 1.2.6 Diagnosis of *Helicobacter pylori*

To diagnose the *H. pylori* infection; certain tests are applied to patients. Those tests are divided into two main groups; invasive and noninvasive. Invasive tests required the endoscopy biopsy specimens which are undergoing histology, culture, rapid urease tests as well as molecular methods (Wang, et al., 2015). On the other hand, noninvasive methods are divided into three groups; serology, urea breath test and stool antigen test and they applied without any requirements to biopsy specimens. Thirdly, molecular methods include both invasive and noninvasive tests. For accurate diagnosis of infection; combination of those tests must be used (Lopes, Vale, & Oleastro, 2014)**.**

**Table 1.1** Summary of *H. pylori* diagnostic methods, adapted from (Lopes, Vale, & Oleastro, 2014).

| | Invasive/noninvasive | Antibiotic resistance detection |
|---|---|---|
| Endoscopy | Invasive | No |
| Histology | Invasive | No |
| Rapid urease test | Invasive | No |
| Culture | Invasive | Yes |
| Molecular methods | Both | Yes |
| Serology | Noninvasive | No |
| Urea breath test | Noninvasive | No |
| Stool antigen test | Noninvasive | No |

#### 1.2.6.1 Invasive Methods

Endoscopy is the most common invasive method that generally used to diagnose *H. pylori* associated diseases like ulcer, gastritis and cancer. Conventional endoscopic process is based on the examination of gastrointestinal tract with miniature video equipment. Before the operation start, the patient is anesthetized. After that, miniature video equipment is inserted through the mouth and down into the gastrointestinal tract which enables to real time observing of esophagus, stomach and duodenum. During observation, biopsy specimens from gastric mucosa can be obtained for further studies on other invasive tests, including rapid urease test, histology, culture, and molecular methods (Wang, et al., 2015).

Histology is considered to be gold standard in directly detecting the *H. pylori* which mainly based on the staining procedures. Hematoxylin and eosin staining, Giemsa, Warthine-Starry, Hp silver stain, toluidine blue, acridine orange, McMullen and Genta, Dieterle and immunohistochemical stain are basic stains that used to detection of bacterium (Wang, et al., 2015). The most sensitive staining procedure is immunohistochemical staining but H&E staining is also sufficient for *H. pylori* diagnosis. However, the sensitivity and accuracy depends on the site, size and

numbers of biopsies and staining methods. By increasing the number of biopsies, accuracy of diagnosis will be increased.

For most of bacterial infection diagnosis, culture is used as standard test. Highest specificity is achieved with this culturing method, however, it is less sensitive and takes a long time. Therefore, even culture methods gives highest specificity, it is not preferential method for diagnosis of *H. pylori.*

Finally, Rapid Urease Test (RUT) is most common test that used in clinical diagnosis of *H. pylori*. It is because, this technique is inexpensive, rapid, easy perform and highly specific (Wang, et al., 2015). Technique is based on the urease activity of the *H. pylori.* If there is *H. pylori* colonization in biopsy specimen, urea test reagent is converted to ammonia. By this way, pH in the environment increase and color change (Wang, et al., 2015). pH directly observe with pH monitor. Most common version of RUT test is CLO test which based on the agar gel usage. It usually takes 24 hour for accurate result.

#### 1.2.6.2 Non-Invasive Methods

Non-invasive methods are based on the examination of urine, whole blood, saliva, serum and expired air samples for diagnosis of *H. pylori*. The advantages of these non-invasive techniques are, recover patients form discomfort of endoscopy and process is not expensive as much as endoscopy.

Urea breath test is based on the urease activity of *H. pylori.* $^{13}C$ or $^{14}C$ labeled urea ingested by the patient and it hydrolyzed to labeled $CO_2$ in stomach (Wang, et al., 2015). That labeled carbon dioxide is absorbed by blood and exhaled by breathing and measured. Tests give almost 95% sensitive results and specificity under standardized procedures (Wang, et al., 2015).

Stool antigen test (SAT) is other non-invasive method based on the detection of *H. pylori* antigen in stool samples. It could be applied by using either immunoassay (EIA) or immunochromatography assay (ICA). EIA based test is more accurate than ICA. It can be safely applied to children too.

In addition to antigen testing, antibody response of patient body could be used in detection of *H. pylori* infection. When body infected with *H. pylori,* there is systemic antibody response of body with rise of specific IgM level and then IgA and IgG. By

ELISA testing, those antibodies existence and amount could be detected from patient serum and urine.

#### 1.2.6.3 Molecular Methods

Molecular methods involve both invasive and non-invasive techniques. By combining both techniques, molecular methods give more accurate and reliable results. As biological samples, gastric biopsies, stool or oral cavity samples could be used. Most common technique is PCR, either conventional or real time. In polymerase chain reaction (PCR), main purpose is detection of *H.pylori* DNA. By using PCR, each virulence factors and target gene could be separately examined. Therefore, sensitivity and specificity is around 95% (Lage, 1995). Reverse Transcription Polymerase Chain Reaction (RT-PCR) could increase the accuracy of the results and multiplex PCR assay help saving time. Also, fluorescence in situ hybridization (FISH) technique is using in detection of infection. Advantageous of this method is; it does not effect from DNA contamination and it allows direct visualization of *H. pylori* in the gastric biopsy specimens.

### 1.2.7 Treatment

The discovery of *H. pylori* related gastrointestinal diseases, eradication of bacterium gains importance. The clearance of infection required combinations of drugs; antimicrobial agents and anti-secretory agents (Yang, Lu, & Lin, 2014).

Anti-microbial agents required for killing the bacteria. Most commons are; clarithromycin, levofloxacin, metronidazole and amoxicillin. The efficiencies of those drugs are depends on the concentration of them. If their plasma concentration are lower than; 0.25μg/mL, 1μg/mL and 8μg/mL in respectively, *H. pylori* could develope resistance against those anti-microbial agents. On the other hand, the efficiency of amoxicillin is also depends the time. Its efficacy is proportional to the the time that the plasma concentration is higher than the minimum inhibitory concentration which is 0.5μg/mL (Yang, Lu, & Lin, 2014).

Anti-secretory agents are useful for elevation of gastric pH which is increased as a result of the *H. pylori* virulence factors. For this purpose; $H_2$-receptor antagonists and proton pump inhibitors (PPIs) can be used. Because of PPIs are more efficient, they are more common in clinical trials. Proton pumps inhibitors, inhibits the $H^+/K^+$

ATPase activity which is a gastric acid pump. $H^+/K^+$ ATPase pump is responsible for the hydrochloric acid secretion. When the pH is low, proton pump inhibitors are protonated and undergo cyclization to form a tetracyclic sulfonamide, which binds irreversibly to cysteines in α subunit of the H+/K+ATPase (Yang, Lu, & Lin, 2014). Therefore, the H+/K+ATPase activity is inhibited.

There are three different treatment regimens exists to manage with *H. pylori* infection (Papastergiou, Georgopoulos, & Karatapanis, 2014). In standard first line therapy; combination of amoxicillin, clarithromycin as an antibiotic and a proton-pump inhibitor (PPI) are applied to eradicate the *H. pylori.* This is the most widely used option of standard first line therapy. However, it is only suitable for the areas with lower than 20% incidence of resistance against the clarithromycin. When first line therapy is failed, second line therapy applied. In second line therapies, dose of amoxicillin could be increased and lexofloxacin preferentially used rather than clarithromycin. Also, it could be the combination of pump inhibitor (PPI), bismuth, tetracycline and metronidazole. Tetracycline is protein synthesis inhibitor that inhibits the translation and Metronidazole inhibits nucleic acid synthesis by disrupting DNA of microbial cells (Papastergiou, Georgopoulos, & Karatapanis, 2014). If second line therapy is failed also, third line therapy is applied. Third line therapy includes the combination of amoxicillin, sitafloxacin and PPI. Sitafloxacin is new generation broad spectrum oral fluoroquinolone that inhibits the topoisomerase II in many gram-positive, gram-negative and anaerobic bacteria (Anderson, 2008). Topoisomerase II leads to DNA fragmentation via nuclease activity.

All anti-microbial agents have side effects. Especially, antibiotics lead to death of normal microbiota of human gastrointestinal tract. Also, *H. pylori* could develop resistance against to antibiotics. Therefore, alternative therapies have been started to use for the treatment of *H. pylori* infection such as phytomedicines and probiotics (Yang, Lu, & Lin, 2014). However, these alternative methods efficiencies are not proven yet.

### 1.2.8 Immune Response to *Helicobacter pylori*

The host immune defense against to *H. pylori* is mediated with innate and adaptive response. In the primary infection, innate immunity gives quick and non-specific reaction against to bacterial pathogens. This rapid recognition of bacteria is mediated

by TLRs (Toll-like receptors) expressing on APCs (antigen-presenting cells). These antigen presenting cells are mainly; monocytes and dendritic cells. When bacteria recognized by APCs, pro-inflammatory cytokines are secreted. Cytokines are small proteins that are functional in signaling and regulate the host responses to infections, immune responses, inflammation and traumas (Dinarello, 2000). Those secreted pro-inflammatory cytokines, generally TNF-α (tumor necrosis factor-a), IL (interleukin) -1β and IL-8, act as local chemo attractant and induces the granulocyte infiltration. If innate immune response against to *H. pylori* is failed, adaptive immune response is mediated by lymphocytes; T-, B- and memory cells. (Janeway, Travers, & Walport, 2001). The adaptive immune response is highly specific to pathogenic antigens. *H. pylori* related inflammations are mediated by the combination of T cells, plasma cells and macrophages. Also, it induces the specific antibody secretion by host cells against to its antigens.

#### 1.2.8.1 The Development of T cells and T cell Types

All T cells are derived from the HSCs (haematopoietic stem cells) that are found in the bone marrow. These T cell precursors are expressed in bone marrow and then migrate to thymus and colonies at there for maturation and selection to specialized T cells. Disparateness of T cells from the other adaptive immune cells is lie on the expression of unique T cell receptor (TCR) on the surface of T cells. TCR production is dictated by thymic selection by rearrangement of TCR genes enable T cells to recognize infinite numbers of foreign antigens (Luckheeram, 2012). In thymus, T cells are isolated from the any foreign antigens and founded as naïve form. To have an active role in adaptive response, naive T cells must be turned to effector T cells. This activation is accomplished in the specialized microenvironments within secondary lymphoid tissues such as peripheral lymph nodes, Peyer's patches and tonsils. Antigen presenting cells captures antigens and presents them to naïve T cells in these microenvironments. T cells develop their specific T cell markers, including TCR, CD3, CD4 or CD8, and CD2. After activation, T cells undergo quick proliferation and migrate to the place where antigen of interest is present. Effector function of T cell could mediated by two distinct way; if T cells directly kills foreign antigen carrying cells, it named as CD8+ cytotoxic T cells, however, if T cells secrete cytokines to affect other immune cells and inflammatory mechanisms, it refers CD+4 helper T cells (Broere, 2011).

### 1.2.8.2 CD4+ Effector T Cells

$CD4^+$ T cell effector function is mediated by four main subgroups of it; T Helper 1 (Th1), T Helper 2 (Th2), T Helper 9 (Th9) and T Helper 17 (Th17). Differentiation on to these subtypes are mainly mediated by the activation signals, cytokine profiles, types of antigen presenting cells, and regulation of co-stimulatory molecules in the microenvironment (Jankovic, Kugler, & Sher, 2014).

Th1 cells are essential for the immune response against the intracellular pathogens, autoimmunity and inflammation. It is transition from naïve T cell to Th1 is mainly mediated by the activation of T-bet transcription factor. Th1 secretes cytokines interferon-γ (IFN-γ), Interleukin 2 (IL-2) and Interleukin 12 (IL-12) (Jankovic, Kugler, & Sher, 2014).

Th2 cells are produced in response to activation of GATA-3 transcription factors. Th2 lymphocytes produce IL-4, IL-5, IL-10, and IL-13 cytokines. These cytokines are responsible in eosinophil activation, and inhibition of several macrophage functions which provides phagocyte-independent immune responses. Also, Th2 cytokines are directly connected to humoral immunity, due to its cytokine secretion causes the strong antibody production by B cells (Romagnani, 1999). Especially, IL-4 secretion promotes IgE (Immunoglobulin E) production which is direct reason of allergic reactions (Wan & Flavell, 2009).

Th17 cells are more recently discovered types of T helper cells that mainly functional in host defense against especially fungal and bacterial infection. Additional Th17 has role in induction and propagation of autoimmunity. Th17 progressed from naïve T cells with the presence of Transforming Growth Factor-beta (TGF-β) and IL-6 or IL-21 (Ruan, et al., 2011). When secreted IL-6 binds to its receptor, it leads to activation of Signal Transducer and Activator of Transcription-3 (STAT3) and this activations followed with the activation of master regulator transcription factors of Th17 which are ROR-α and ROR-γt; orphan nuclear receptors. In between these two transcription factors; Orphan Nuclear Receptor gamma t (RORγt) is preferentially expressed in the thymus and it is accepted as lineage specific marker of Th17 (Ruan, et al., 2011). RORγT could be activated with co-secretion of IL-21 and TGF-Beta which is independently from IL-6 secretion. IL-21 cytokine could activate STAT3 and leads RORγT expression too (Zhang, 2018). Th17 cells secrete the 6 members of IL-17

cytokines; IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, IL-17F, and IL-21 and IL-22 (Wan & Flavell, 2009). In those cytokines, IL-17 family is highly associated with the autoimmune diseases. Also, there is a new hypothesize about T helper 17 has role in tumor immunity (Bailey, Nelson, Himes, Li, Mehrota, & Paulos, 2014). Th17 is highly sensitive to cancer cells. However, it is not proved yet.

Th9 is the subset of the CD4+T cells that progressed as a result of the action of the STAT6 transcription factors and express IL-9. It is mainly specialized in response to inflammation, allergies, and parasite clearance. Finally, Th22 is produced with the STAT3 transcription factor activation. It produces TNF alpha, IL-23, IL-22 and functional in pathogen clearance as like as Th9.

#### 1.2.8.3 Regulatory T Cell (Treg)

Regulatory T cells (Treg) are immune suppressive cells that functional in self-tolerance, immune homeostasis with Th16, tissue repair and regeneration (Li, Tan, & Lui, 2018). They are normally colonized in the lymphoid organs, but if there is any organ damage, they migrate to the injury site to regulate immune response already progressing and regenerate the damaged sites. In the case of *H. pylori* colonization in the gastric epithelium, regulatory T cells accumulate in the injury sites to balance response of host to bacteria (Kao, 2010). By this regulatory action, *H. pylori* could increase its durability in the stomach. Treg cell specific marker is Forkhead Box P-3 (FOXP-3) which is a major transcription factor of it.

### 1.3 Programmed Death 1 (PD-1)

Programmed Death 1 (PD-1) is a surface receptor that acts as immune check point protein. PD-1 Receptor is expressed on the surface of CD4+ T cells, CD8+ T cells, natural killer T cells, B cells and activated monocytes (Sharpe, Wherry, Ahmed, & Freeman, March). Normally, it is absent on resting T cells (Simon & Labarriere, 2018). Induction of PD-1 expression is maintained by TCR, BCR and TNFs. Receptor PD-1 has a monomeric structure with single Ig variable domain and cytoplasmic domain. The ligands of the PD-1 are Programmed Death-Ligand 1 (PD-L1) and Programmed Death-Ligand 2 (PD-L2). PD-L1 which is also known as B7-H1 mainly expressed on T cells, B cells, macrophages, monocytes, dentritic cells

(DCs), and non-lymphoid cells including endothelial cells, syncytiotrophoblasts in the placenta, muscle, and pancreatic islands (Wang S. &., 2004).

PD-1 and its ligand PD-L1 are negative co-stimulary molecules that regulate the balance between the stimulatory and inhibitory signals, which required for the adequate immune response against microbes and maintenance of self-tolerance (Sharpe, Wherry, Ahmed, & Freeman, March). Individually, PD-1 has importance on progression of T cells during thymocyte maturation. Initially, precursors of effector T cells, are expressed PD-1 in adequate level. Expression level increase when TCR ligated to CD4+CD8+ double-positive thymocytes for controlling the TCR signaling thresholds on T cells (Sharpe, Wherry, Ahmed, & Freeman, March). After the activation of T cell; engagement of PD-1 with its ligand PD-L1 has potential to lead T cell apoptosis, anergy, exhaustion, IL-10 expression. PD-L2 also has same suppressive signal effect on T cells; however, it is not critical as much as the PD-L1 because of the relatively rare expression (Zou, Wolchok, & Chen, 2016).

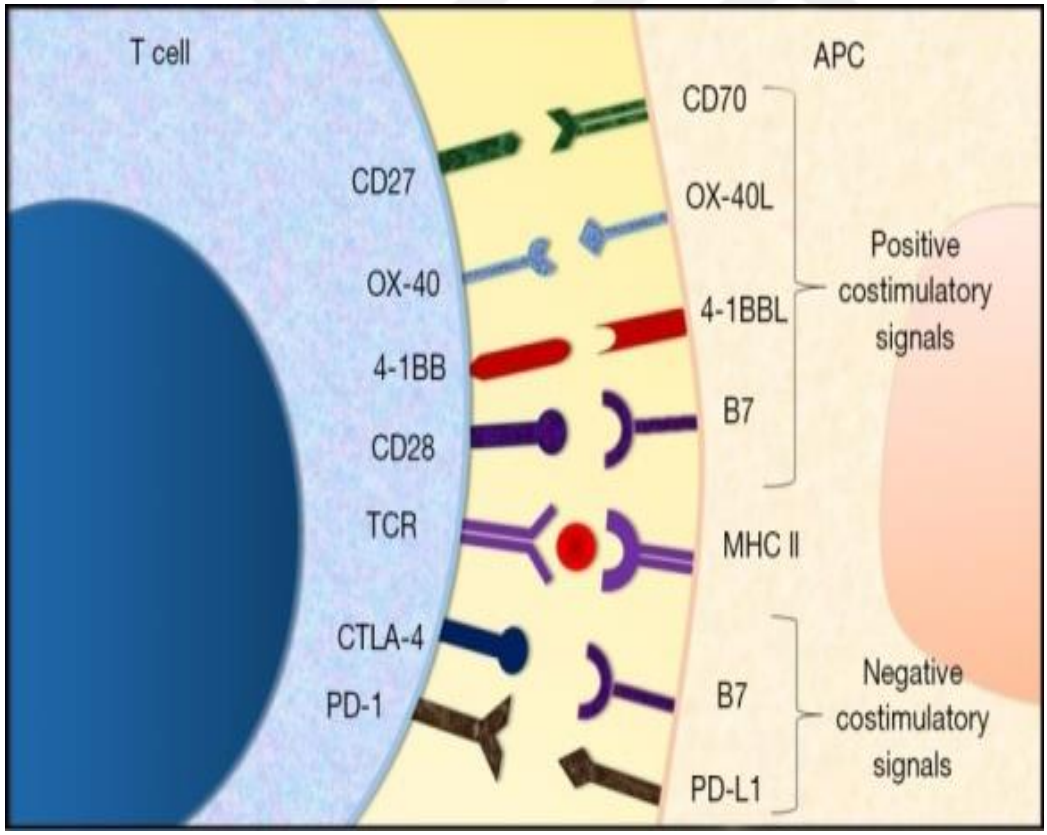


**Figure 1.4 :** Description of costimulatory signals between T cell and antigen presenting cells **(Chae, 2017)**.

PD-1 has direct effect on interaction with infectious disease and cancers. First of all, many infectious microorganisms used the PD-1-PD-L1 interaction pathway to escape the immune effector response. In the initial phase of viral infection, PD-1 expression on $CD8^{+}$Tcells increase. Increase of PD-1 and its binding to PD-L1 negatively regulates the activity of effector T cells against the viruses and it increases the durability of infection on host. If infection treated clinically, PD-1 expression lowered, if not it remains high. Additionally, cancer cells evaluate a mechanism to break away the immune response based on the PD-1 and PD-L1 interaction. There is elevated level of PD-1 expression observed in the patients with different cancer types (Zou, Wolchok, & Chen, 2016). Based on suppression of T cell activity by PD-1 and its ligand PD-L1, cancer cells mimics the PD-L1 (Wu, et al., 2017). Especially, malign cancer cells are becoming able to synthesize PD-L1. By this mechanism, cancer cells could bind the active T cells and suppress its function. Immunohistochemical analysis already shows the PD-L1 expressing ability of tumor cells and tumor microenvironments.

### 1.4 Precancerous Lesions

Gastric Cancer is forth most common diagnosed malignant disease at all around the world (Kim & Oh, 2018). On the other hand, because of the subclinical progression feature of disease, it is diagnosed mostly in later stages. This high frequency and late diagnose end with the high mortality rate. Gastric cancer is second leading cause of cancer related deaths all around the world (Kim & Oh, 2018). According to Lauren classification, gastric cancer can be divided into two subtypes; intestinal type adenocarcinoma and diffuse type adenocarcinoma (Woude, et al., 2003). These two histological type shows differences in prognosis and prevalence (Mabogunje, Subbuswamy, & Lawrie, 1978). Only for intestinal type, we could refer histological precancerous lesion progression order from normal stomach to gastric cancer. This stepwise order is; chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and intestinal type adenocarcinoma (Woude, et al., 2003). Risk of disease progression from one step to later step is given around 2% to 3% percent (Sipponen & Maaroos, 2015).

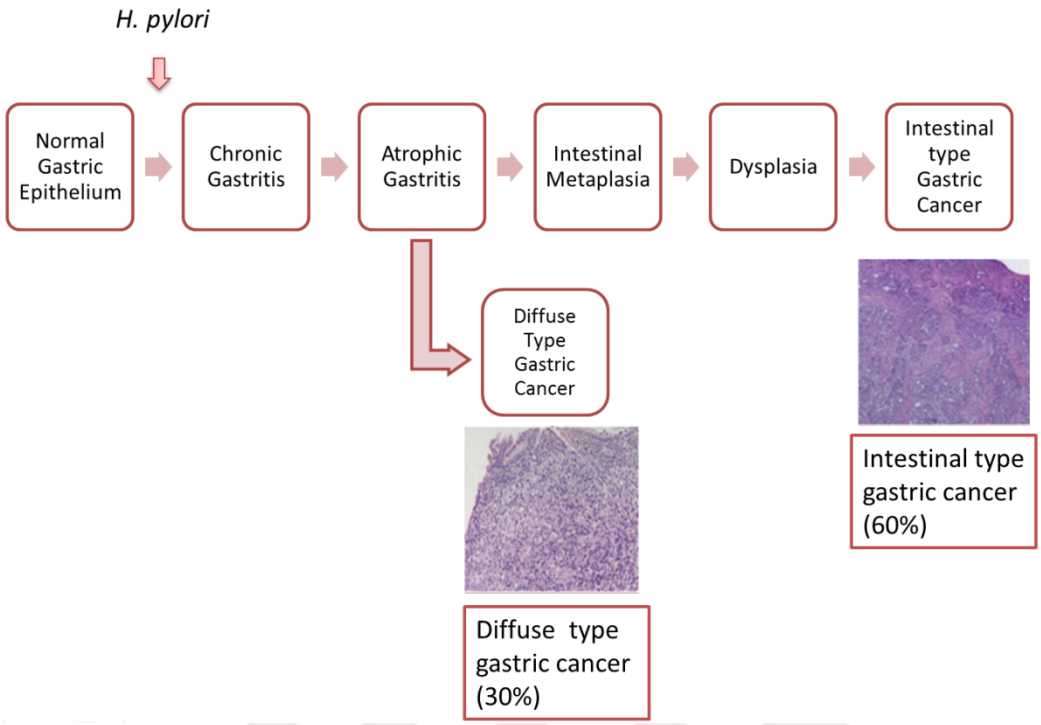


**Figure 1.5 :** Stepwise developmental mechanism of gastric adenocarcinoma (Conteduca, et al., 2012).

### 1.4.1 Chronic Gastritis

Chronic gastritis is a lifelong chronic disease that generally begins with childhood *H. pylori* infection. After infection, *H. pylori* could adapt the acidic environmental conditions of human stomach and could overcome the body immune response. *H. pylori* adhesion into host gastric epithelium causes the recruitment of B, T lymphocytes, macrophages, plasma cells and neutrophils (Conteduca, Sansonno, Lauletta, Russi, Ingravallo, & Dammacco, 2012). This multistep, progressive chronic disease coexists with active neutrophilic inflammation (Sipponen & Maaroos, 2015). This inflammation level and progression of disease is generally determined by cytotoxicity of the bacterium. When the cytotoxicity of bacteria strain increases, risk for progression to atrophic gastritis is increased. Due to the fact that there is only inflammation without changing the physiology of human stomach in chronic gastritis, eradication of bacterium in this step is one of the most important step to prevent Gastric Cancer.

### 1.4.2 Atrophic Gastritis

The effect of many environmental and epigenetic factors could progress the chronic gastritis to atrophic gastritis and other precancerous lesions. Around 50% of patients who diagnosed with *H. pylori* infected chronic gastritis, may develop atrophic gastritis (Sipponen & Maaroos, 2015). However, development of disease is slow process may takes lifelong period. Continuing inflammation already causes the superficial changes in host stomach epithelium, in atrophic gastritis, these changes extend deeper. Inflammation could infiltrate around the tubule thereby, loss of normal gastric glands is histologically observed (Correa, 1988). Based on the quantification of number of glands losses, atrophic gastritis classified three subgroup; mild, moderate and severe. When there is only one or two glands loss, it is mild atrophy. If all glands are damaged histologically, it called as severe atrophy. All forms in between these two are referred as moderate atrophy (Whitehead, Truelove, & Gear, 1972).

### 1.4.3 Intestinal Metaplasia

Intestinal metaplasia is replacement of gastric columnar epithelial cells with the cells which has an intestinal morphology (Conteduca, Sansonno, Lauletta, Russi, Ingravallo, & Dammacco, 2012). Gastric intestinal metaplasia has histological two subgroups; incomplete and complete. There is colonic epithelium cells with variable sized irregular mucin droplets in cytoplasm of incomplete intestinal metaplasia patients (Tang, Wu, & Bhaijee, 2012). On the other hand, complete intestinal metaplasia includes small intestinal epithelium with eosinophilic enterocytes, goblet cells and variable Paneth cells (Tang, Wu, & Bhaijee, 2012). *H. pylori* infection mostly gives rise to complete type intestinal metaplasia. One person could develop either complete type or incomplete type intestinal metaplasia or both at the same time. Intestinal metaplasia is highly associated with intestinal type gastric cancer. Risk of intestinal type gastric cancer development from intestinal metaplasia is around 0.25% percent (Jencks, Adam, Borum, Koh, Stephen, & Doman, 2018).

### 1.4.4 Gastric Dysplasia

Gastric dysplasia is the term that refers to intraepithelial neoplasia in the human gastric which is the high risk marker for gastric cancer (Appelman, 2005). In general definition, neoplasia is the abnormal growth of normal cells or tissues. In gastric

epithelial dysplasia, unequivocal neoplasm is shown (Baek, et al., 2015). Therefore in dysplasia, histological changes could be observable under microscops. These observable changes are; variation in epithelial cell sizes, shapes and orientations, enlargement of nucleus (Conteduca, Sansonno, Lauletta, Russi, Ingravallo, & Dammacco, 2012). Also, distortions of glands are changed. Gastric epithelial dysplasia has two subtypes; intestinal type and foveolar type. Both types are currently associated with adenocarcinoma. Although the prevalence of dysplasia in gastric epithelium is directly associated with the prevalence of *Helicobacter pylori*, at this point eradication of bacterium is not sovereign treatment for dysplasia (Baek, et al., 2015). There is no current treatment implemented specifically for dysplasia.

### 1.4.5 Intestinal Type Gastric Cancer

*H. pylori;* give rise to two types of cancer; adenocarcinoma and lymphoma. Lymphomas are very rare but, adenocarcinoma is one of the most diagnosed types of cancer at the world. Adenocarcinoma has two subtypes; diffuse type adenocarcinoma and intestinal type adenocarcinoma. For diffuse type adenocarcinoma, no finding to prove any histological progression order is followed by disease. Only 30% of adenocarcinomas are diffuse type. The rest of 70% of adenocarcinomas are intestinal type (Conteduca, Sansonno, Lauletta, Russi, Ingravallo, & Dammacco, 2012). For intestinal type, we could refer a stepwise development mechanism; normal, atrophic gastirits, intestinal metaplasia, dysplasia and cancer. Due to this stepwise development, intestinal type gastric cancer has all those precancerous lesions features. It has neoplastic epithelium cells as in dysplasia; mimics small intestine mucosa likes intestinal metaplasia (Leivo, 2017).

Based on histopathological observations, intestinal type adenocarcinoma has five subgroups; papillary, colonic, solid, mucinous, and mixed subtypes (Leivo, 2017). That much subtype indicates that, it is a heterogeneous type of cancer which coexists with many genetic mutations. Therefore, acquired prognosis and treatment is still not available. This is the reason why disease related mortality rate is still high.

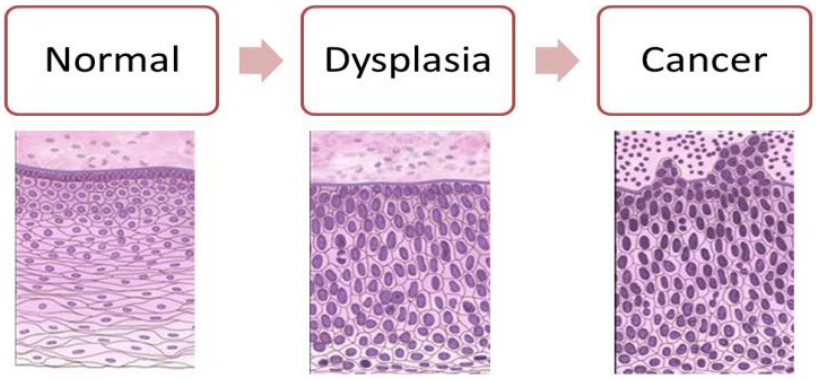


**Figure 1.6 :** Histopathological features of normal, dysplastic and cancerous cells in gastric epithelium (National Cancer Institute).

## 1.5 Hypothesis

*Helicobacter pylori (H. pylori)* is infected more than half of the world's human population and highly associated with upper gastrointestinal tract diseases; gastritis, peptic ulcer, gastric carcinoma and MALT. Especially, for developing carcinoma, *H. pylori* accepted as primary carcinogen. These pathogenicity of *H. pylori* is directly associated with the virulence factors of bacterium. Virulence factors and chronic gastirtis relation is already known. Important virulence factors that might be correlated with the progression of precancerous lesions are; cytotoxin associated geneA *(cagA)*, vacuolating cytotoxinA (*vacA*), outer inflammatory proteinA (oipA), blood group antigen-binding adhesion (babA), putative neuraminyllactose-binding hemaggluttinin homologA (*hpaA*), neutrophil activating proteinA (napA), duodenal ulcer promoting geneA (*dupA*), urease subunit A (*ureA)* and urease subunitB (*ureB*). Host adaptive immune response has important role in this disease progression. Especially, $CD4^+$ T cell response (Th1, Th17 and Treg) increased in *H.pylori* infected patients. PD-1 and its ligand PD-L1 are negative co-stimulary molecules that regulates the immune responses against the bacterial pathogens. The influence of virulence factors on CD4+ T cells; Th17 and PD-1 have not been elucidated in detail.

In this study, firstly, we aimed to characterize status of *H. pylori* and nine major *H. pylori* specific virulence factors; *CagA, VacA, OipA, BabA, HpaA, NapA, DupA, UreA, UreB,* in precancerous lesions and gastric cancer. Second aim of this study was determination of *H. pylori* related immune responses of Th17 cells with detailed examination of RORγT, IL-17 and PD-1 progression in *H. pylori* infected patients.

Final aim of this study was understanding the mechanism of PD-1 and RORγT, IL-17 expression and progression mechanism in development of precancerous lesions to intestinal type gastric adenocarcinoma.

# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Collecting Gastric Biopsy and Resection Specimens

All fresh gastric biospy and resection specimens are collecting into the DNase-RNase free collection tubes which fullfilled with Ambion, RNAlater® RNA Stabilization Solution. Ambion RNAlater was stored in room temperature.Reagent for RNA stabilization and supplier companies are given in Table 2.1.

**Table 2.1. :** Reagent for collecting tissue specimens and supplier company.

| | Stabilization Solution | Volume | Company |
|---|---|---|---|
| Endoscopic Biopsy Specimens | RNAlater® | 250 µL/each | Ambion |
| Resection Biopsy Specimens | RNAlater® | 500 µL/each | Ambion |

### 2.1.2 *Helicobacter pylori*

*Helicobacter pylori* G27 strain was kindly provided by Dr. Sinem Öktem Okullu from Acıbadem University.

### 2.1.3 Equipments and materials

Laboratory equipments and materials that were used in this study are listed with their supplier companies in Table 2.2 and Table 2.3, respectively.

**Table 2.2 :** General laboratory equipments used in this study

| Equipment | Company |
|---|---|
| Laminar Air Flow Cabinets | FASTER BH-EN 2003 |
| Pipettes | 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl Socorex |
| Electronic pipette | CappAid |
| Centrifuges | Centrifuge Scanspeed 1730 R<br>Labogene Scanspeed mini |
| Vortex | Mixer Uzusio VTX-3000L,LMS |
| Nanodrop 2000 Spectrometer | ThermoScientific |
| Step One Plus Real Time PCR | Applied Biosystem |
| Speed Mill Plus Homogenization System | Analytic Jena |
| Basic Gel Electrophoresis | BioRad Powerpac |
| T100 Thermal Cycler | BioRad |
| Chemidoc MP Imaging System | BioRad |
| Tissue-Tek VIP 6 AI Vacuum Infiltration Processor | Sakura |
| Shandon Histocentre 3 | ThermoFisher Scientific |
| COP 30 Cooling Plate | Medite |
| Water Bath | Denmark LTD |
| Incubator | Nüve FN400 |
| Finesse Me+ Microtome | ThermoScientific |
| Tissue Tek Film System | Sakura |
| Weight | Precisa |
| Refrigerators | Altus (+4 0C)<br>Siemens (-20 0C)<br>Panasonic (-80 0C) |

**Table 2.2 (continued) :** General laboratory equipments used in this study

| Equipment | Company |
|---|---|
| Ice Machine | Scotsman AF10 |
| Panoramic Scanner Flash III (P250) | 3D Histech |

**Table 2.3 :** General laboratory materials used in this study

| Material | Company |
|---|---|
| Gloves | Tenty |
| Erlenmeyers | Isolab |
| Falcon Tubes (15mL, 50 mL) | Isolab |
| Eppendorf tubes (0,6 ml, 1,5 ml, 2 ml) | Interlab |
| Slide | Interlab |
| CoverSlip | Interlab |
| MicroAmp® Fast Optical 96-Well Reaction Plate, 0.1 mL | Thermo Fischer |
| FG-Optical Adhesive Coverslip | Thermo Fischer |
| Serological pipettes | Sarstedt |

### 2.1.4 General Chemicals

General chemicals that were used in this study are listed with their supplier companies in Table 2.4.

**Table 2.4 :** General chemicals used in this study

| Chemical | Company |
|---|---|
| Ethanol | Merck |
| β-Mercaptoethanol | Sigma-Aldrich |
| Isopropanol | Sigma-Aldrich |
| Agarose | Sigma-Aldrich |

**Table 2.4 (continued) :** General chemicals used in this study

| Chemical | Company |
|---|---|
| Formaldehyde | Sigma-Aldrich |
| Xylene | Merck |
| Paraffin | Sigma-Aldrich |
| TAE Buffer | ThermoFischer |
| Hematoxylin and Eosin | Richard-Allan Scientific™ |

### 2.1.5 Primers

Primers that were used in this study and their properties were given in the table 2.5 and Table 2.6 respectively.

**Table 2.5 :** Conventional and multiplex PCR primers that used in this study

| DNA region(s) | Primer Name | Sequences (5′→3) PCR amplified | Product Size (bp) |
|---|---|---|---|
| *ure A* | ure A-F<br>ure A-R | TGA TGG GAC CAA CTC GTA ACC GT<br>CGC AAT GTC TAA GCG TTT GCC GAA | 244 |
| *ure B* | ure B-F<br>ure B-R | AGT AGC CCG GTG AAC ACA ACA TCCT<br>ATG CCT TTG TCA TAA GCC GCT TGG | 645 |
| *cag A* | Cag A-F<br>Cag A-R | AGAGCAAGCGTTAGCCGATCTCAA<br>TTTCCCTACACCACCCAAACCACT | 415 |
| *hpa A* | hpa A-F<br>hpa A-R | TAG TGG GAT GCA GCC CGC ATA TTA<br>CGC TAT GGC TTG AAT GGG TGG TTT | 534 |
| oip A | Oip A-F<br>Oip A-R | GTT TTT GAT GCA TGG GAT TT<br>GTG CAT CTC TTA TGG CTT T | 401 |
| bab A | Bab A-F<br>Bab A-R | AAT CCA AAA AGG AGA AAA AGT ATG AAA<br>TGT TAG TGA TTT CGG TGT AGG ACA | 832/601 |
| *dup A* | dupA-F<br>dupA-R | TGA GCG TGG TAG CTC TTG AC<br>GAG CGC GTT AGC GAT ATA GG | 584 |
| nap A | napA-F<br>napA-R | GAA TGT GAA AGG CAC CGA TT<br>ATC GTC CGC ATA AGT TAC GG | 304 |
| *vac A s1/s2* | vac A s1/s2-F / vac A s1/s2-R | ATG GAA ATA CAA CAA ACA CAC<br>CTG CTT GAA TGC GCC AAA C | 259/286 |
| *vac A m1/m2* | vac A m1/m2-F /vac A m1/m2-R | CAA TCT GTC CAA TCA AGC GAG<br>GCG TCTVAAA TAA TTC CAA GG | 567/642 |

**Table 2.6 :** Quantitative RT-PCR primers that used in this study.

| Primer Name | Sequences (5′→3) PCR amplified | Product Size (bp) |
|---|---|---|
| IL-17-F | CCTGGGAAGACCTCATTGGT | 219 |
| IL-17-R | ATTCCAAGGTGAGGTGGATCG | |
| RORγt-F | CTGCAAAGAAGACCCACACC | 170 |
| RORγt-R | GCAGTTCTGCTGACGGGT | |
| 18s rRNA-F | GGCCCTGTAATTGGAATGAGTC | 146 |
| 18s rRNA-R | CCAAGATCCAACTACGAGCTT | |
| hPD-1 F | CAGTTCCAAACCCTGGTGGT | 115 |
| hPD-1 R | TGGCTCCTATTGTCCCTCGT | |

### 2.1.6 Commercial Kits

Commercial kits that were used in this study are listed with their supplier companies in Table 2.7.

**Table 2.7 :** Commercial Kits used in this study

| Kit | Company |
|---|---|
| quick-DNA miniprep isolation kit | ZYMORESERACH |
| Nucleospin RNA | Macherey-Nagel |
| High Capacity cDNA Reverse Transcription Kit | Thermo Fischer |
| Taq DNA Polymerase with ThermoPol Buffer | Biolabs |
| Power SYBR® Green PCR Master Mix | Applied Biosystems |

## 2.2 Methods

### 2.2.1 Patient Selection

Gastric biopsy specimens were obtained from the patients who underwent endoscopy at the Gastroentorology Department of Acibadem Hospital Groups, in Istanbul, Turkey. Resection specimens were obtained from patients who undergo total or sub-resection of stomach due to the gastric cancer complaints at General Surgery Department of Acibadem Hospital Groups, in Istanbul, Turkey. In total 60 patients

were selected and categorized based on the histological diagnosis at pathology reports. 15 patients has normal stomach pathology, 16 patients with *H. pylori* positive active chronic gastritis, 4 patients with *H. pylori* negative active chronic gastritis, 5 patients with *H. pylori* positive intestinal metaplasia, 4 patients with *H. pylori* negative intestinal metaplasia, 3 patients with *H. pylori* positive inactive chronic gastritis and 14 intestinal type adenocarcinoma patient selected who fulfilled the following criteria for obtaining expulsion: older 18 years or under 80 years old patients are selected. Whole participants are informed about the study by providing written informed constent; "Patient Information Form" and this study is approved by the ethical committee of Acıbadem University and Istanbul Technical University.

### 2.2.2 Gastric Biopsy and Resection Specimens

Three gastric biopsy specimens were taken from the antrum of the stomach of the patients during endoscopy and resection, one for DNA, one for RNA isolation and one for hematoxylin and eosin staining. Resection biopsy specimens are collected after the determination of tumor sites by macroscobic examinations. Datas verified by hematoxylin and eosin staining and histological diagnosis. Fresh biopsy and resection specimens were taken into a preservative solution (Ambion, RNAlater® RNA Stabilization Solution), and were keping overnight at + 4°C, and then placed at – 80°C until the isolation process.

### 2.2.3 DNA Extraction from Gastric Biopsies

To study virulence factors of *Helicobacter pylori (H. pylori)*, DNA was extracted by using a DNA isolation kit (quick-DNA miniprep isolation kit, ZYMORESERACH). Firstly, 200 µL Genomic Lysis buffer was added to tissue specimens and incubated 10 minutes at room temperature. Supernatants were transferred to Zymo-Spin™ IC Columns and centrifugated for 1 minute at 10.000g. Then, Zymo-Spin™ IC Columns were transferred into the new collection tubes and 200 µL DNA Pre-Wash Buffer added to the spin column and centrifugated for 1 minute at 10.000g. After that, 500 µL g-DNA wash buffer was added to the spin column and centrifugated for 1 minute at 10.000g. Then, spin columns were transffered into the new microcentrifuge tubes and 17 µL DNA Elution buffer was added and incubated 5 minutes at room temperature. Finally, centrifugated at maximum speed for 1 minute. Eluted DNA was stored at – 80 °C deep freezer for later usages. All DNA samples quantification were

completed by NanoDrop Spectrometer (ND-2000, Thermoscientific) and DNA integrities were examined by gel electrophoresis.

### 2.2.4 RNA Extraction from Gastric Biopsies

To study mRNA expression levels of PD-1, IL-17, RORγt, RNA was isolated from RNA-Later stabilized human precancerous lesions and gastric cancer patients tissue specimens. For RNA isolation Macherey-Nagel Nucleospin RNA isolation kit used. Firstly 350 µL RA1 Buffer and 3.5 µL s-mercaptoethanol was added directly and tubes were vigorously vortexed. For homogenization of biopsy and resection specimens homogenization system (SpeedMill PLUS, Analytikjena) was used. After that for filtration of lysate and reducing the viscosity, Nucleospin Filter® was placed onto a Collection Tube (2 mL) and mixture applied to filter and centrifuged for 1 minutes at 11.000g. Then, filter removed from the collection tubes and 350 µL ethanol was added and pipetted up and down for 5-6 times. Lysate was loaded into NucleoSpin® RNA Column and centrifugated 30s at 11.000g. For desalting the silica membrane, 350 µL Membrane Desalting Buffer (MDB) was loaded onto the lysate and centrifugated 1 minutes at 11.000g. 95 uL DNase reaction was applied directly onto the center of the silica membrane of the column and incubated 15 minutes at room temperature. Membrane Desalting Buffer increases the efficiency of rDNase. For washing of the membrane 200 µL of RAW2 Buffer added to the NucleoSpin® RNA Column and centrifugated 30s at 11.000g. By this rDNase were inactivated. Flow through was removed, then, membrane were washed second time with 600 µL RA3 buffer and centrifugated at 11.000g for 30 seconds. Then, for final washing step, flow through of second wash was removed and 250 µL of RA3 buffer was added and centrifugated for 2 minutes at 11.000g to dried membrane completely. Finally, each column were placed onto Nuclease-free Collection tubes and 35 µL RNase-free $H_2O$ added for elution. Centrifugate the samples at 11.00 g for 1 minutes. All eluted RNA samples were kept in-80°C deep freezer and RNA samples quantification happened by using NanoDrop Spectrometer (ND-2000, Thermoscientific).

### 2.2.5 cDNA Synthesis

For synthesis of cDNAs from RNA, High Capacity cDNA Reverse Transcription Kit, Applied Biosystems (Thermoscientific) was used. By following the manifacturers

insturaction 2x master mix was prepared and in total 1 µg of RNA was used. The colume of the components and the conditions of the thermal cycler for cDNA synthesis reactions were given at Table 2.8 and Table 2.9 respectively.

**Table 2.8 :** The amount of the cDNA synthesis assay components

| Component | Amount |
|---|---|
| 10X RT Buffer | 2 µl |
| RT dNTP (10 µM) | 0,8 µl |
| 10X RT Random Primers | 2 µl |
| Reverse Transcriptase | 1 µl |
| RNase Inhibitor(Riboblock) | 1 µl |
| Nuclease-free H2O | 4,07 µl |
| RNA | Depending on RNA concentration |

**Table 2.9 :** Thermal cycler conditions for cDNA synthesis

| | Step 1 | Step 2 | Step 3 | Step 4 |
|---|---|---|---|---|
| Temperature(°C) | 25°C | 37°C | 85°C | 4°C |
| Time | 10 min | 120 min | 5 min | ∞ |

### 2.2.6 Conventional PCR and Multiplex PCR Assay

Primers that used in this study were obtained from Assist. Prof. Sinem Öktem Okullu, Acıbadem University. *H. pylori* G27 strain genomic DNA is used as positive control template for conventional PCR and multiplex PCR assay for virulence factors characterization. Reaction is progressed in total volume of 25µl by using Biolab PCR Kit. Reaction amounts and conditions are given in Table 2.10, Table 2.11 respectively.

**Table 2.10 :** The amount of the conventional PCR assay components

| Component | Amount |
|---|---|
| 10X DreamTaq Buffer (includes 20 mM MgCl2), | 2,5 µl |
| dNTP (200 µM) | 2,0 µl |
| Frw Primer (20 µM) | 1 µl |

**Table 2.10 (continued) :** The amount of the conventional PCR assay components

| Component | Amount |
|---|---|
| Rev Primer (20 µM) | 1 µl |
| Dream Taq DNA polymerase (0,65 U) | 0,69 µl |
| PCR Grade water | 18,81 µl |

**Table 2.11 :** Thermal cycler conditions for cDNA synthesis

| | Initial Denaturation | 45 cycles | | | Final Extension | Hold |
|---|---|---|---|---|---|---|
| Temperature(°C) | 95°C | 95°C | 60°C | 72°C | 72°C | 4°C |
| Time | 3 min | 45 sec. | 45 sec. | 2 min | 5 min | ∞ |

### 2.2.7 Real-Time PCR Assay

For quantitative determination of mRNA expression levels of PD-1, IL-17 and RORγt, quantitative real time PCR (qRT-PCR) was performed. Optimum annealing temperatures of primers were determined by gradient conventional PCR assay. qRT-PCR was performed by using SYBR® Green PCR Master Mix and amplified in Step One Plus RT PCR System. All quantitative Real Time PCR experiments were carried out in dublicate with the total reaction volume of 10 µl. Each assay was carried with one dublicated negative control without cDNA. Amplification efficiencies were optimized for each primer pair by using serial dilutions of cDNA. Relative gene expressions were calculated by using standard curves equations. qRT-PCR gene expression results were normalized by using internal control 18s rRNA housekeeping gene. PCR conditions and the amounts of components are given in Table 2.12 and Table 2.13 in respectively.

**Table 2.12 :** The amount of the qRT-PCR assay components

| Component | Amount |
|---|---|
| Power Syber Green Master Mix (2x) | 5 µl |

**Table 2.12 (continued) :** The amount of the qRT-PCR assay components

| Component | Amount |
|---|---|
| Frw Primer (10 µM) | 0.5 µl |
| Rev Primer (10 µM) | 0.5 µl |
| PCR Grade water | 1.5 µl |
| cDNA | 2.5 µl |

**Table 2.13 :** Reaction conditions for qRT-PCR reaction

| Step | Temperature | Time |
|---|---|---|
| Holding Stage | 95°C | 10 minutes |
| Cycling Stage (40 Cycles) | 95°C | 15 seconds |
| | Depending on primers | 30 seconds |
| | 68°C | 1 minutes |
| Melt Curve Stage | 95°C | 15 seconds |
| | 60°C | 1 minutes |
| | 95°C | 15 seconds |
| Holding | 72°C | 5 minutes |

### 2.2.8 Immunohistochemistry (IHC)

The Hematoxylin and Eosin (H&E) immunohistochemical staining experiments were performing in the Acıbadem Maslak Hospital; Pathology Laboratories for examination, histologically diagnosis of gastric tissues and microscopic visualization of *H. pylori*. After the macroscopic examination and dissection of tissue specimens were completed, tissue processing was performing by using Tissue-Tek VIP 6 AI Vacuum Infiltration Processor System. In this system, gastric tissue specimens were firstly fixed with formalin 2 times for 30 seconds. Then, for dehydration, samples were washed with increased concentration of ethanol; 70%, 85%, 90%, 96% respectively. Increased concentration of ethanol ensures the total dehydration and clearence of tissue specimens. Next, 3 times for 40 seconds, samples were placed into the xylene. By this steps, tissue processing proceddures were completed.

Afterward, for final fixation of specimens, tissues were embedded into the paraffin. Embedding proceddures were performing at 63 $^{o}$C for being sure paraffin was in liquid phase. Immediately after, tissues with in paraffins were freezed by cooling plate at -11$^{o}$C by this way paraffin blocks obtained. Then these blocks were cutted at slice which 2 μm thickness by using Finesse Me+ Microtome. Next, freshly cutted gastric tissue sections placed in water bath at 45 ºC and mounted onto the positively charged glass slides. By these methods, tissue sections could be preserved without any degradation till the next staining experiment.

For hematoxylin and eosin staining, formalin fixation steps followed by backwards. Slides heated in etuve at 80 $^{o}$C for half an hour as a drying step. Next, Tissue Tek Film System (SAKURA) used for staining procedure. This staining procedure steps are given at Table 2.14.

**Table 2.14 :** Hematoxylin and eosin stainig staining procedure steps, solutions and steps

| STEP | SOLUTION | TIME |
|---|---|---|
| 1 | Xylene | 0:03:00 |
| 2 | Xylene | 0:03:00 |
| 3 | 100% Ethanol | 0:01:00 |
| 4 | 80% Ethanol | 0:02:00 |
| 5 | 70% Ethanol | 0:02:00 |
| 6 | Washing | 0:01:30 |
| 7 | Hematoxylin | 0:08:00 |
| 8 | Washing | 0:02:00 |
| 9 | Acid Ethanol | 0:00:30 |
| 10 | Washing | 0:02:00 |
| 11 | Lithium | 0:03:00 |
| 12 | Wash Station | 0:01:30 |
| 13 | 75% Ethanol | 0:01:00 |

**Table 2.14 (Continued) :** Hematoxylin and eosin stainig staining procedure steps, solutions and steps

| STEP | SOLUTION | TIME |
|---|---|---|
| 14 | Eosin | 0:01:30 |
| 15 | 50% Ethanol | 0:00:50 |
| 16 | 50% Ethanol | 0:01:00 |
| 17 | 25% Ethanol | 0:02:00 |
| 18 | Drying Station | 0:10:00 |
| 19 | End Station | -: --: -- |

Finally, hematoxylin and eosin staining slides were scanned by Panaromic Scanner Flash III(P250) System. For detailed visualization, 40X-extended magnification objectives were used and images were taken by using Case Viewer Programme.

# 3. RESULTS

## 3.1 Detection of *Helicobacter pylori* Virulence Factors by conventional PCR

To detect which virulence factors are carried in genome of *Helicobacter pylori* (*H.* pylori) strain that infected our patients with gastric pathologies, 19 clinically *H. pylori* positive diagnosed patients with 4 subgroups of precancerous lesions are observed for each of 10 virulence factors. We used *Helicobacter pylori* G27 strain as a positive control for this assay.

First of all, to determine the status of *H. pylori* in gastric biopsy specimens of patients, we examined the *ureA* and *ureB* by PCR assays. We examine 60 patients for the existence of *ureA* and *ureB.* According to pathology reports of those patients, 23 of them were infected with *H. pylori;* 3 of them were suffered from inactive chronic gastric pathology, 16 of them were clinically diagnosed as active chronic gastiritis and 5 of them were suffered from intestinal metaplasia. Clinical pathology reports were prepared based on the histopathological stainings of gastric biopsy specimens of patients. Based on our conventional PCR assay results, in total 21 of 60 patients were detected as *H. pylori* infected. One active chronic gastritis patient who was diagnosed as *H. pylori* negative in clinically, detected as bacterium positive. That patient was carrying *ureA* virulence factor but negative for *ureB.* We found that all of 3 inactive chronic gastritis diagnosed patients were not infected with *H. pylori*. In total, 10 of 21 *H. pylori* positive patients were carrying only *ureA,* 2 of them are carrying only *ureB* and 9 of them carrying both of them.

To analyze the accuracy of conventional-urease PCR, we compare the results with histopathological stainings (Table 3.1). We calculate the Cohen's kappa (κ) coefficient value. This coefficient value is measuring the inter-rater agreement of different qualitative factors. When cohen's kappa coefficient value is equal to 1, it means a complete agreement of two compared groups. If Cohen's kappa coefficient is in between " κ=0.00-0.20", it means slight agreement; "κ = 0.21-0.40" fair

agreement; “κ = 0.41-0.60” moderate agreement; “κ=0.61-0.80” substantial agreement; and; “κ=0.81-1.00” almost perfect agreement. We calculated the Cohen’s kappa coefficient as, κ=0.84 which means almost perfect agreement in between the conventional urease PCR assay and histopathological stainings (Table 3.1).

**Table 3.1 :** Comprasion of conventional urease PCR assay and histopathological staining results. Cohen’s kappa coefficent was used to determine the inter-rater agreement.

| Urease PCR vs Histopathological Staining | | |
|---|---|---|
| | Urease PCR | Histopathological Staining |
| Present | 21 | 23 |
| Absent | 39 | 37 |

κ=0.84 (“almost perfect” agreement)

Next, to detect the distribution of virulence factors with in these 21 *ureA* or *ureB* positive patients based on classifiying their clinical outcomes, we examined each virulence factor individually (Figure 3.1). Most abundant virulence factor gene for both active chronic gastritis and intestinal metaplasia patients were *ureA*. 88% of active chronic patients and 100% of intestinal metaplasia patient were carrying *ureA.* For active chronic gastritis patients next most abundant was *napA* gene, 81% of active chronic gastritis patients were positive. Less abundant virulence factor for both pathogenesis was *oipA*. In total only 3 patients were carrying *oipA* positive bacterium strain and all of them were diagnosed as active chronic gastritis. There were not any intestinal metaplasia patients detected as *hpaA* negative (Table 3.2). Additionally, 80% of intestinal metaplasia patients were infected by *H. pylori* strain that carry genome for *vacA s1/s2* virulence factor.

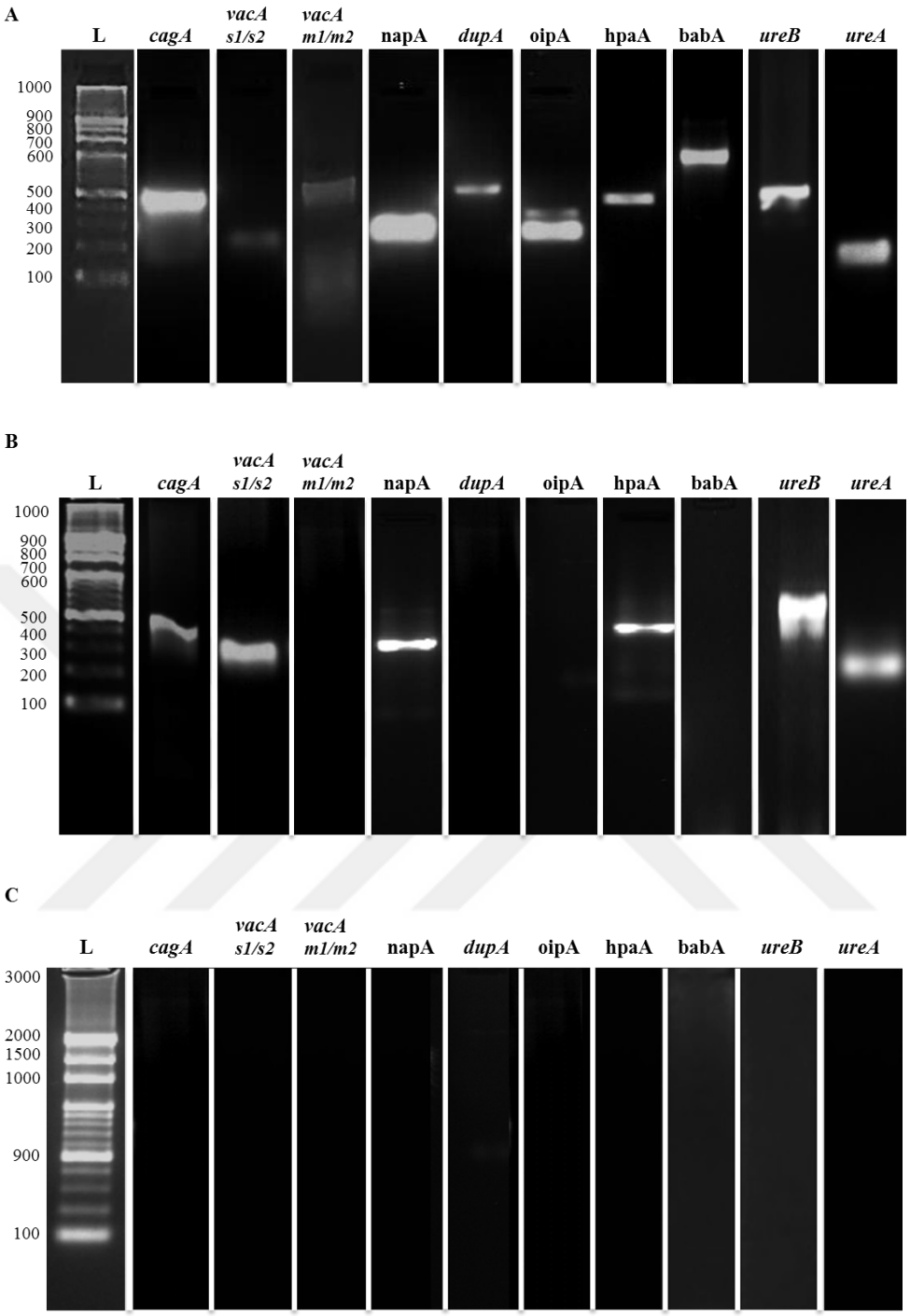


**Figure 3.1 : Analysis of virulence factor genes status in patients with gastric malignancies.** Virulence factors conventional PCR assay results were run on %2 Agarose gel. *cagA* (415bp); *vacS1/S2* (259-289bp); *vacm1/m2*(567-642bp); napA (384bp); *dupA* (584bp); oipA (401bp); hpaA (534bp); babA(832bp); *ureB* (645bp); *ureA* (244bp). A) Representative PCR data for amplification *H. pylori* virulence genes in positive control *H. pylori* G27 strain. Lane L, 1000 bp- marker (ThermoSCIENTIFIC, Gene Ruler) B) Representative PCR data for amplification *H. pylori* virulence genes in intestinal metaplasia patient. Lane L, 1000 bp- marker (ThermoSCIENTIFIC, Gene Ruler) C) Representative PCR data for amplification *H. pylori* virulence genes in uninfected patient. Lane L, 3000 bp- marker (ThermoSCIENTIFIC, Gene Ruler).

No significant difference was detected for these 10 virulence factors in between patients with active chronic gastritis and intestinal metaplasia (Table 3.2). This statistical significance analysis were calculated by two-sided Fisher's exact test.

**Table 3.2 :** Comprasion of distrubiton of virulence factor genes of *H. pylori* strains from the patients who were clinically diagnosed as active chronic gastiritis and intestinal metaplasia.

| | | Strain Type | | | | |
|---|---|---|---|---|---|---|
| | | Active Chronic Gastiritis | | Intestinal Metaplasia | | |
| Virulence Factors | | n | % | n | % | P-value |
| *ureA* | Absent | 2 | 12 | 0 | 0 | 1,000 |
| | Present | 14 | 88 | 5 | 100 | |
| *ureB* | Absent | 9 | 56 | 1 | 20 | 0,311 |
| | Present | 7 | 44 | 4 | 80 | |
| *cagA* | Absent | 9 | 56 | 2 | 40 | 0,635 |
| | Present | 7 | 44 | 3 | 60 | |
| *vacA S1/S2* | Absent | 7 | 44 | 1 | 20 | 0,607 |
| | Present | 9 | 56 | 4 | 80 | |
| *vacA m1/m2* | Absent | 16 | 100 | 5 | 100 | 1,000 |
| | Present | 0 | 0 | 0 | 0 | |
| *hpaA* | Absent | 7 | 44 | 0 | 0 | 0,125 |
| | Present | 9 | 56 | 5 | 100 | |
| *oipA* | Absent | 13 | 81 | 5 | 100 | 0,549 |
| | Present | 3 | 19 | 0 | 0 | |
| *dupA* | Absent | 14 | 88 | 5 | 100 | 1,000 |
| | Present | 2 | 12 | 0 | 0 | |
| *babA* | Absent | 15 | 94 | 5 | 100 | 1,000 |
| | Present | 1 | 6 | 0 | 0 | |
| *napA* | Absent | 3 | 19 | 3 | 60 | 0,115 |
| | Present | 13 | 81 | 2 | 40 | |

## 3.2 Detection of RORγT Expression in Patients with Precancerous Lesions and Gastric Cancer

The upregulation of Th17 cells in peptic ulcer diseases and its association with gastiritis has already been studied (Bagheri, et al., 2018). However, the change in of Th17 cell distrubition in response to progression of gastric pathogenesis has not sufficiently been investigated yet. RORγt is a transcription factor that has important role in differentiation of Th17 cells by regulating the expression pattern of genes in Th17 cells (Castro, et al., 2017). For determining the relation between Th17 cells and gastric pathologies, we examined quantitative expression of RORγt by qRT-PCR assay. Firstly, we tested the expression pattern of RORγt in human gastric tissue

specimens from 60 patients who were suffered from gastric-related diseases. From these 60 patients, 15 had normal (uninfected with *H. pylori* and uneffected) gastric pathologies, 20 suffered from active chronic gastritis, 3 had inactive chronic gastiritis, 9 had intestinal metaplasia and 14 had gastric adenocarcinoma. The most high expression pattern was observed in the normal groups. However, there were no significant change in the mRNA expression of RORγt when we compare the 5 subgroups of gastric pathologies (Figure 3.2 A). Then, we decided to divide cancer resection tissue specimens into two subgroups based on their histological grades. From 14 gastric adenocarcinoma patients, 7 of them were pathologically reported as grade 2 adenocarcinoma and the rest 7 of them were reported as grade 3. Then, we re-analyzed the mRNA expression level of RORγt. We detected that, there were significant increase of RORγt expression in grade 2 cancer patients when compared to grade 3 cancer and intestinal metaplasia patients. Lowest expression of RORγt was observed in grade 3 gastric cancer patients and highest in normal gastric tissue (Figure 3.2 B). Finally, we re-analyzed the data of mRNA expression levels of RORγt, by dividing them into three subgroups; normal, precancerous lesions and gastric cancer. Normal groups were used as uninfected, uneffected control groups for gastric malignancies. The expression pattern in between normal, precancerous lesions and gastric cancer analyzed but there was no significant difference detected(Figure 3.2 C).

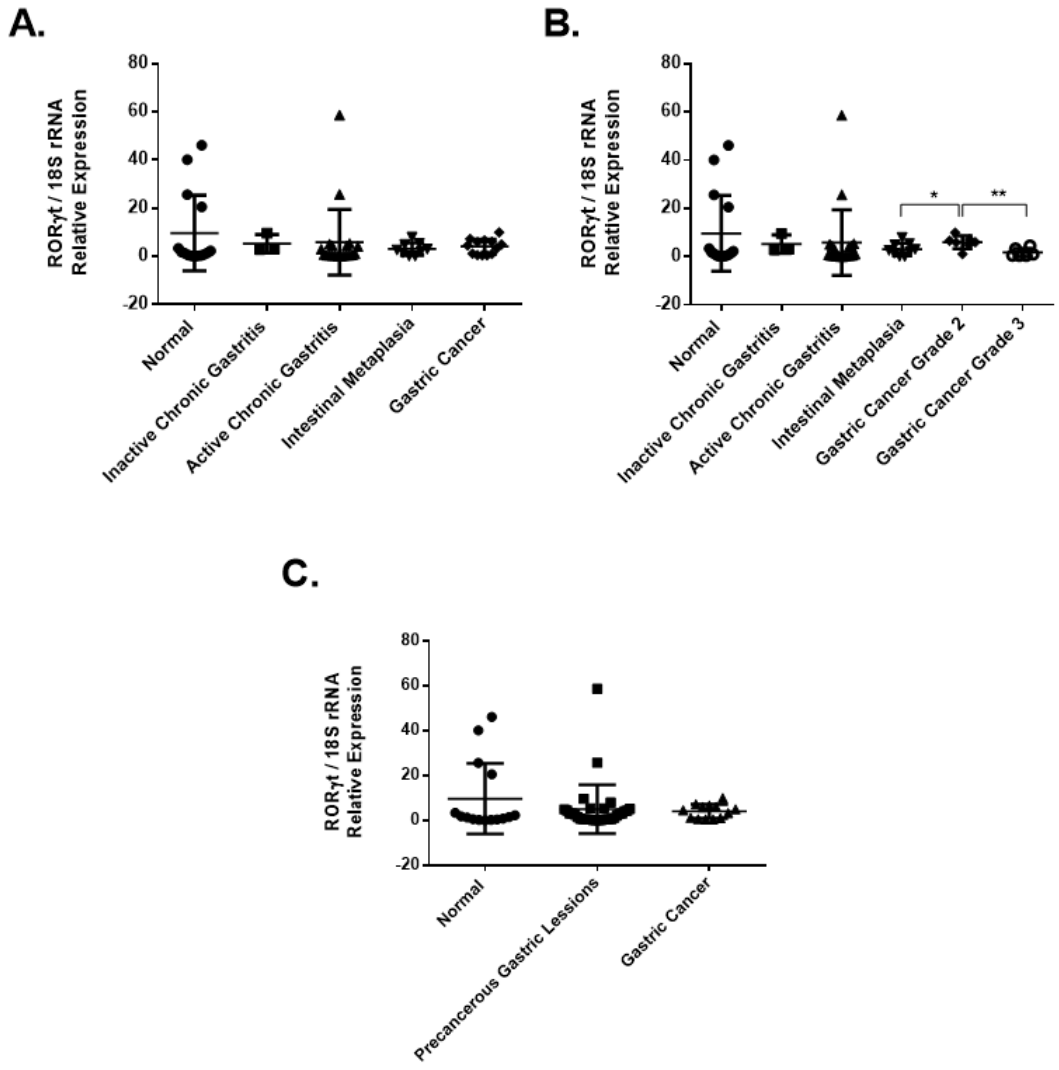


**Figure 3.2 : Expression level of RORγT in gastric pathologies evaluated by quantitative real time PCR**. A) RORγT expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia and gastric cancer. B) RORγT expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia, gastric cancer grade 2 and gastric cancer grade 3 patients. C) RORγT expression was compared in between normal, precancerous lesions and gastric cancer. Level of expressions were normalized to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.3 Correlation of *H. pylori* and RORγT in Patients with Precancerous Lesions and Gastric Cancer

Immune host response against *H. pylori* infection is important to understand the mechanism of disease development. To determine the response of Th17 cells to *H. pylori* infection; we compared RORγT mRNA expression in between *H. pylori*,

infected and uninfected, 60 gastric biopsies of 5 subgroups; normal, inactive chronic gastritis, active chronic gastirits, intestinel metaplasia and gastric cancer. In total, 21 and 39 of total patients were infected and uninfected by *H. pylori* respectively. From these 39 uninfected patients, 24 of them had one of gastric pathogenesis (inactive chronic gastritis, active chronic gastritis, intestinal metaplasia or gastric adenocarcinoma) and rest 15 were uninfected and uneffected by *H. pylori*, used as control groups. Nevertheless, we could not detect significant difference in expression levels of RORγT in patients based on their *H. pylori* infection status (Figure 3.3).

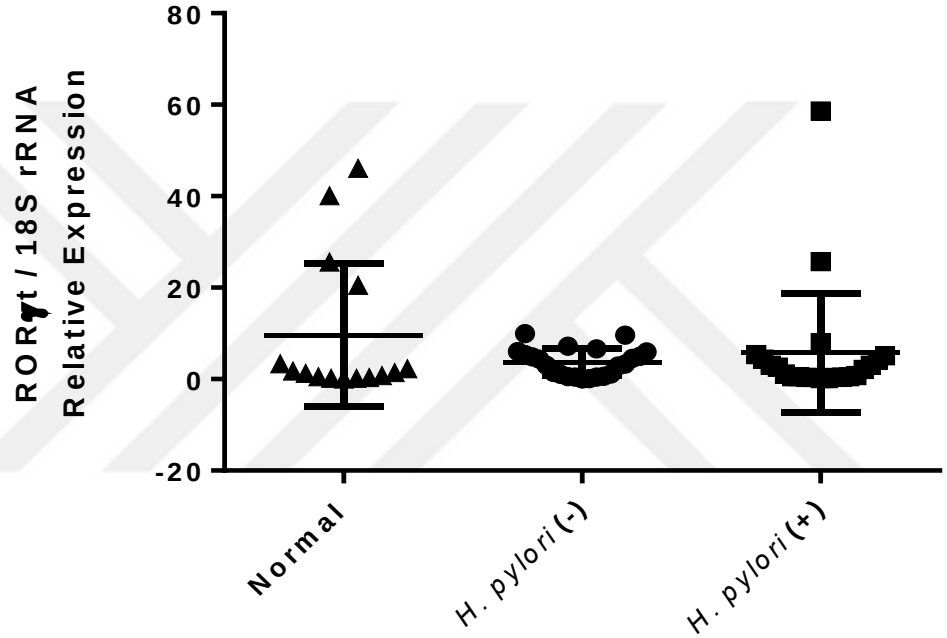


**Figure 3.3 : Comprasion of expression level of RORγT in *Helicobacter pylori* infected and uninfected patients with gastric pathologies**. Normal groups were used as uninfected, uneffected control groups for gastric malignancies.Level of RORγT expressions were normalized to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.4 Detection of IL-17 Expression in Patients with Precancerous Lesions and Gastric Cancer

After detecting expressing profile of main regulatory transcription factor of Th-17 cells, we focused on the expression status of IL-17 which is a major cytokine expressed by Th17 cells. The expression profile of IL-17 in different types of gastric cancers and in gastiritis patients have already been examined (Błogowski, Madej-Michniewicz, Marczuk, Dołęgowska, & Starzyńska, 2016). However, no investigation focused on the expression profile of IL-17 in gastric carcinoma and how it is changed during the progression of gastric pathogenesis from normal to gastric cancer. Normal groups were used as uninfected, uneffected control groups for gastric malignancies. To determine the level of IL-17 expression in different gastric pathologies, we examined expression of IL-17 by qRT-PCR assay (Figure 3.4). The highest expression of IL-17 was determined in normal gastric pathology groups. There was a significant decrease of IL-17 mRNA expression between normal and gastric cancer patients. Also, there was a significant decrease from inactive chronic gastritis to intestinal metaplasia and gastric cancer (Figure 3.4 A). When we analyzed the expression profile in two different gastric cancer grades, there was no significant difference in between them. We could observe the significant decrease of IL-17 cytokines from inactive chronic gastritis to grade 3 gastric adenocarcinoma (Figure 3.4 B). While examining the precancerous lesions together, the significant decrease of IL-17 from normal to precancerous lesions and gastric cancer was observed (Figure 3.4 C). Based on this results, both the transcrition factor (RORγt) and the cytokine (IL-17) of Th17 cells were showed highest expression level in normal group.

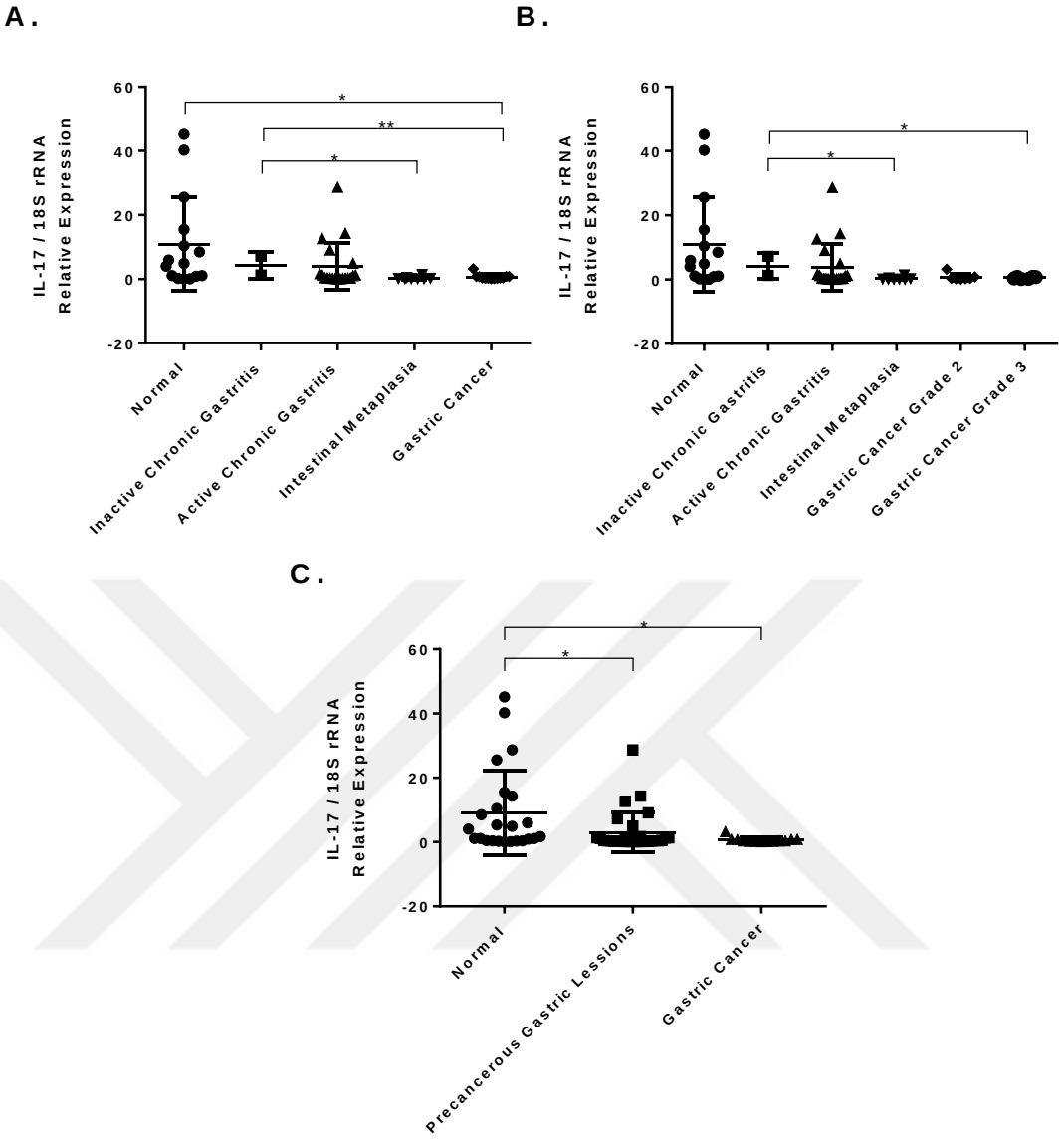


**Figure 3.4 : Expression level of IL-17 in gastric pathologies examined by quantitative qRT-PCR assay**. A) IL-17 expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia and gastric cancer. B) IL-17 expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia, gastric cancer grade 2 and gastric cancer grade 3 patients. C) IL-17 expression was compared in between normal, precancerous lesions and gastric cancer. Level of expressions were normalized according to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.5 Correlation of *H. pylori* and IL-17 in Patients with Precancerous Lesions and Gastric Cancer

For detailed understanding of host immune response against *H. pylori* infection based on status of Th17 cells, we compared the qRT-PCR results of IL-17 in between *Helicobacter pylori* -infected and -uninfected 45 gastric patients (Figure 3.5). 15 gastric biopsies from normal groups were used as uninfected, uneffected control groups for gastric malignancies. We could not detect any significant difference of IL-17 expression between *H.pylori* infected or uninfected patient. We detected signifcantly highest IL-17 expression in normal; uninfected and uneffected control group.

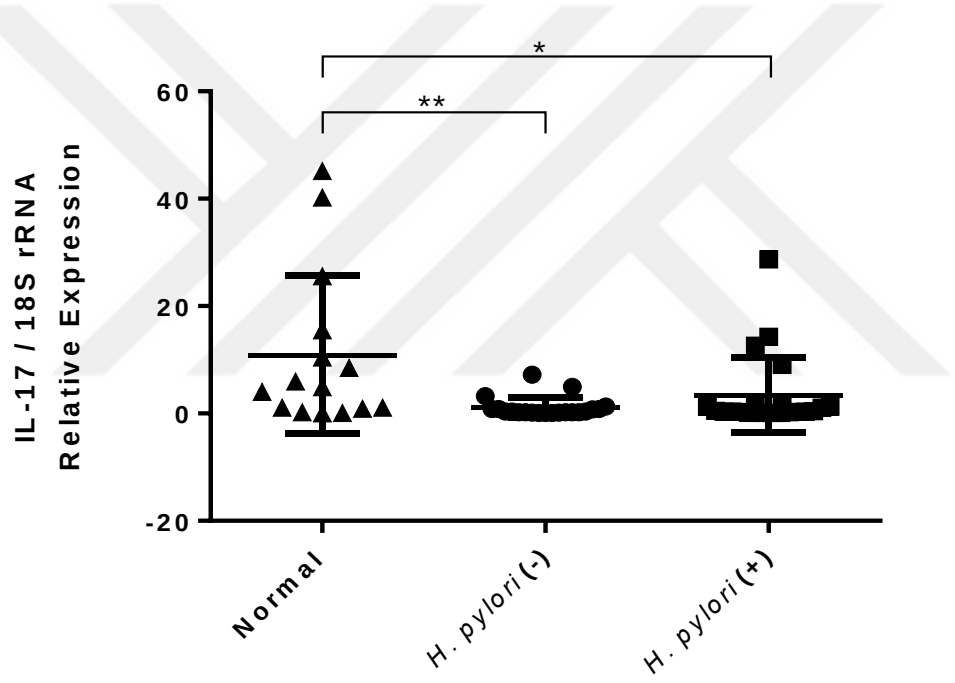


**Figure 3.5 : Comparison of expression level of IL-17 between *H. pylori* infected and uninfected patients with gastric pathologies**. Level of expressions were normalized to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.6 Detection of PD-1 expression in Patients with Precancerous Lesions and Gastric Cancer

The expression profile of PD-1 in gastric adenocarcinoma has been already studied. There is numerous studies about the mechanism of PD-1 and its possible therapeutic usage for gastric adenocarcinoma. However, the expression of PD-1 during the progression of normal gastric tissue to gastric cancer has not been studied yet. To determine whether there is an association between progression of gastric pathologies and PD-1, we examined the expression level of PD-1 in patients by quantitative RT-PCR (Figure 3.6). Normal groups were used as uninfected, uneffected control groups for gastric malignancies. We detect that expression level of PD-1 was highest in active chronic gastritis patients. There was a significant increase of PD-1 mRNA levels from normal to active chronic gastiritis patients. Even though the expression of PD-1 was increased when normal gastric tissue and with intestinal metaplasia was compared, the difference was not statistically significant. Besides, a significant decrease in PD-1 expression in gastric cancer patients was detected when compared to active chronic gastritis (Figure 3.6 A). For detailed analysis of expression pattern of PD-1 in gastric cancer, we compared the expression of PD-1 between two histological grade of adenocarcinoma; grade 2 and grade 3. We observed that there was significant increase of PD-1 expression from normal gastric pathology to gastric cancer grade 2. However, there was a significant decrease of PD-1 in grade 3 adenocarcinoma patients compare to grade 2 (Figure 3.6 B). In general comparison of PD-1 expression in normal, precancerous lesions and gastric cancer, we observed significant increase from normal pathology to active chronic gastritis. However, there was not a significant increase of PD-1 expression in gastric cancer (Figure 3.6 C).

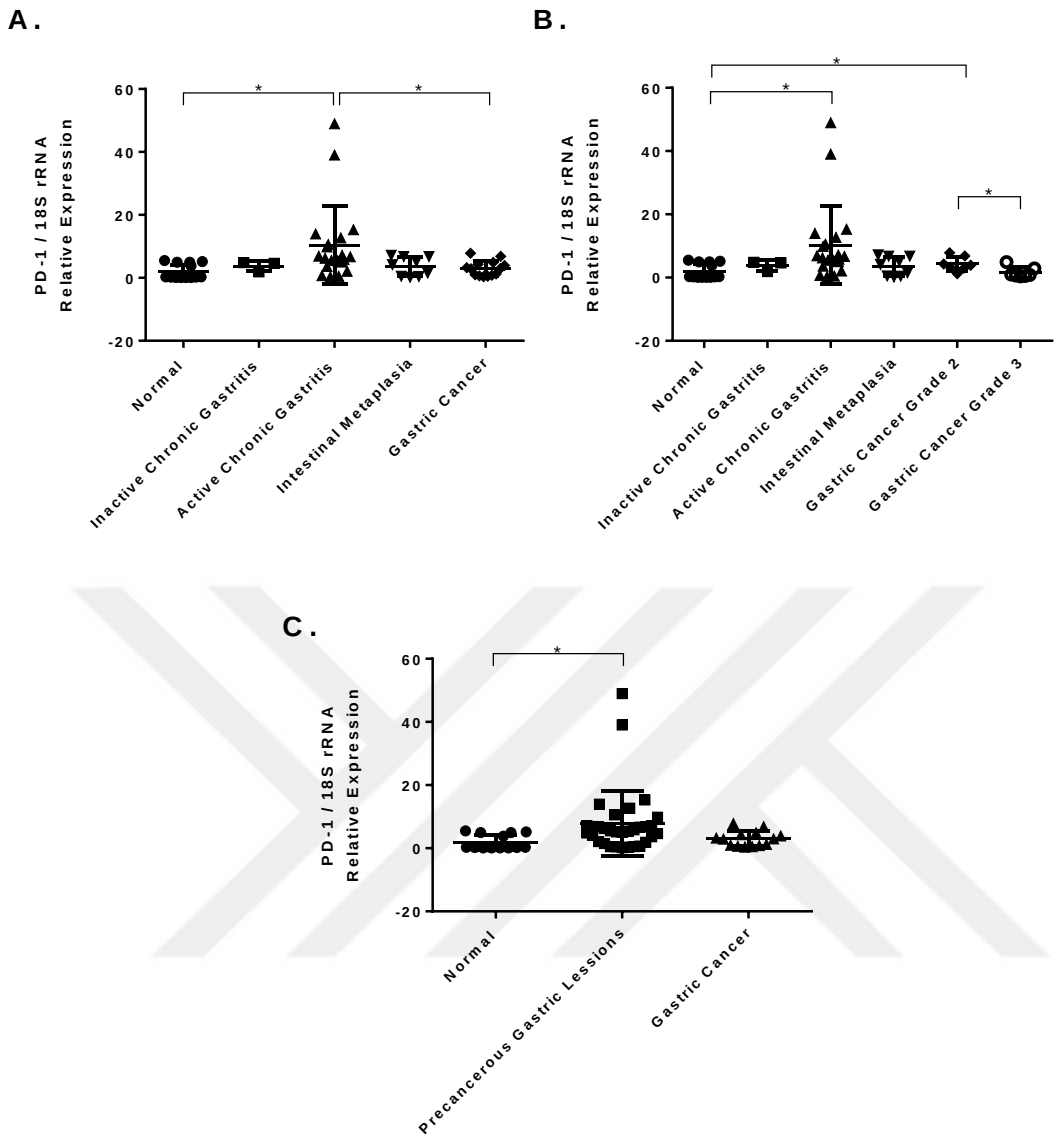


**Figure 3.6 : Expression level of PD-1 in gastric pathologies examined by quantitative real time PCR assay**. A) PD-1 expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia and gastric cancer respectively. B) PD-1 expression was compared in between normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia, gastric cancer grade 2 and gastric cancer grade 3 patients. C) PD-1 expression was compared in between normal, precancerous lesions and gastric cancer. Level of expressions were normalized according to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.7 Correlation of Virulence Factors and PD-1 in Patients with Precancerous Lesions and Gastric Cancer

To determine the expression pattern of PD-1 in respect to *H. pylori* infection status of patients, we compared PD-1 qRT-PCR results in *H. pylori* -infected and -uninfected 60 patients. Normal groups were used as uninfected, uneffected control groups for gastric malignancies. We determined a significant increase of relative PD-1 expression in *H. pylori* -infected patients compared to uninfected counterparts and normal control group (Figure 3.7).

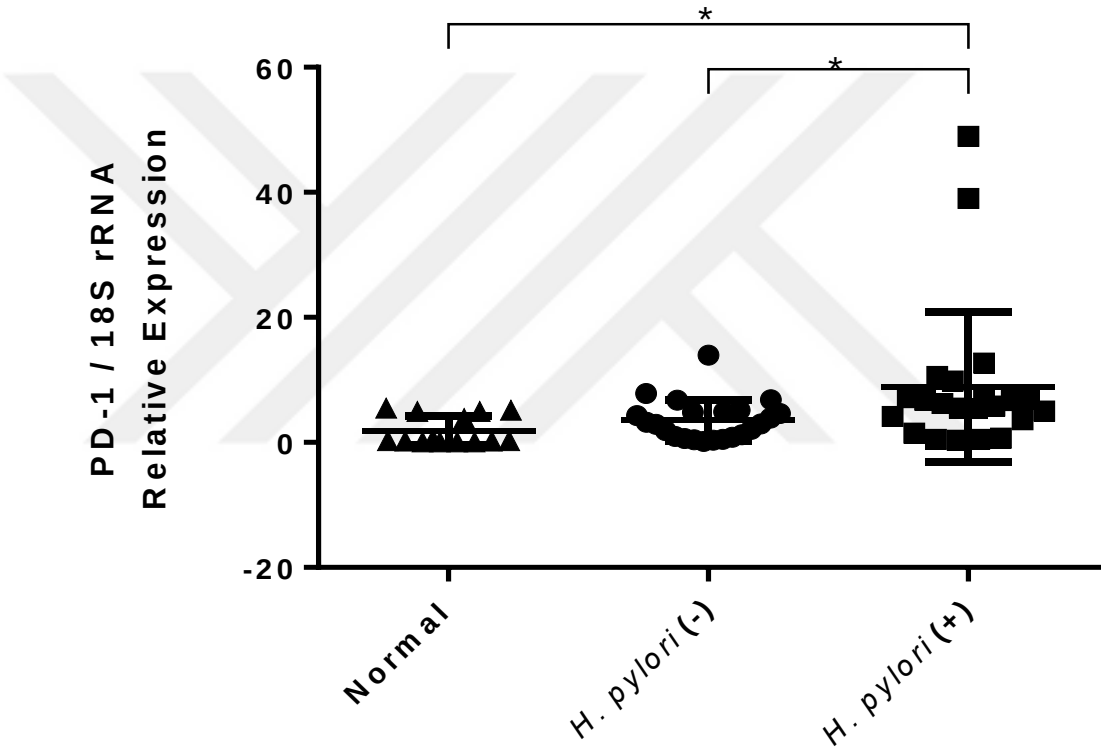


**Figure 3.7** : **Comparison of expression level of PD-1 in *H. pylori* infected and uninfected patients gastric pathologies**. Level of expressions were normalized according to 18S rRNA expression. Graphs were prepared by using GraphPad Prism 6. Each value represents the mean ±SEM of two replicates analyzed by Student's *t* test. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

### 3.8 Immunohistochemical Depiction of Precancrous Lesions

Tissue sections from FFPE blocks of endoscopic biopsy specimens were stained with hematoxylin and eosin in order to show the pathological changes in precancerous lesions and screening the *Helicobacter pylori* in infected gastric tissues. For this

purpose, we examined 4 different patients with different gastric pathologies; normal, inactive chronic gastritis, active chronic gastritis and intestinal metaplasia respectively. In normal gastric tissue staining results, we could detect the proper glands of epithelial cells and without any *H. pylori* colonization(Figure 3.8 A-C). In inactive chronic gastritis biospy samples, there were minimal changes in glands of epithelial cells, without detectable inflammation or increase in T cell counts (Figure 3.8 D-F). In active chronic gastritis, additional to detectable glandular changes, we also observed an increase of lymphocytes (Figure 3.8 G-I). Also, in 100x magnification, we could detect colonization of *H. pylori* in epithelial glands (Figure 3.8 I).Here, bacterium colonization was limited to epithelial glands. In intestinal metaplasia specimen, we observed the loss of epithelial glands. Also this patient had increased number of *H. pylori* colonization. Colonization of bacterium was not limited to glands (Figure 3.8 J-L).

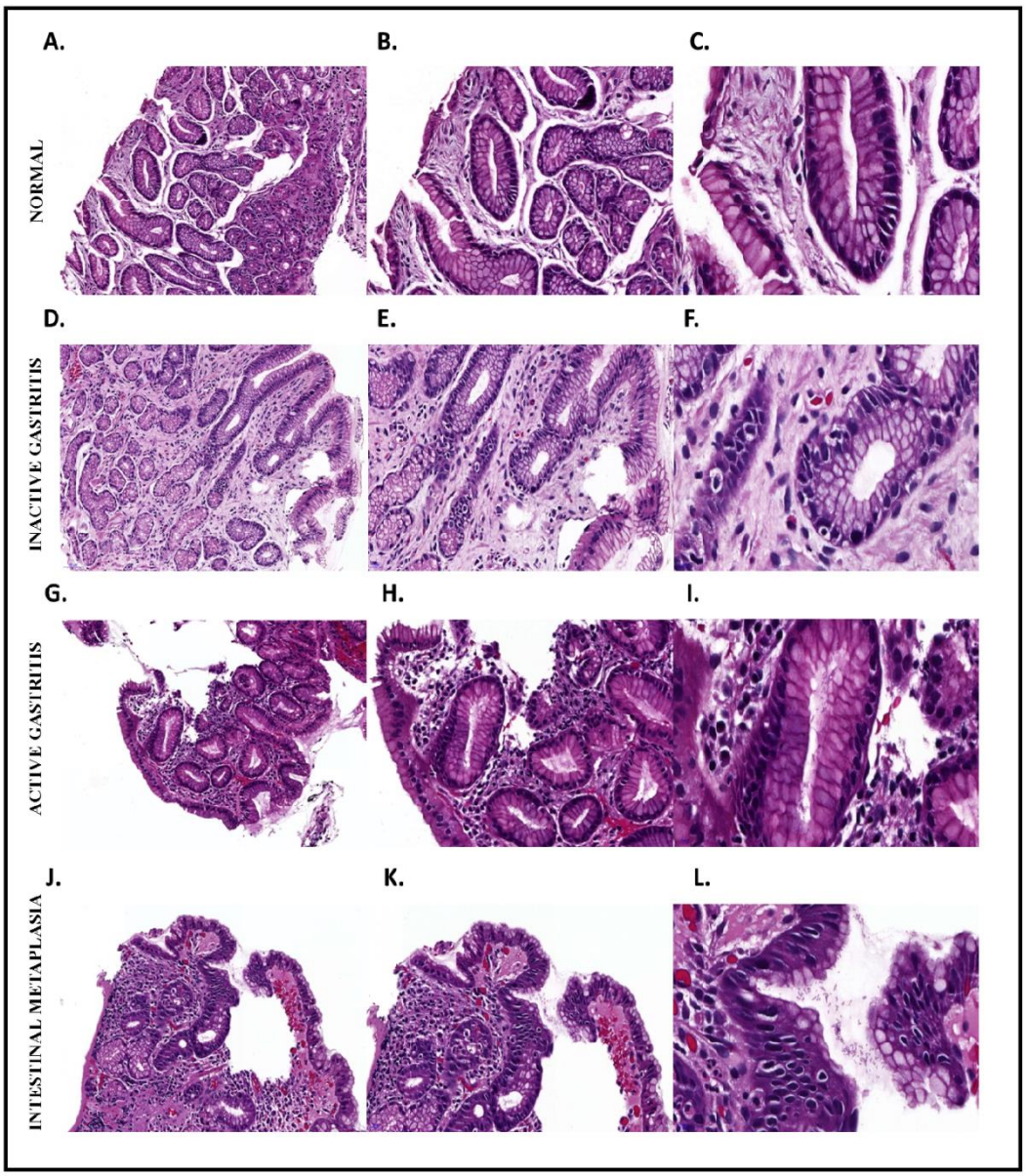


**Figure 3.8 : Immunohistochemical stainning by hematoxylin and eosin**. Nucleuses were stained (dark purple-blue) with hematoxylin and cytoplasms were (pink) stained with eosin. (A-C) Patient with normal gastric tissue; 20x, 40x, 100x respectively. (D-F) Images of gastric tissue of inactive chronic gastritis pathology; 20x, 40x, 100x respectively.( G-I) Images of gastric tissue of active chronic gastritis pathology; 20x, 40x, 100x respectively.(J-L) Images of intestinal metaplasia pathology ; 20x, 40x, 100x respectively. Slides were scanned by Panaromic Scanner Flash III(P250) System. For detailed visualization, 40X-extended magnification objectives were used and images were taken by using Case Viewer Programme.

## 4. DISCUSSION

Pathogenecity of *Helicobacter pylori (H. pylori)* is directly related to virulence factors that bacterium encodes in its genome. Cytotoxicity, colonization and persistance to the host immune response levels are proportional with this virulence factors. Therefore, examining the virulence factors are crucial in understanding the disease formation caused by *H. pylori* infection.

In this study, we aimed to investigate the relation between degree of genetic heterogenecity in *H. pylori* genome with precancancerous lesions in gastric pathologies. *H. pylori* is one of the main reason for development of several diseases in gastrointestinal tract. In literature; nine (9) virulence factors were shown to be most important for disease development which are *ureA, ureB, cagA, vacA, hpaA, dupA, oipA, babA, napA.* By using convantional PCR technique, we are able to detect the status of each virulence factors in the gastric biopsies which are clinically diagnosed as *H. pylori* positive. Every *H. pylori* strain must encodes *ureA* or *ureB.* Therefore, first of all we checked the existence of these virulence factors in *H. pylori* strains that infected 24 patients with three different types of pathological precancerous lesions; inactive chronic gastritis, active chronic gastritis and intestinal metaplasia. In clinical reports, there were three inactive chronic gastritis patients who were infected. However, in our conventional urease PCR test we diagnosed them as uninfected patients. It was showns in literature that if *H. pylori* is in its coccoid form, its urease activity of it is lower than its spiral form (She, 2001). Coccoids forms of *H. pylori* strains shows lower urease activities and it sometimes cannot be detected in PCR assays. In order to rule out this possibility, we stained the gastric biopsy samples of the patients without detected *ureA* or *ureB* with hematoxylin and eosin. However, we did not detect any *H.* pylori in stained specimens. Therefore, we categorized these three patient as uninfected patients. Afterwards, we investigated the remaining 7 virulence factors in 21 patients, who were diagnosed as infected by our conventional PCR assay. These 21 patients were pathologically diagnosed as

active chronic gastritis or intestinal metaplasia. Then, we compare the virulence factors' distributions between these two precancerous lesions. Even though, we could not detect any significant correlation between active chronic gastritis and intestinal metaplasia, we detected that patients with intestinal metaplasia were dominantly infected by *hpaA* positive *H. pylori* strain. *hpaA* is main adhesion protein of bacterium. In literature it was shown that, even *H. pylori* infection is important factor for gastric cancer progression, prevelance of *H. pylori* infection decreased in gastric adenocarcinome compared to normal (Ando, Goto, Maeda, Watanabe, Ishiguro, & Goto, 2006). In this study, *H. pylori* infection prevelance was highest in active chronic gastritis patients. Within 5 intestinal metaplasia patients, all of them was infected by *H. pylori* that was *hpaA* positive. As a future prospects, the number of the intestinal metaplasia patients may be increased to investigate this preliminary finding regardin the correlation between *hpaA* and survival of bacterium. In previous studies, the significance in *napA* gene expression in *Helicobacter pylori* infected ulcer patients was reported (Rautelin, 1993). Also, in recent study from our group, the significant increase of *napA* gene expression in gastritis patients when it is compared to ulcer was reported (Sin151). Here, we investigated this correlation between the infected active chronic gastritis patients *napA* profile with intestinal metaplasia. Even though, the expression of *napA* was higher in gastritis patients, there was no any significant change between these two precancerous lesions. When we compared our urease PCR results with Acıbadem Maslak Hospital pathology reports which were prepared based on the histological staining, we got ($\kappa$=0.84) almost perfect agreement. This supports the relability of our method.

To understand host immune response in gastric cancer is important to determine cause and effect of disease. There are several publications about the characteristics of T-helper (Th) cells in gastric cancer. However, the analysis about the Th17 expression profiles in precancerous lesions and gastric cancer are limited. Intestinal type gastric cancer progressed from chronic gastritis, followed by atrophy and intestinal metaplasia (Conteduca, Sansonno, Lauletta, Russi, Ingravallo, & Dammacco, 2012). The contribution of Th17cells in this progress is unknown. Therefore, we studied the RORγT, IL-17 which are Th17 cell signatures in gastrointestinal disease progression. RORγT acts as molecular determinant for Th17 polarization and necessary for IL-17 expression (Soutto, 2017). Previous studies

shown that, both RORγT and IL-17 expression was higher in gastric cancer compared to normal groups (Peng, et al., 2010). However, we found that, both of them expressed higher in normal patients compared to gastric cancer. For RORγT expression, there was no significant difference between gastric cancer patients and precancerous lesions. Lowest RORγT expression was detected in gastric cancer. This decrease in RORγT expression may be due to partial gastroctemy process. There is a recent study showing an inbalance between Th17/Treg expression as a result of the gastric surgery (Dong, Zheng, Wang, Wang, & Zou, 2018). They found that, after partial gastroctemy RORγT expression in mice decreased. In our patients, they were first undergo partial gastroctemy by endoscopic operation. After the analysis of this tissues by pathology, gastric resection take place. In literature it was shown that the increase number of Th17 cells detected in actively inflammated patients due to *H. pylori* infection (Bagheri, Azadegan-Dehkordi, Shirzad, Rafieian-Kopaei, Rahimian, & Razavi, 2015). As we expected, we detected the increase of RORγT expression in active chronic gastritis patients which is active inflammation disease. IL-17 were shown to be expressed significantly higher in normal and inactive chronic gastritis patients compared to gastric cancer patients. We examined little number of patients from inactive chronic gastritis groups. Therefore, an increase in patient number for this group is required for detailed analysis. The low level IL-17 and RORγT expression when normal and gastric cancer patients are compared could be due to an increase in regulatory T cells and in general immunosuppressive microenvironment of cancer.

After detection of *H. pylori* strains of infected patients with gastric pathologies, we focus on the host immune response against it. When there is a *H. pylori* infection; Th17 cells and its cytokine IL-17 increased due to the its function in autoimmunity and protection against extracellular bacterium (Ruan, et al., 2011). In here, we were examine RORγT and IL-17 expression levels in *H. pylori* infected and uninfected patients in great detail. However, we could not observed any significant difference in expression profiles of IL-17 and RORγT in these patients. In literature it was shown that, *H. pylori* infection mainly induce the T Helper 1 (Th1) and Th17 cell response (Sun, et al., 2018) (Oktem-Okullu, et al., 2015). Also, in our experiment groups, dominant inflammation group could be Th1. To understand this mechanism more detail we may analyze Tbet (Th1 specific transcription factor) expression.

PD-1 is a negative co-stimulatory molecule which interacts with its ligand PD-L1. Many microorganisms and cancer cells used PD-1/PD-L1 interaction to escape the immune response of host cells. When PD-1 binds to its ligand, this leads to inhibition of the cytotoxic T cell activities so that protects tumors from T cell microenvironment. In this study, we examined PD-1 in two way. First of all, we compared the expressions in normal, inactive chronic gastritis, active chronic gastritis, intestinal metaplasia and gastric cancer. We observed the increase of PD-1 expression in gastric cancer patients compared to normal patients, which is consistent with the literature (Saito, et al., 2018). When we grouped our gastric cancer patients into two grades, grade 2 and grade 3, based on the histopathological evaluation, we detected a significantly higher expression of PD-1 in grade 2 when compared to both grade 3 and normal patients. This difference in between two grades of cancer may be highly due to the cancer therapies. Most of the Grade 2 gastric cancer patients did not undergo pre-treatment before resection, however, some of Grade 3 patients underwent two or three doses of neoadjuvant chemotherapy. In literature, the effect of chemotherapy on T cells has been already studied; Dr. Ulrichs reports showed that, after neoadjuvant therapies, there was a observable decrease of lymphocytes and CD3+ T-cells (Beutner, et al., 2008). So that, the decrease of PD-1 expression in grade 3 patients may be partially due to the decrease of PD-1 expressing T cells as result of chemotherapies. Secondly, we investigated the differences in expression of PD-1 in response to *H. pylori* infection. There was a significant increase of PD-1 expression in infected patients. We also, detected an increase in PD-1 expression in active chronic gastritis groups that undergo high inflammation. In literature it was shown that, both *H. pylori* infection and active chronic gastritis are causes the inflammation in gastritis (Qadri, Rasool, Gulzar, Naqash, & Shah, 2014). This inflammation increases the T cell activation in inflammation sites (Jarbrink, Lundin, Löroth, & Svennerholm, 2001). Increase of PD-1 expression in active T cells was also detected (Simon & Labarriere, 2018). So that, increase of PD-1 expression in active chronic gastritis and *H. pylori* infected patients are expected results.

Finally, in immunohistochemistry experiment, we detected the histological changes in normal, inactive chronic gastiritis, active chronic gastritis and intestinal metaplasia patients. In inactive chronic gastritis patients, (even it was reported as infected patients by pathology), we could not observe any *H. pylori*. Also, our PCR data

verified this result. The number of counted lymphocytes were not increased significantly in inactive chronic gastritis patients in hematoxylin and eosin stained tissue sections. However, in active chronic gastritis patient slides, we could observe the increase number of lymphocytes in the environment. When the *H. pylori* infection becomes chronic, host response against it was increased and also inflammation level was increased. High inflammation could cause erosion in the environment and the glandular structure was started to degrade. Since there was not a total degradation and resistance of *H. pylori*, colonization is only be limited in epithelial glands. This situation was changed in further stages of disease. In intestinal metaplasia, we observed the total degradation of epithelial structures. *H. pylori* could spread all around the gastric environment and could change the environment with it favor. If the bacterium is not eradicated till this time, atrophic changes become irreversible in antrum part of human stomach (Kim & Han, 2013).

In conclusion, our study revealed that *H. pylori* infection induces expression of PD-1 significantly. Secondly, early phase gastric cancer patients shows higher T cell and PD-1 expression than latter stages. There is no significant expression profiles of Th17 and IL-17 in between normal, precancerous lesions and gastric cancer. Correlation between the *H. pylori* infection and PD-1 can be a tool for treatment of bacterium related gastrointestinal diseases.

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