THE ANALYSIS OF WNT PATHWAY IN GASTRIC CANCER

by

Gürkan ŞEN

A thesis submitted to

the Graduate Institute of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

May 2009
Istanbul Turkey

APPROVAL

Student **:** Gürkan ŞEN

Institute **:** Institute of Sciences and Engineering

Department **:** Biology

Thesis Subject **:** The Analysis of Wnt Pathway In Gastric Cancer

Thesis Date **:** May 2009

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Asist. Prof. M. Serdal SAKÇALI
Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Asist. Prof. Meliha Burcu Irmak YAZICIOĞLU
Supervisor

Examining Committee Members

Asist. Prof. Meliha Burcu Irmak YAZICIOĞLU …….

Prof. Fahrettin GÜCİN …….

Asist. Prof. M. Fatih ABASIYANIK …….

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate Institute of Sciences and Engineering.

Assist. Prof. Nurullah ARSLAN
Director

THE ANALYSIS OF WNT PATHWAY IN GASTRIC CANCER

Gürkan ŞEN

M.S Thesis – Biology

May 2009

Thesis Supervisor: Asist. Prof. Dr. Meliha Burcu Irmak YAZICIOĞLU

ABSTRACT

Gastric cancer is one of the most dangerous cancer types ranking as the second cancer related causative of death after lung cancer. Carcinogenesis develops due to deregulation of signal transduction pathways in the cells. Among these pathways disturbance in Wnt signalling has contributions to many cancer types including gastric cancer. However, the exact molecular mechanism leading to gastric carcer is still unclear.

In this study, the involvement of the components of Wnt pathway in gastric cancer tissues and cell lines was analyzed. RT-PCR analysis was performed to find out and compare the expression profiles of wnt ligands, frizzled receptors, and β-Catenin gene in 61 pairs of normal and tumor gastric biopsies and gastric cancer MKN-45 and 23132/87 cells. In addition to this, MKN-45 and 23132/87 cells were infected with H. pylori which is an important risk factor for gastric cancer development in order to

determine how it affects the cellular response in term of gene expression, protein localization, apoptosis, and proliferation.

Expression profiling of wnt pathway components results indicate that, there is significant correlation between Frizzled, Wnt, and β-Catenin genes that will be result in activation of the Wnt pathway. H. pylori infection results demonstrated that there are many changes in morphological, apoptotic, proliferative, gene expression.

These data help to understand the underlying mechanism of the gastric cancer. However, obtained data are not sufficient to clarify the exact underlying molecular mechanism, due to complexity of the signaling pathway in the cell.

Keywords: Gastric cancer, Cancer, Helicobacter Pylori, Wnt Signaling Pathway.

MİDE KANSERİNDE WNT SİNYAL YOLUNUN ANALİZİ

Gürkan ŞEN

Yüksek Lisans Tezi: Biyoloji

Mayıs 2009

Tez Yöneticisi: Yrd. Doç. Dr. Meliha Burcu Irmak YAZICOĞLU

ÖZ

Mide kanseri akciğer kasnerinden sonra kanser ölümlerine sebep olan en tehlikeli kanser türlerinden biridir. Birçok kanser türünde olduğu gibi mide kanserinde de birçok sinyal yolunda bozukluklar görülür. Wnt, cancerde rolü olan en önemli sinyal yollarından biridir. Önceki çalışmlarda Wnt sinyal yolunun mide kasnerine büyük katkısının olduğu gösterilmiş fakat altında yatan moleküler mekanizma tam olarak açıklanmamıştır.

Biz bu çalısmada, mide kanseri dokularında ve hücrelerinde Wnt sinyal yolu aktivitesini analiz etmek amaçlandı. Bunun için öncelikle 61 mide kasneri hastasından normal ve tümörlü doku çiflteri toplandı, bu örneklerde Frizzled, Wnt, β-Catenin ve iç kontrol olarak GAPDH genleri RT-PCR yöntemi ile çoğaltıldı ve Wnt, Frizzled ve β-

Catenin ekspresyonları kaşılaştırıldı. Buna ilaveten, mide kasnerindeki en onemli risk faktörü olan Helicobacter Pylori etkisi apoptoz, proliferasyon, Wnt sinyal yolu aktivasyonu ve gen ekpresyonu değişikliği olmak uzere 4 parametre altında mide kasneri hücreleri olan MKN45 ve 23132/87 hücreleri üzerinde analiz edildi.

Wnt sinyal yolu elemanlarının ekspresyon profili analiz sonuçlari, Frizzled, Wnt ve β-Catenin genleri ekspresyonları arasında Wnt sinyal yolunun aktivasyonu ile sonuçlanacak onemli derecede korelasyon olduğunu gösterdi. Helicobacter Pylori enfeksiyon sonuçları da hücrede birçok morfolojik, apoptotik, proliferatif ve gen eskpresyonu seviyelerinde değişiklik olduğunu gösterdi.

Bu bilgiler mide kasneri altında yatan moleküler mekanizmayı anlamamıza yardımcı oldu. Ancak hücre içindeki sinyal yollarının karmaşıklığından dolayı bu bilgiler tam mekanizmayı anlamamız için kendi başina yeterli olmadı.

Anahtar Kelimeler: Mide kanseri, Kanser, Helicobacter Pylori, Wnt Sinyal Yolu.

Dedicated to my parents.

ACKNOWLEDGEMENT

I express sincere appreciation to Asist. Prof. Dr. Meliha Burcu Irmak YAZICIOGLU for their guidance and insight throughout the research.

I wish to express my appreciation to Salih Gencer for all his help and valuable contribution to my thesis.

I would also thanks to the other faculty members.

I would like to thanks to Hakan OCAK, Tuğba OGRETMEN and Raif Cumhur YAMAN for their contribution to my experiments.

I would like to express great appreciation to Asist. Prof. Dr. M. Fatih ABASIYANIK for his supporting and guidance.

Especially, I would like to express my thanks and appreciation to Asist. Prof. Dr. Duran USTEK for giving me the opportunity to complete my thesis in his lab and for his great support.

Particularly, I express my thanks and appreciation to my family for their endless understanding, motivation and patience.

Also I would like to express great thanks to Tubitak [105S352 (SBAG-K-110)] and Fatih University (P50030703) for their funding my thesis.

Lastly, but in no sense the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

FZD	Frizzled Receptors
Wnt	Wnt Ligands
H. pylori	Helicobacter Pylori
bp	Base pair
cagA	Cytotoxin-associated gene A
CLO	*Campylobacter* like organism
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylenedinitrilo tetraacetate
kb	Kilobase
kDa	Kilodalton
m	Middle region
M	Molar
MALT	Mucosa associated lymphoid tissue
µl	Microliter
PAI	Pathogenicity island
PCR	Polymerase chain reaction
pmol	Picomole
rpm	Rotation per minute
s	Signal region
TBE	Tris-Boric acid-EDTA
vacA	Vacuolating-associated gene A
DNA	Deoxiyribonucleic Acid
RNA	Ribonucleic Acid
ng	nanogram
$MgCl_2$	Magnesium Chloride

mRNA	Messanger Ribonucleic Acid
NaOH	Sodium Hydroxide
DMSO	Dimethylsulfoxide
ddH_2O	Double distilled water
CO_2	Carbondioxide
SDS	Sodium Dodecyl Sulphate
Tris	(hydroxymethyl)aminomethane
U	Unit
TAE	Tris- Acetic Acid- EDTA
EtBr	Ethidium Bromide

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. There are more than 100 different types of cancer and subtypes of tumor in specific organs. Cancer is caused by both external factors such as tobacco, chemicals, radiation, and infectious organisms and internal factors such as inherited mutations, hormones, immune conditions, and mutations that occur from metabolism. These contributory factors may act together or in sequence to initiate or promote carcinogenesis [1].

Carcinogenesis process has many steps that require the accumulation of numerous genetic alterations in a cell. These alterations might be show the differences according to cancer type but the altered genes are generally related to six essential properties of cells such as: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these physiologic changes novel capabilities acquired during tumor development [2].

1.1.1 Self-Sufficiency in Growth Signals

The growth signaling pathway regulation can be abrogated by some alterations. These alterations might be in different step of signaling cascade. During transition of signal from extracellular area to intracellular are some alterations might be cause cancer formation such as increased production of ligands, increased levels of EGFR protein, EGFR mutations giving rise to constitutively active variants, defective down regulation of EGFR and cross-talk with heterologous receptor systems. During transduction of signal through intracellular components of pathway, many molecules have oncogenic potential such as RAS, RAF, MAPK, and in last step during transcription regulation, transcription factor may oncogenic role in cancer formation [3-5].

1.1.2 In Sensitivity of Anti-Growth Signal

Tissue homeostasis is maintained by helping numerous type of antigrowth signal molecules. These signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. Proliferation inhibition by these molecules can be occur via 2 ways: These molecules can be undergo to cells to quiescent (G0) from active proliferative cycle from which they may reemerge on some future occasion when extracellular signals permit or cells may be induced to permanently relinquish their proliferative potential by being induced to enter into post-mitotic states, usually associated with acquisition of specific differentiation-associated traits.

Most common alterations in this pathway occur on RB gene and TGF b signaling pathway components. Alteration in RB gene may be cause passing of cell from G0 to G1 phase. These transitions might be caused cancer formation due to loss of sensitivity to anti-growth signal.

1.1.3 Evading Apoptosis

Apoptosis, a controlled form of cell death, plays a pivotal role in many physiological processes, including maturation and effectors mechanisms of the immune system [6, 7], embryonic development of tissue, organs and limbs [8], development of the nervous system (9, 10) and hormone-dependent tissue remodeling [11]. Another form of cell death that results from acute tissue injury and provokes an inflammatory response named necrosis, carried out in a unregulated way in contrast to apoptosis. The apoptotic cell death is associated with typical morphological features like cell shrinkage, chromatin condensation and cytoplasmic membrane blebbing.

Unregulated apoptosis has been implicated in a variety of diseases. Increased level of apoptosis can cause cell-loss disorders, while decreasing level of apoptosis results in uncontrolled cell proliferation, leading to cancer [15].

There are two well-characterised apoptosis pathways in mammalian cells. The first one, also called the “extrinsic” pathway, and second one is called “intrinsic” pathway. Both pathways cause the activation of “initiator” caspases, which then activate “effector” caspases [15]. These deregulations which cause cancer formation can be occurring in many different steps of these two different ways from beginning cell membrane to nucleus. In addition inappropriate regulation of apoptosis has been indicated in different pathological conditions such as ischemia, stroke, heart disease, cancer, AIDS, autoimmunity, hepatotoxicity and degenerative diseases of the central nervous system [12-14].

1.1.4 Limitless Replicative Potential

Until finding of telomeres it was believed that normal cells had an unlimited capacity to replicate and aging was thought to have little to do with intracellular events

but then it is found out that normal cells do have a limited replicative capacity [16]. Human telomeres are TTAGGG repeats containing sequences which are located specialized end of chromosomes. Their function is protecting chromosomes from degradation, fusion and recombination. Conventional DNA polymerases need to RNA primer to initiate DNA synthesis due to 5'-3' replication direction of linear DNA molecules. End of replication, RNA primers are removed by an enzyme that results inf DNA loss with each cell division. Most dividing cells are shown telomeres length shortening that result in cell senescence when critically short telomeres are reached. In addition to that some type of cell has limitless replicative potential such as stem cells. These properties of stem cells are maintained by an enzyme which is named telomerase, which has been identified as a ribonucleoprotein that can synthesize telomeric repeats onto chromosomes [17]. Moreover increased telomerase activity can be increase the capability of replication. Therefore if a cell has increased telomerase activity, this might be cause cancer formation. Introduction of the telomerase catalytic protein component into normal telomerase-negative human cells results in restoration of telomerase activity and extension of cellular life span [18].

1.1.5 Sustained Angiogenesis

Tumor angiogenesis is the proliferation of a network of blood vessels penetrating into the cancerous growths to supply nutrients and oxygen and remove metabolic waste products from tumors. Tumor angiogenesis is a complex process and involves the tight interaction of tumor cells, endothelial cells, phagocytes and their secreted factors, which may act as promoters or inhibitors of angiogenesis. The angiogenic promoters send signals to the surrounding normal tissues in which specific gene activation and protein expression encourages growth and proliferation of new blood vessels [19]. More than a dozen different proteins (such as VEGF, bFGF, IL8, etc.), as well as several smaller molecules (such as adenosine, PGE, etc.) have been identified as angiogenic factors secreted by tumor cells to mediate angiogenesis.

1.1.6 Tissue Invasion and Metastasis

Malignant disease is one of the most common causes of death. Most cancer patients do not die from local complications of their primary tumor growth, but rather from the development and spread of the tumor. Therefore, the main cause of death of cancer patients is metastasis.

In order to obtain tumor cell characteristics, metastasis steps consist of a complex serried of linked, sequential steps as follows:

1. Disconnection of intercellular adhesions and separation of single cells from solid tumor tissue.
2. Escape from anoikis.
3. Proteolysis of extracellular matrix.
4. Locomotion of tumor cells in the extracellular matrix.
5. Invasion of lymph-and blood vessels.
6. Immunologic escape in the circulation.
7. Adhesion to endothelial cells.
8. Extravasation from lymph-and blood vessels.
9. Proliferation and induction of angiogenesis [20, 21].

In cancer formation, to obtain the invasion, metastasis, angiogenesis characteristic, main group of proteolytic enzymes which is named MMPs are involved [22,23]. Previously most reported human MMPs in various malignant tumors are MMP-2 (gelatinase A/ Mr 72,000 type IV collagenase) and MMP-9 (gelatinase B/ Mr 92,000 type IV collagenase), and MMP-7 (Matrylsin) [24]. Therefore, they are considered key enzymes for tumor invasion and metastasis [25].

1.2 Gastric Cancer

Despite the decreasing incidence and mortality rates observed worldwide over the last 50 years, gastric cancer (GC) still ranks as one of the most frequent and lethal cancers worldwide [26]. Today, GC is the fourth leading cancer type in incidence accounting for almost a million new cases diagnosed annually (International Agency for the Research on Cancer-IARC 2002) [27]. According to Lauren's histological classification gastric cancer is divided in two groups, the better differentiated intestinal carcinomas and the poorly differentiated diffuse-type cancers. The genetic changes underlying the initiation and progression of gastric cancer are not well defined [28].

1.2.1 Risk Factors

1.2.1.1 Diet

Diet has been hypothesized to play a role in the etiology of gastrointestinal cancer for a long time [29]. Distinct variations in the incidence and mortality of gastric cancer, over time, between and within countries, in differing socioeconomic groups, and in migrants and their offspring suggest that diet may be etiologically important and its role has been extensively investigated with inconclusive results [30].

1.2.1.2 Salt

Ingestion of sodium chloride is thought to promote gastric carcinogenesis. Some studies indicate that, nutrition with high salted foods such as salted fish have reported the increased risk of gastric cancer associated to salt. But in that case, there is a critical point while considering results; intake of salted foods commonly correlates with the intake of secondary amines and of fresh vegetables due to that role of salt cannot

explain exactly. But this is known that salt induces gastritis by directly damaging the gastric mucosa and increasing the rate of mitosis [34]. Damage caused to the gastric mucosa by salt is therefore one plausible explanation for the association with risk of gastric cancer.

1.2.1.3 Vegetables and Fruits

The strongest dietary hypothesis is that fresh fruits and vegetables, or contained micronutrients, are protective against gastric cancer. Numerous studies have shown, almost uniformly, a protective association with fresh fruits and vegetables, independent of other dietary factors [35].

1.2.1.4 Vitamins and Minerals

Possible protective micronutrients include vitamins C (ascorbate) and E (alpha-tocopherol), carotenoids (particularly beta carotene), and selenium [36]. The evidence is strongest for vitamin C, with an approximate halving of risk associated with high intake vs. low intake demonstrated in case– control studies [37].

N-nitroso compounds are potent carcinogens formed in vivo by the nitrosation of amides or amines in the stomach by nitrites, a process that is inhibited by vitamin C in gastric juice. However, a 5-year intervention trial, involving 30,000 40 to 69 year olds in China, did not show any change in risk of gastric cancer in subjects receiving supplemental vitamin C [38].

The speculation that this approach might increase protection against gastric cancer is supported by the results of a randomised controlled trial conducted in Columbia, in which treatment of patients at high risk of gastric cancer with a combination of vitamin C, beta-carotene and H. pylori eradication successfully promoted the regression of pre-malignant lesions [39].

1.2.1.5 Nitrate, Nitrite, Nitrosamines

Nitrate can be converted into nitrite and in a nitrosation reaction to N-nitroso compounds (e.g. nitrosamines); these are carcinogenic in animal [40].

These potent carcinogens are found in foods such as cured meat, cured fish, and fried or grilled bacon. The majority are, however, formed *in vivo* through nitrosation by nitrites of amines or amides in the stomach [41]. Nitrosation is facilitated by low acidity, and is catalysed by bacteria at neutral pH values. Nitrites in the stomach are mainly produced from food and drinking water. The acidity of gastric juice also influences the formation of nitrites from nitrates in the stomach [42].

In Japan, almost all ingested nitrate comes from vegetables, with a negligible amount from drinking water. Adverse effects of a number of dietary exposures (particularly cured or salted meat and fish), are thought to be linked to the N-nitroso model of gastric carcinogenesis. N-nitroso compounds are potent carcinogens formed in vivo by the nitrosation of amides or amines in the stomach by nitrites, a process that is inhibited by vitamin C in gastric juice. In the stomach, nitrites are mainly derived from food and water sources, with the proportions varying according to dietary pattern and water source [43].

1.2.2 Age, Sex, and Race

The incidence of gastric cancer rises progressively with age, with most patients being between the ages of 50 and 70 years at presentation. Cases in patients younger than 30 years are very rare.

Noncardia cancer is more common in males than females by a ratio of approximately 2:1. Gastric cardia cancer has a higher male-to-female ratio, of up to nearly 6:1 in U.S. Whites [44].

1.2.3 Genetic Susceptibility

Acetaldehyde derivatives are carcinogen for human. Therefore, acetaldehyde is detoxified by Mitochondrial class II aldehyde dehydrogenase (ALDH2) via oxidation of acetaldehyde compounds. In individuals who have genetically deficient ability to detoxify acetaldehyde and heavy drinking has higher risk to digestive tract cancer [45].

The ALDH2*2 allele is the result of a single point mutation in chromosome six, where the normal ALDH2*1 allele is coded. Individuals homozygous for the mutated ALDH2*2 allele lack ALDH2 activity, whereas heterozygous individuals with the ALDH2*1/*2 genotype have 30–50% of the activity of ALDH2*1 homozygotes [46]. Some Asian populations show high frequencies of the ALDH2*2 allele, e.g. about 50% of the Japanese population are ALDH2-deficient, while this is extremely rare in Caucasian populations [47].

ALDH2-deficient individuals may show some unpleasent symptoms after alcohol ingestion like flushing of the face and body, tachycardia, drop in blood pressure, headache and nausea due to elevated blood acetaldehyde levels [48]. As a result of these adverse reactions totally ALDH2-deficient individuals are protected against alcoholism [49]. However, heterozygotic subjects may become heavy drinkers, or even alcoholics [50]. Many recent epidemiological studies have shown that the risk of alcohol-related digestive tract cancers is markedly increased in Asian subjects who are deficient in the ALDH2 enzyme [51, 52].

1.2.4 Alcohol and Smoking

One of the strongest risk factors for gastric cancers is excessive alcohol consumption. In addition, heavy drinking may also increase cancer risk in other parts of the gastrointestinal (G1) tract. The exact mechanism of alcohol-related cancers has remained unclear, since ethanol is not a carcinogen by itself. However the first metabolite of ethanol oxidation is acetaldehyde which has strong carcinogenic effects on cells. Evidence suggests that acetaldehyde formed from ingested alcohol by microbes representing normal gut flora or by salivary glands is also a local carcinogen in humans [31-33]. However, alcohol consumption may be associated with several indirect tumor promoting effects such as malnourishment (decreased intake of cancer-protective fruits, vegetables, trace elements such as zinc and selenium and vitamins such as folic acid, riboflavin, retinol, ascorbic acid and alfa-tocopherol), increased exposure to other carcinogens, metabolic activation of procarcinogens via cytochrome P-450-dependent metabolic pathways to carcinogens and the local effects of strong alcoholic beverages [53-55].

1.2.5 Helicobacter Pylori Infection

Helicobacter pylori (*H. pylori)* is a Gram-negative, microaerophilic bacterium which expresses an abundant amount of urease. Infection with this bacterium is very common in the world. Incidence increases with age, but show differences quite dramatically between populations [56-60].

Moreover, *H pylori* infection is the most important acquired risk factor for both the diffuse and intestinal types of gastric cancer [61], and the bacterium itself is classified as a class I carcinogen by the World Health Organization and International Agency for Research on Cancer Consensus Group [62].

Host genetic factor and virulence factor of *H.* Pylori play a major role in increased risk of cancer [63].

H. pylori infection can induces gastric inflammation, and the diseases that include chronic gastritis, peptic ulcers and, more rarely, gastric cancer. Host susceptibility, environmental factors and the genetic diversity of the organism might be determine the progression of the infection. By that way, why a minority of patients with H. pylori develops gastric cancer could is explained. Moreover, the mechanisms by which H. pylori triggers hyperproliferative processes and takes direct command of epithelial cell signalling, including activation of tyrosine kinase receptors, cell–cell interactions and cell motility are reviewed [64].

1.2.5.1 Virulance Factors

Bacterial virulence factors, such as the cag pathogenicity island (PAI) type IV secretory system [65] and genetic polymorphisms in proinflammatory and immunoregulatory cytokines [66], have been linked to an increased risk of developing gastric atrophy or gastric cancer.

1.2.5.2 Urease

H. pylori are specifically adapted to colonize and survive in the hostile acidic gastric environment [67] Several factors including the urease enzyme and urea transport protein are key to maintaining a neutral pH micro-environment and survival of the organism [68,69].

1.2.5.3 Cag Pathogenicity Island

The 40-kb DNA insertion segment of *H. pylori* genome known as the cag pathogenicity island (cag-PAI) has been acquired from other bacteria. The cag PAI contains 31 putative genes, 6 of which are thought to encode components of a bacterial type IV secretion system, which specializes in the transfer of a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells [70,71,72].

1.2.5.3.1 CagA Gene

H. pylori strains carrying the cytotoxin-associated gene A (*cagA)* gene are strongly associated with an increased risk of gastric adenocarcinoma**.** CagA is the 120–130 KDa protein, encoded by the *cag*A gene, located on the 40 kb cag-PAI, interacts directly with host epithelial cells. CagA and other bacterial proteins are delivered to into host cell cytoplasm by a specific Type IV secretion system.

CagA induces epithelial cell proliferation and division after tyrosine phosphorylation and binding with the Src homology 2 (SH-2) domain, by activation of nuclear factor kappa B (NF-kB) and secretion of interleukin 8 (IL 8). A negative feedback system, whereby 20% of tyrosine phosphorylated CagA binds a carboxyl-terminal Src kinase (Csk) and activates Csk rather than Src family kinases, promotes persistence of infection and chronic inflammation. Additional reported effects of the Cag-PAI encoded proteins include rearrangement of the actin cytoskeleton, inhibition of apoptosis, epithelial cell DNA damage, activation of transcription factor AP-1, increased expression of the proto-oncogenes *c-fos* and *jun* and increased expression of Prostaglandin E2 [73-77].

There are two distinct types of CagA which are called Western and Eastern [78]. Identified polymorphisms in cagA, tyrosine phosphorylation sites, give Eastern forms a higher binding affinity for SHP-2, than western CagA varieties. The increased binding affinity has been related with more strict and active gastritis, gastric atrophy and has been linked to gastric cancer [79]. The grade of gastric atrophy (and therefore gastric cancer risk) is higher in patients with Eastern *cagA*-positive strains than in those with *cagA*-negative or Western *cagA* –positive strains. Of interest is that atrophy grade varies even among patients with East Asian *cagA*-positive strains[80], and that most *H pylori*-infected subjects in fact develop no significant disease, remaining asymptomatic throughout their lives. Bacterial virulence factors not sufficient explanation for this issue. For that reason, genetic factors of the host should also be considered to play a role in *H pylori*-induced outcomes. [81-85].

1.2.5.3.2 VacA gene

Vacuolating cytotoxin A encoded by the vacA gene induces vacuole formation in gastric epithelial cells and stimulates apoptosis. VacA enables organic anions to enter the cell by attaching to cell membranes and acting as a voltage dependant channel. Inducing apoptosis results in a complimentary increase in cellular proliferation a key element of *H. pylori* induced gastritis. Additional reported effects of VacA include disruption of the cytoskeletal architecture, through gene modifications, damage to cell-cycle related genes and induction of the inflammatory response [86,87]. Unlike cagA, the vacA gene is present in all *H. pylori* strains, but functional expression is variable. Variations within the vacA gene have been identified, which affect expression.

The vacA gene contains two variable regions. The signal s-region exists as s1 or s2 allelic types. Among type s1 strains, subtypes s1a, s1b, and s1c have been identified. The middle m-region exists as the m1 or m2 allelic type [88-91]. Among type m1, three subtypes have been identified, designated m1a, m1b, and m1c [92,93]. Production of the vacuolating cytotoxin is related to the mosaic structure of vacA. In general, type s1/m1 and s1/m2 strains produce high and moderate levels of toxin, respectively, whereas s2/m2 strains produce little or no toxin [88].

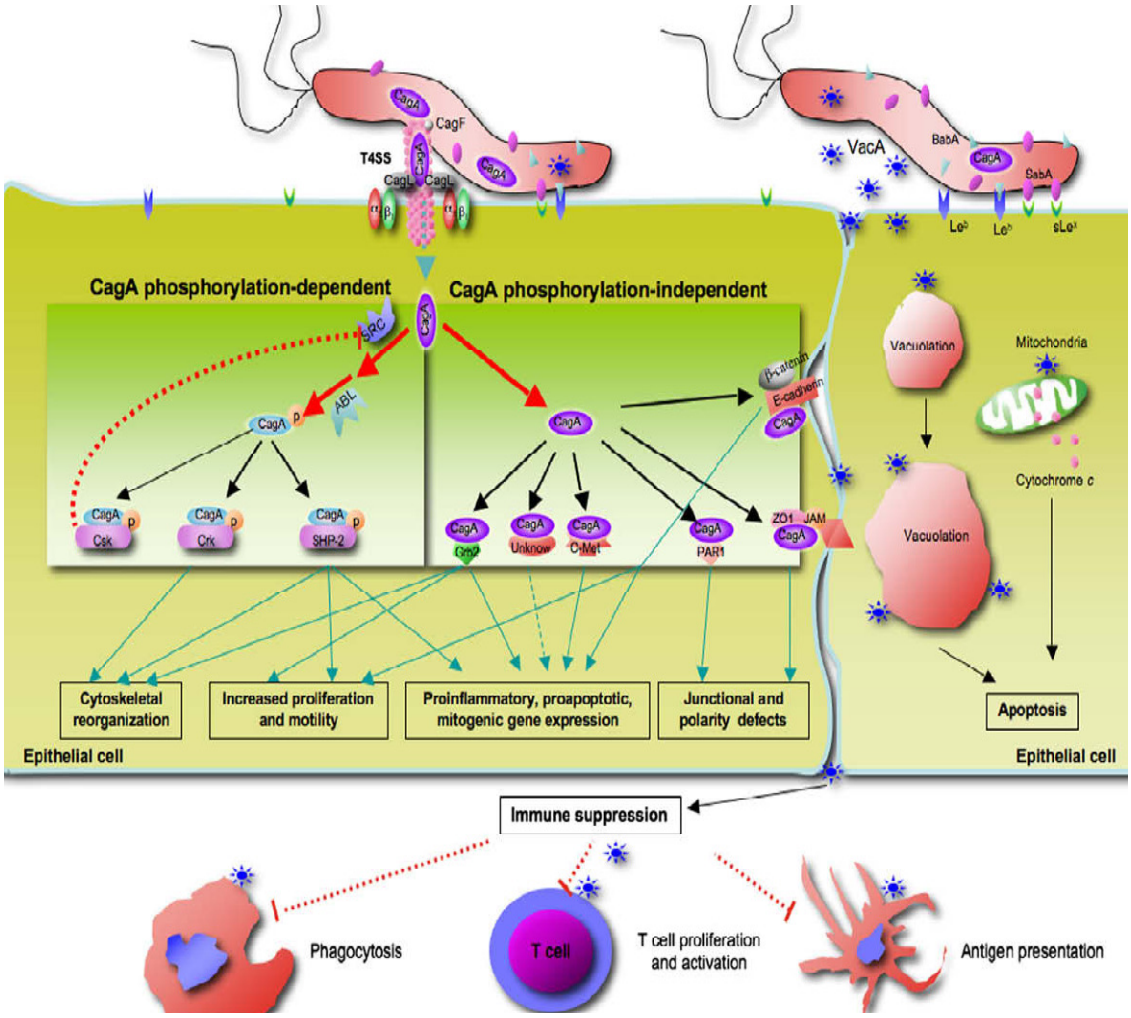
A recent study has demonstrated that strains with genotype vacA s1/m1, but without s2/m2 mediate channel activity and cellular vacuolation in intoxicated cells, which results in a detectable reduction of mitochondrial transmembrane potential and the release of cytochrome c. This suggests a functional correlation between VacA channel activity and changes in mitochondrion membrane permeability [92,94].

1.2.5.4 ROS Production and Helicobacter Pylori Infection

Reactive oxygen species (ROS) has an important role in carcinogenesis due to inducing DNA damage. In a study, this is found that fraction of expressed CagA protein localizes to mitochondria and produce significant amount of ROS in the cells via transfecting cagA gene in gastric epithelial cells [95]. In addition, increased ROS production might lead to acceleration of cell cycle and subsequent cell proliferation [96]. The underlying mechanism of CagA induce ROS production in gastric epithelial

cells are not known. However, CagA localize to mitochondria may deregulate the function of mitochondria electron transport chain and might be cause production of primarily super oxide (O^{-2}).

As described, CagA induces ROS production in mitochondria. The mitochondrial DNA (mtDNA) is more vulnerable to ROS damage than nuclear DNA because of its proximity to the electron transport chain and it's deficient in protective histones or DNA-binding proteins [97]. In addition, some cell has little amount of repair enzymes



S. Wen, S.F. Moss, Helicobacter pylori virulence factors in gastric carcinogenesis, Cancer Lett. (2008) [107]

Figure 1.1 Infection mechanism of Helicobacter Pylori. Helicobacter pylori infection cause many changes in signaling pathway that lead cells to many physiological response such as apoptosis, cytoskeletal reorganization, proliferation.

so mtDNA damage cannot repaire sufficiently. [98]. Consequently, the respiratory enzymes containing the defective mtDNA encoded protein sub-units may exhibit impaired electron transport function and thereby increase the electron leak and ROS

production, which in turn elevate the oxidative stress and oxidative damage to mitochondria. As a result, CagA might induces oxidative stress to the gastric mucosa, and may damage cellular components, containing polyunsaturated fatty acids, proteins and mtDNA, which may enhance nuclear DNA damage, and possibly result in the pathogenesis of gastric carcinogenesis. [99]

In addition to that, intracellular ROS induce 8-OHdG production and this cause point mutation in the DNA, thus disrupting the expression and function of several tumor suppressing genes such as p53, which might contribute to the pathogenesis of gastric cancer [100]. Therefore, in younger patients with cagA-positive H. pylori, DNA oxidative damage in the gastric mucosa is accumulated in earlier stage of their life, and may cause more extensive gastric mucosal derangement [101,102].

1.2.5.5 Host Immune Response

Toll-like receptor (TLR) molecules play a frontline role in the defence of the host against infection by microbial pathogens. These molecules, together with the recently described Nod family proteins, have been shown to trigger innate immune responses in host cells via the recognition of highly conserved microbial structures. TLR4, which is the best-characterised of these “pathogen-recognition molecules” (PRMs), was the first to be shown to recognise a specific microbial component: the lipopolysaccharide (LPS) from Gram-negative bacteria [103].

It appears likely that epithelial cells play an important role in innate immune signalling to *H. pylori*. Moreover, *H. pylori*–epithelial cell interactions may condition the type of immune responses induced by the bacterium. Indeed, it is well known that in Western populations, certain *H. pylori* strains with the capacity to alter normal epithelial cell functions are more frequently associated with severe forms of gastric inflammation, peptic ulceration and an increased risk of gastric cancer [70,104-106].

1.3 Wnt Pathway

Wnt pathway was originally described in Drosophila as Wingless pathway and is highly conserved among flies, frogs, and mammals. The combined effort of genetic, biochemical and developmental research has led to the comprehensive understanding of the Wnt pathway. More than 20 years ago, the oncogenicity of a Wnt ligand was revealed in a series of experiments derived with random proviral integration in mice. The most studies are realted with part of this pathway which is referred to as the canonical Wnt pathway that leads to transcriptional activation of specific genes. The significance of Wnt signaling in human cancer has since been supported by the identification of mutations in genes coding for the Wnt pathway components Axin, APC, and b-catenin. [109-111].

There are 19 Wnt proteins known only some of which, such as Wnt1, Wnt3a and Wnt8 activate the canonical Wnt/β-catenin pathway. Wnts ligands bind to seven pass transmembrane receptors named frizzelds and to co-receptors LRP-5 and LRP-6, which are essential for signal transmission [112-114]. After activation of Wnt signaling dishevelled becomes recruited to the plasma membrane probably through interaction with phospholipids [122]. It is thought that this step leads to activation of dishevelled which can then interfere with β-catenin degradation. The exact nature of the activation mechanism of dishevelled is not known although phosphorylation of dishevelled might be important [123-125]. The levels of cytoplasmic β–catenin are normally controlled by a multiprotein destruction complex which targets β–catenin for degradation in proteasomes [120,126]. This complex is assembled over the scaffold component axin or its homologue conductin, which contain binding domains for β–catenin, the tumor suppressor APC (adenomatous polyposis coli), the serine/threonine kinases GSK3β and casein kinase 1α/ε [127-132]. The main function of the destruction complex is to promote phosphorylation of β–catenin which is required to trigger ubiquitination of β–catenin and its subsequent degradation in proteasomes [133]. Unphosphorylated ß-catenin then binds to transcription factors of the T-cell factor/lymphoid-enhancer factor (Tcf/Lef) family and activates the transcription of specific target genes including c-Myc, cyclin D1, MMP7, gastrin and ITF-2 [134] (Figure 1.2).

In the absence of WNT ligands, β-catenin is recruited into a 'destruction complex' that contains adenomatous polyposis coli (APC) and AXIN, which assist the phosphorylation of β-catenin by casein kinase 1 (CK1) and then glycogen synthase kinase 3 (GSK3). This leads to the ubiquitylation and proteasomal degradation of β-catenin. In the nucleus, potential target genes of the pathway are kept in a suppressed state by interacting with T-cell factor (TCF) and lymphoid enhancer-binding protein (LEF) transcription factors,with associated co-repressors. So, in the 'off state', cells maintain low cytoplasmic and nuclear levels of β-catenin, although β-catenin is associated with cadherins at the plasma membrane, an association that spares it from the degradative pathway [115].

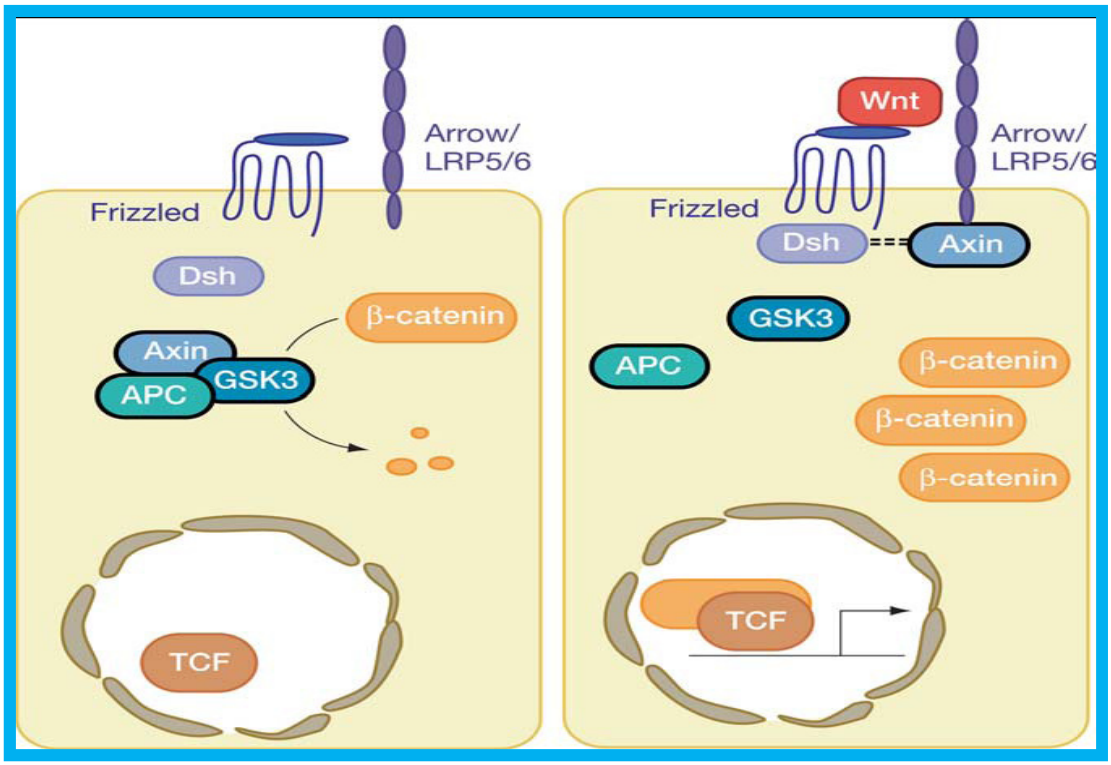


Figure 1.2 Overview of the Wnt pathway. In the absence of a Wnt signal, b-catenin binds the APC/Axin/GSK complex, is phosphorylated, and subsequently degraded. Wnt binding causes phosphorylation of Dsh, which leads to dissociation of the APC/Axin/GSK complex. Free b-catenin can accumulate, translocate to the nucleus, and after binding to TCF it activates transcription of a number of target genes, including cyclin D1, c-Myc and matrix metalloproteinase (MMP-7).

Wnt pathway defect is reported in 70–80% of colorectal cancers case [116, 117]. Most frequently APC mutations are found, but a subset of tumors wild-type for APC carry β-catenin mutations. The current model of colorectal carcinogenesis predicts that at least four mutations in critical genes are required for the evolution of colorectal cancer [118, 119]. The earliest adenomatous stages are associated with inactivating APC mutations, followed by mutations of the oncogene K-Ras and inactivation of tumor-suppressor genes containing p53. Approximately 20–30% of hepatocellular carcinomas (HCCs) carry mutations in the Axin or the β-catenin gene [120] also cause deregulation of Wnt signaling. The epidemiological importance of cancers associated with defects in the Wnt pathway is evident. Colorectal cancer is one of the leading causes of cancer-related morbidity and mortality in Western countries. In Europe alone more than 210,000 new cases and 110,000 deaths are reported each year [121] and the risk of developing colorectal cancer during a lifetime is about 5– 6%. While colorectal cancer is much more common in developed than in developing countries, HCC is the most frequent cancer in some regions of the world [121].

The Wnt family consists of at least 19 cysteine rich glycoproteins. In animal models and tissue cell lines, Wnts have been shown to have a direct involvement in neoplastic transformation. For example, a number of head and neck squamous cell carcinoma cell lines have increased levels of the mRNA of Wnt 1, 7a, 10b, and 13 [135]. Blocking Wnt 1 signalling has been shown to lead to reduced cell proliferation and increased apoptosis. These effects were accompanied by reduced expression of b-catenin and cyclin D1 [135]. Wnt11, a polypeptide containing 354 amino acids, whose mRNA is normally expressed in adult liver, heart, pancreas, and skeletal muscle, is upregulated in the human gastric cancer cell line, MKN45 [136]. Changes in Wnt levels have been described in some tumours. For example, Wnt 2 mRNA is detectable in colon cancer, but is not found in normal colonic mucosa [137]. In most human tumours there is no direct evidence linking the Wnt proteins themselves to carcinogenesis, but mutations mimicking Wnt stimulation lead to activation of the pathway during carcinogenesis.

Most Wnt proteins can bind to multiple Frizzled receptors. The co-receptors LRP-5 and LRP-6 are members of the single transmembrane low density lipoprotein receptor related protein (LRP) family [138]. Disruption in the synthesis of either receptor leads to a dramatic reduction in signalling [139]. In Drosophila, LRP (known as arrow) is essential for Wnt signalling. It has been shown that canonical Wnt signalling is only initiated when Wnt is complexed with both FZD and LRP [138,140,141]. In Xenopus embryos, LRP activates Wnt signalling and induces Wnt-responsive genes [138,140]. Mouse embryos mutant for LRP6 show developmental defects similar to those caused by mutations in Wnt genes [141].

Some data also exist on the expression of Frizzled receptors in tumour tissues. In colonic tumours, it has been shown that while FZD 1 and 2 are not expressed in normal colonic mucosa or in well differentiated tumours, there is a high degree of expression in poorly differentiated tumours, especially at the margin of cellular invasion [137]. This would suggest that receptor expression may be associated with tumour invasion and may therefore be useful as a prognostic marker.FZD10 has also been shown to be upregulated in some cases of colon cancer [142]. Similarly, FZD 2 and 3 are upregulated in both oesophageal and gastric tumours, associated with increased nuclear staining of b-catenin and cyclin D1 expression [143,145]. Other FZDs that have been shown to be upregulated in gastric cancer are FZD 7, 8, 9, and 10 [143,144].

1.3.1 The non-canonical Wnt pathways

Wnt binding may also lead to signal transduction via two other pathways. First, binding to some FZD receptors leads to intracellular calcium release and activation of protein kinase C (PKC). Alternatively, Dsh also activates the jun-N terminal kinase (JNK) pathway. JNK translocates to the nucleus where it regulates the activity of transcription factors such as c-jun, ATF 2, Elk1, DPC4, and p53.

In addition to a potential role in b-catenin/TCF-induced tumour formation, Rho proteins and their regulators influence cell motility and polarity by interacting directly

with components of Wnt signalling pathways. The Racspecific GEF Asef was cloned because of its ability to interact with the armadillo repeat domain of APC [146]. This interaction leads to the activation of the GEF function of Asef, through relief of its auto-inhibition. Overexpression of Asef decreases E-cadherin-mediated cell–cell adhesion and promotes the migration of MDCK cells [147].

Additionally, it was shown recently that Wnt/Fz signalling activates Rac and Rho independently of each other and of b-catenin/TCF signalling. The adaptor protein Dvl plays an important role in this Wnt-induced activation of Rac and Rho, which controls morphogenetic movements during vertebrate gastrulation [148]. The GEFs involved in Dvl-mediated activation of Rac and Rho are still unknown, and whether or how these pathways regulate motility of tumour cells or other aspects of tumourigenesis still remains to be established.

1.4 Objectives

In many gastric cancer cases, increased level of Wnt pathway activation is observed. As explain above, there are many studies to explain the exact underlying molecular mechanism of wnt pathway activation in gastric cancer. However previous studies are not sufficient to clarify.

In order to show the pathway activation, Kirikoshi et al. performed a study that is showing the increased level of some frizzled genes in gastric cancer patients and gastric cancer cell lines in 2001. However, in this study is completed only 10 gastric cancer tissues that is not enough to obtain statistically reliable results. Therefore, we decided to increase the tissue number to obtain statically important data. Additionally, in order to show the pathway activation we decided to analyze the β-catenin gene expression level as well. Theoretically, if frizzled receptors, wnt ligands, and β-catenin genes are expressed, pathway will be activated. To show that, we decided to analyze 7 members

of Frizzled genes those are FZD1, 2, 3, 4, 7, 8, 10, and 8 members of Wnt ligands those are Wnt 1, 2b, 3, 4, 5a, 5b, 6, 9a and β-catenin gene.

As explain above, Helicobacter Pyori is the one of the most important risk factor for the gastric cancer. Previously, many studies were performed in order to understand the role of Helicobacter Pylori infection in gastric cancer. However, these data did not provided satisfactory information about the underling molecular mechanism in cell. In fact, some studies were conducted with effect of Helicobacter Pylori on cellular signaling mechanism, but most researches were conducted with the effect of Helicobacter Pylori on MAPK signaling pathway. As elucidated above, Wnt in gastric cancer cases, Wnt pathway is the one of the most active signaling pathway. Therefore, we also aimed to clarify effect of Helicobacter pylori on wnt singling pathway.

On the other hand, this is well known that Helicobacter pylori trigger the cancer formation. However, some previous studies showed that Helicobacter pylori infection lead to cells to apoptosis. But, as it well known, while apoptosis mechanism is evaded, proliferation mechanism should be activated in cancer formation. Considering these previous information, we decided to find out whether Helicobacter pylori infected cells going under apoptosis or proliferation.

CHAPTER 2

MATERIAL and METHODS

2.1 MATERIALS

2.1.1 General Reagents

All laboratory chemicals were analytical grade from Sigma Biosciences Chemical Company (USA), Merck (Germany), Applichem(Germany) and Fluka(Germany).

2.1.2 Tissue Samples

All patient samples were collected by gastric surgery in Cerrahpasa Medical School. Collected cancerous and normal tissues were stored -80 °C in Cerrahpasa Medical School pathology laboratory. They were transferred to Fatih University.

2.1.3 Tissue Culture Reagents

Dulbeco's Modified Eagle's Medium (DMEM), RPMI 1640 Medium, nonessential amino acids, penicillin/streptomycin, tyripsyn/EDTA, and fetal bovine serum (FBS) were purchased from Biochrom, Berlin, Germany. Tissue culture petri

dishes, 15 and 50 ml polycarbonate centrifuge tubes with lids and cryotubes were from Grainer Bio-One Corp., Germany.

2.1.4 Oxidative Stress Reagents

2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was from Sigma, Germany. Anti-8dOHG monoclonal antibody was purchased from Cosmo Bio Corp. Ltd, Japan.

2.1.5 Apoptosis Assay Reagents

Caspase 3 Colorimetric Assay Kit was from Sigma, Saint Louis, Missouri, USA, In Situ Cell Death Detection Kit was from Roche Applied Science, Mannheim, Germany, and DNA extraction Kit was From Machery-Nagel, Germany.

2.1.6 Immunochemistry and Immunofluorescence Reagents

Paraformaldehyde, Bovine Serum Albumin (BSA), and Hoechst 33258, TritonX-100 were purchesed from Sigma, Germany. Mouse anti β-Catenin monoclonal antibody was from Abcam, England. Mouse ant Ki-67 (Ki-67) and CyclinD1 (A-12) monoclonal antibody was puchased from SANTA CRUZ BIOTECHNOLOGY, INC. and immunochemistry kit which is including Horse Redish Peroxidase conjugated secondary antibody was purchased from DAKO, CA, USA.

2.1.7 Cell Lines

Two gastric adenocarsinoma derived, namely MKN45 and 23132/87 cell lines were obtained from German Resource Centre for Biological Material (DSMZ).

2.1.8 Polymerase Chain Reaction Reagent

Polymerase chain reaction kit was from Fermentas, Germany. Primers used were synthesized by IONTEK (Istanbul, TURKEY). Sequence of the primers used is given in Table 2.3 and Table 2.4.

2.2 Equipment

Autoclave	: CERTO CLAW A-4050 Traun, Austria
Balance	: Sartorius, Wender Landstrasse 94-108 D-37075 Goettingen, Germany
Centrifuge	: Hettich, Mikro 22
Electrophoresis Equipment	: Bio-Rad Sub Cell, GT
Power Supplies	: Bio-Rad Power PAC-300
Thermocyclers	: TECHNE TC-512
Transilluminator	: Bio-Rad GelDoc 2000
Vortex	: IKA LABOTECHNIK
Water Purification System	: Millipore
Inverted Light Microscope	: Zeiss

Fluorescent Microscope	: Zeiss
Laminar Flow Hood	: Esco, Kotterman
Microplate Reader	: Bio-Tek
CO_2 Incubator	: Thermo, Sanko
Spectrophotometer	: Shimadzu
Digital Photograph Machine	:Canon

2.3 Solutions and Media

2.3.1 Tissue Culture Reagents

Standart Medium	2 mM L-Glutamine, 0,1 mM nonessential amino acid, 100U/ml penicilin, 100 µg/ml streptomycin and supplemented with 10% FBS in DMEM or RPMI 1640 medium.
Freeze Medium	65% RPMI 1640 control medium, 25% FBS, and 10% DMSO in 1 ml per cell line.
10X PBS	80 gram NaCl, 2 gram KCl, 14,4 gram Na_2HPO_4, 2,4 gram KH_2PO_4 in 1 lt dd H_2O, pH 7.2. Diluted working concentration of 1X.

2.3.2 Oxidative Stress Reagents

DCFH-DA Solution	10 mM HEPES (pH 7.5), 10 mM glucose, and 1 µM DCFH-DA (dissolve in methanol) in 1X PBS. Prepared freshly.

2.3.3 Apoptosis Assay Reagents

2.3.3.1 TUNEL Assay Reagents

In Situ Cell Death Detection Kit	Bottle 1 (enzyme solution) and Bottle 2 (label solution). Store at -20 °C.
Tunel Reaction Mixture	50 µl from bottle 1 is added to 450 from bottle 2, mixed and kept on ice. Prepared freshly.
Permeabilization solution	0,4% TritonX-100 in 1X PBS.
Fixation Solution	4% paraformaldehyde or 4% formaldehyde.

2.3.3.2 Protein Assay Reagents

Bio-Rad Protein Assay Dye Stock	100 mg Coomassie Brilliant Blue G-250 in 40 ml 95% methanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved. Kept in dark at 4 °C.
Bio-Rad Protein Assay Working Solution	Bio-Rad Protein Assay Dye Stock was diluted 400 times with water and filter through a whatmann filter paper just before use.

2.3.3.3 Caspace3 Assay Reagents

5X Lysis Buffer	250 mM HEPES, pH 7.4, 25 mM CHAPS, 25 mM DTT
10X Assay Buffer	200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, 20 mM EDTA
Caspase 3	Reconsitution with 50 µl of 17 megohm water will give a solution of 100 µg/ml caspase 3 in 50 mM HEPES, pH 7.4, 0.1% CHAPS, 10 mM DTT, 100 mM NaCl, 1 mM EDTA and 10% sucrose Specific Activity : >1 unit per mg protein. Unit

	definition: One unit is the amount of enzyme that will cleave 1.0 μmol of the substrate Ac-DEVDpNA per minute at pH 7.4 at 25 °C.
Ac-DEVD-pNA Substrate,	Acetyl-Asp-Glu-Val-Asp p-nitroanilide
Ac-DEVD-CHO Inhibitor,	Acetyl-Asp-Glu-Val-Asp-al
p-Nitroaniline Standard,	
Water (17 megohm)	

2.3.3.4 DNA Fragmentation Assay Reagents

MN Nucleospin Tissue Kit Components	Lysis Buffer, Buffer B1, Buffer B2, Wash Buffer B5, Wash Buffer BW, Elution Buffer BE, Proteinase K (lyophilized), Proteinase Buffer PB.

2.3.4 Immunochemistry and Immunofluorescence Reagents

Fixation Solution	4% paraformaldehyde (4 gram paraformaldehyde in 10 ml 1X PBS. Stir 4 hours at 58 C and alliquot) or 4% formaldehyde, store at -20 °C.

Permeabilization solution	0,4% Triton X-100 in 1X PBS
Hoechst 33258 solution	Stock solution of 300 µg/ml in ddH_2O. Diluted to working concentration of 3 µg/ml. Store at 4 °C.
BSA 1%	BSA (1 gram in 100 ml) in 1X PBS.
Antibody Diltion	Antibodies were diluted to ratio from 1/100 to 1/500 with 1% BSA in 1X PBS.

2.4.5 RT-PCR Reagents

2.4.5.1 RNA Isolation Reagents

MN-Nucleospin RNA II kit components	Lysis Buffer RA1, Wash Buffer RA2, Wash Buffer RA3, Membrane Desalting Buffer MDB, Reaction Buffer for rDNase rDNase, RNase-free (lyophilized), RNase-free Water.

2.4.5.2 cDNA Synthesis

RevertAidTM M-MuLV cDNA syhthesis Kit (Fermentas) kit components:

Reverse Transcriptase (200u/µl)	120 µl of the enzyme solution in storage buffer: 50mM Tris-HCl(ph8,3), 0,1M NaCl, 1mM EDTA, 5mM DTT, 0,1% Triton X-100 and 50% glycerol.
RiboLock™ Ribonuclease Inhibitors (20u/µl)	120 µl of the enzyme solution in storage buffer: 20mM HEPES-NaOH (ph7,5), 50 mM NaCl, 8mM DTT, 0,5mM ELUGENT Detergent and 50% glycerol.
5X Reaction Buffer Tris-	500 µl of 5X reaction buffer: 250mM HCl (pH 8,3 at 25 C), 250mM KCl, 20mM MgCl2, 50mM DTT.
10mM dNTP Mix	250 µl of 10mM aqueous solution of each dGTP, dATP, dCTP, dTTP.
Oligo$(dT)_{18}$ Primer	120 µl of 0,5 ug/µl ($15A_{260}$ units/ml) aqueus solution.
Random Hexamer Primer aqueus	120 µl of 0,2 ug/µl ($6A_{260}$ units/ml) solution.
Control Primer	40 µl of 10pmol/µl ($1,7A_{260}$ units/ml) 17-mer aqueus solution.

Control RNA	40 µl of 1,1kb RNA with 3'-poly(A) tail, 0,5 ug/µl.
DEPC-Treated Water	2X1,5ml of water deionized on a Milli-Q system and treated with DEPC.

2.4.5.3 PCR Reagents

PCR kit component (Fermentas):

10X Taq Buffer with KCl	100mM Tris-HCl (pH 8.8 at 25°C), 500mM KCl, 0.8% Nonidet P40.
10X Taq Buffer with (NH4)2SO4	750mM Tris-HCl (pH 8.8 at 25°C), 200mM (NH4)2SO4, 0.1% Tween 20. $MgCl_2$ 25mM $MgCl_2$
dNTP mix	1,5ml of 2mM aqueous solution of each dGTP, dATP, dCTP, dTTP.
Primers	10 pmol/reaction.
Tag Polymerase	5U/µl in 20mM Tris-HCl (pH 8.0), 1mM DTT, 0.1mM EDTA, 100mM KCl, 0.5% Nonidet P40, 0.5% Tween 20 and 50% glycerol.

2.3.5.4 Agarose Gel Electrophoresis

10X TAE	48.4 g Tris and 2.92 g EDTA were balanced dissolve in ddH_2O and Glacial Acetic Acid 11.2 ml was added after dissolving completely volume was adjusted to 1 lt and pH was adjusted to 8,4.
6X Loading Dye	10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA.
GeneRuler™ 100 bp DNA Ladder	100 µl (0.5µg/µl) 100 sized DNA fragments in 10mM Tris-HCl (pH 7.6), 1mM EDTA.

2.3.6 Bacteria Culture Reagents

Transfer Solution (0,9 % saline)	4,5 gram NaCl was dissolved in 500 ml ddH_2O and sterilized by autoclaving.
Clo Test	0,75 gram bacto agar dissolved in 45 ml water and autoclaved. 1,45 gram Urea Agar base and 1 gram urea were dissolved in 5 ml ddH_2O, sterilized by filter. Solutions were mixed to each other.

Helicobacter Pylori Selective Supplement (Oxoid)	1 vial is composed of 5.0mg Vancomycin 2.5mg Trimethoprim, 2.5mg Cefsulodin, 2,5 mg Amphotericin B.

2.4 Methods

2.4.1 Tissue Culture

2.4.1.1 Growth Conditions

Cell lines were cultured at 37 °C under 5% CO_2 in control medium unless otherwise specified.

2.4.1.2 Thawing Cell Lines

Previously frozen cell in liquid nitrogen was taken out from the tank, immediately soak into pre-warmed water bath and incubate in water bath at 37 °C until totally thawed. Thawed cell gently mixed by pipetting and transferred to 15 ml tube which is containing 5 ml growth medium. Than cells were centrifuged at 1500 rpm for 5 to 8 min and supernatant carefully aspirated. Lastly cell pellet was resuspended in 8 ml growth medium and transferred to sterile culture flask. Culture flask size is determined according to cell pellet size. Culture flask is incubated at CO_2 incubator under humidified microaerofilic condition with 5% CO_2. Following day cells were observed under invert microscope and if necessary cells were subcultured according to their confluency or medium is replaced with fresh growth medium after washing PBS.

2.4.1.3 Sub-Culture of Cell Lines

Cultured cells were observed under microscope and if the confluency is about 70-80% and there is no contamination or other abnormal formation, cells are determined to sub-culture. Culture medium is aspirated and cells were washed for 3 times with pre-warmed PBS at 37 °C. After washing cells were detached by treatment with Tyripsin/ EDTA solution for 3-5 min until all cells detached. 3 volume growth medium is added to detached cells to stop the tyripsin/EDTA activity. Than cells were splited and diluted dependen to aim, transferred to new culture flask, and incubated in CO_2 incubator at 37 °C under humidified condition.

2.4.1.4 Cryopreservation of Cell Lines

Medium of the cells that are 70-80% confluent was aspirated, the cells were washed with pre-warmed PBS for 3 times, treated with 2,5 ml tyripsin/EDTA, and incubated at 37 °C for 3-5 min to be detached. In order to inactivate tyripsin/EDTA, 7,5 ml growth medium was added. Cells wer counted with hemacytometer and centrifuged at 1500 rpm at 4 °C for 5 min, the cell pellet was gently resuspended in freezing medium in cryovials. The vials were kept in -80 °C overnight and transferred to liquid nitrogen tank the next day.

2.4.2 Detection of Oxidative Stress in Gastric Adenocarsinoma Cell Lines

2.4.2.1 DCFH-DA Staining

1.5X 10^5MKN45 and 23132/87 cells were plated on 6 well plate. Cells were incubated in 5% CO_2, 37 °C with DMEM growth medium overnight. Next day cells growth medium was aspirated and fresh medium is added which contain Helicobacter Pylori and incubated for 24 and 48 hours. After incubation medium and unbounded

bacteria were removed via aspirating medium and washed for 2 times with PBS. Following washing DCFH-DA reaction mixture is was added to cells and incubated for 15 min. Cells were washed pre-warmed PBS for 3 times and analyzed under fluorescent microscope. The non-fluorescent DCFH-DA can diffuse cell membranes into cytoplasm where it is deacetylated and entrapped as DCFH (2'-7'-dichlorodihydrofluorescein) from. Creation of highly fluorescent product, DCF consequent to oxidation of DCFH facilitates the detection of the cells exposed to oxidative stress under fluorescent microscope **[**108**].**

2.4.2.2 Staining with Mouse Anti- 8-dOHG Antibody

$1x10^5$ MKN45 and 23132/87 cells were plated on sterile glass slide in six well plate. Cells were incubated in 5% CO_2, 37 °C with DMEM growth medium overnight. Next day cells growth medium was aspirated and fresh medium is added which contain Helicobacter Pylori and incubated for 24 and 48 hours. After incubation medium and unbounded bacteria were removed via aspirating medium and washed for 2 times with PBS. After washing cells were fixed with 4% paraformaldehyde for 10 min on ice, washed with chilled PBS for 3 times and permeabilized with 0,4% TritonX-100 on ice for 5 min. After washing with PBS, endogenous peroxidase activity is blocked by treating with 3% H_2O_2 for 5 min to 30 min. Than mouse anti 8-dOHG antibody is diluted 1/40 in 1% BSA containing 1X PBS, treated cell with primary antibody for 2 hours. Following primary antibody incubation DakoCytomation LSAB2 System-HRP kit is used. The cells were incubated with biotinylated goat anti-rabbit and goat anti-mouse IgG for 30 min at room temperature and with streptavidin-HPR solution for 30 min at room temperature. Between incubations, cells were washed with 1X PBS (pH7.4). 3-3'- diamino benzidin tetrahidrochloride (DAP) was used as HRP substrate and sections were incubated with substrate-chromogen solution for 5 min. Finally, counterstaining was performed with Mayer's hemotoxylin to visualize nucleus.

2.4.3 Apoptosis Assay

2.4.3.1 TUNEL Assay

TUNEL assay was performed using In Situ Cell Death Detechtion Kit according to manufacturer's recommendation. Cells were seeded on sterile cover slip in six well plates. Cells were incubated in 5% CO_2, 37 °C with DMEM growth medium overnight. Next day cells growth medium was aspirated and fresh medium is added which contain Helicobacter Pylori and incubated for 24 and 48 hours. After incubation medium and unbounded bacteria were removed via aspirating medium and washed for 2 times with PBS. After washing cells were fixed with 4% paraformaldehyde for 10 min on ice, washed with chilled PBS for 3 times and permeabilized with 0,4% TritonX-100 on ice for 5 min. Tunel reaction mixture was prepared as recommended in kit manual. 1 volume enzyme solution and 9 volume label solution is mixed, 50 µl reaction mixture was added to all cover slip, to avoid the evoparation seal the top of glass slide with parafilm or with another cover slip, incubate for 60 min in 37 °C at humidified atmosphere under dark. Following incubation slide was washed with PBS and observed under fluorescent microscope.

2.4.3.2 DNA Fragmentation Assay

Cells were plated to six well plate and infected with H. Pylori for 24 and 48 hours. 1.10^5 cell were cultured MKN-45 and 23132/87 cells were cultured in six well plates. Firstly old medium was removed from cultured flask and PBS is added to cells for washing. Washing step is repeated for 3 times. Cells were collected with scraper and put in a micro centrifuge tube. DNA extraction was performed according to MN-Nucleospin tissue kit protocol step by step as describe below:

- 1 x10^5 cells was resuspended in a final volume of 200 µl Buffer T1. 25 µl Proteinase K solution and 200 µl Buffer B3 were added. Samples were incubated at 70°C for 10-15 min.

- In order to adjust bindign condition 210 µl ethanol (96-100%) to the sample was added and vortexed vigorously.

- For each sample, one NucleoSpin® Tissue Column was placed into a Collection Tube. The sample was loaded to the column, was centrifuge for 1 min at 11,000 x *g*. Flow-through was discarded and place the column back into the Collection Tube.

- 500 µl Buffer BW was added, centrifuged for 1 min at 11,000 x *g*. Flow-through was discarded and placed the column back into the Collection Tube.

- 600 µl Buffer B5 was added to the column and centrifuged for 1 min at 11,000 x *g*. Flow-through was discarded and placed the column back into the Collection Tube.

- In order to dry and removethe residual ethanol from membrane empty column was centrifuged for 1 min at 11,000 x *g*.

- NucleoSpin® Tissue Column was placed into a 1.5 ml microcentrifuge tube and 100 µl prewarmed Elution Buffer BE (70°C) was added, incubated at room temperature for 1 min, centrifuged 1 min at 11,000 x *g*.

- Highly pure DNA is extracted at the end of procedure and eluted DNA concentration and purity is calculated using spectrophotometer.

  Extracted DNAs were run on 1% agarose gel. Observed under Bio-Rad Transimilator.

2.4.3.3 Caspase3 Assay

1, 5 x 10^6 cells were plated on culture flask. Caspase 3 enzyme activity assay was performed whether cells going under apoptosis or not. To show that Caspase 3 Assay Kit (Sigma) was used and assay was performed as manufecturer's recommendation as describe below step by step:

- Apoptosis was tested in a cells of MKN45 and 23132/87 cells (at least 10^6 cells) by infection of Helicobacter Pylori final concentration of 100 MOI. Sample of non-induced cells for a zero-time control was reserved.
- Incubated for 2.5 to 3 hours at 37 °C in a 5% CO_2 atmosphere.
- The infected cells and the control cells were pelleted by centrifugation at 600 x *g* for 5 minutes at 4 °C.
- Supernatant was removed the by gentle aspiration.
- Cell pellets were washed once with 1 ml of PBS, centrifuged, and removed the supernatant completely by gentle aspiration.
- Cell pellets were suspended in 1X lysis buffer at a concentration of 100 µl per 10^7 cells.
- Cells were incubated on ice for 15-20 minutes.
- Lysed cells were centrifuged at 16,000 to 20,000 x *g* for 10 to 15 minutes at 4 °C.
- Supernatants were transfered to new tubes.
- Lysates was freezed in liquid nitrogen and store in aliquots at –70 °C.

Protein concentration of collected cell lysates were determined by using Commassie Brillinant Blue based Bio-Rad Protein Assay kit is used according to manufecturer's recommendation as describe below:

In order to perform the protein assay BSA standard was prepared as shown in Table 2.1.

In parallel, protein samples were prepared by mixing 160 µl Bio-Rad working solution with ddH_2O (30-38 µl) and sample (2-10 µl) in a tube. All BSA standards and samples were mixed and incubated at room temperature for from 5 to 30 min. than absorbance of samples was read at 595 nm using a UV-visible spectrophotometer in disposable plates. By using Microsoft Excel graphic program, a standard curve is

obtained, BSA concentration on x-axis and absorbance on y-axis, with a line formula of y=mx+c. The concentrations of the samples of interest are calculated according to the line formula.

Table 2.1 BSA standard preparation chart.

Tubes	b	1	2	3	4	5
ddH_2O(µl)	40	38	36	34	32	30
BSA(1mg/ml) (µl)	0	2	4	6	8	10
Bio-Rad Working Solution(µl)	160	160	160	160	160	160

After collection of cell lysate, 96 well assay procedure was applied as describe below:

- 5 µl of cell lysate or Caspase 3 Positive Control was placed in the appropriate wells as indicated in Table 2.2.
- 1X Assay Buffer was added to each of the wells as indicated in Table 2.2.
- Caspase 3 Inhibitor was added to the appropriate wells.
- Reaction was started by adding 10 µl of caspase 3 substrate to each well and mixed gently by shaking. Tried to avoid forming bubbles in the wells.
- Plate was covered and incubated at 37 °C for 70 to 90 minutes.
- Absorbance was read at 405 nm.
- Results were calculated using a p-nitroaniline calibration curve.

Table 2.2 Caspase 3 enzyme activity reaction scheme for 96 well plate.

	Cell lysate	Caspase 3 5 µg/ml	1x Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO 200 µM	Caspase 3 substrate Ac-DEVD-pNA 2 mM
Reagent blank	----	----	90 µl	----	10 µl
Non-induced cells	5 µl	----	85 µl	----	10 µl
Non-induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Induced cells	5 µl	----	85 µl	----	10 µl
Induced cells + inhibitor	5 µl	----	75 µl	10 µl	10 µl
Caspase 3 positive control	----	5 µl	85 µl	----	10 µl
Caspase 3 positive control + inhibitor	----	5 µl	75 µl	10 µl	10 µl

Calibration curve is prepared as recommended kit manual as describe below:

- A series of p-nitroaniline solutions were prepared at the concentration range of 10 to 200 µM by diluting the p-nitroaniline stock solution in 1X Assay Buffer.
- 100 µl of each dilution was added to a well, include 100 µl of assay buffer as a blank.
- Absorbance was read at 405 nm.
- Calibration curve was prepared of the absorbance values versus the concentrations of the p-nitroaniline solutions. Alternatively, plot the OD405 values versus the amount of p-nitroaniline per well, in µmol, using the following table 2.3.

Table 2.3 Calibration curve preparation scheme for p-Nitroaniline.

µM p-Nitroaniline	µmol p-Nitroaniline per 100 µl
10	0.001
20	0.002
50	0.005
100	0.01
200	0.02

2.4.3.4 Hoechst 33258 Staining

Cells were plated to six well plate and infected with H. Pylori for 24 and 48 hours. 1.10^5 cell were cultured MKN-45 and 23132/87 cells were cultured in six well plates. Firstly old medium was removed from cultured flask and PBS is added to cells for washing. Washing step is repeated for 3 times. Cell were fixed with methanol for 10 min. After fixation cell were washed for 3 times with PBS 3µM Hoechst 33258 dye was added and incubated for 5 minute.

Cells were washed with PBS. Finally excess amount of staining was removed by incubating ddH_20.

2.4.4 Immunocytohemistry

2.4.4.1 Staining of 23132/87 Cells

$1x10^5$ 23132/87 cells were plated on sterile glass slide in six well plate. Cells were incubated in 5% CO_2, 37 °C with DMEM growth medium overnight. Next day cells growth medium was aspirated and fresh medium is added which contain Helicobacter Pylori and incubated for 24 and 48 hours. After incubation old medium and unbounded bacteria were removed via aspirating medium and washed for 2 times with PBS. After washing cells were fixed with 4% paraformaldehyde for 10 min on ice, washed with chilled PBS for 3 times and permeabilized with 0,4% TritonX-100 on ice for 5 min. After washing with PBS, endogenous peroxidase activity is blocked by treating with 3% H_2O_2 for 5 min to 30 min. Than mouse anti β-Catenin, CyclinD1 and Ki67 antibodies were diluted from 1/250 to 1/1000 in 1% BSA containing 1X PBS, cell were treated with primary antibody for 2 hours. Following primary antibody incubation DakoCytomation LSAB2 System-HRP kit was used. The cells were incubated with biotinylated goat anti-rabbit and goat anti-mouse IgG for 30 min at room temperature

and with streptavidin-HPR solution for 30 min at room temperature. Between incubations, cells were washed with 1X PBS (pH7.4) for 3 replicate. 3-3'- diamino benzidin tetrahidrochloride (DAP) was used as HRP substrate and sections were incubated with substrate-chromogen solution for 5 min. Finally, counterstaining was performed with Mayer's hemotoxylin to visualize nucleus.

2.4.4.2 Fixation of MKN 45 Cells for Staining

MKN45 cells have lost their adhesion capacity to culture flask after infection with Helicobacter Pylori due to their tendency to clumps formation and disruption ability of Helicobacter Pylori on cell adhesion proteins.

Therefore MKN45 cells were cultured in culture flask, following infection, medium was transferred a 15 ml tube and centrifuged to collect clump formed cells. Subsequently cell pellet was resuspended in DMEM and put one drop to sterile poly-lysine coated or positively charged glass slide, incubated at room temperature until dry. Then the immunocytochemistry protocol was performed as explained above (Section 2.4.4.1).

2.4.5 Helicobacter Pylori Culture

2.4.5.1 Preparation of Columbia Based Blood Agar with Helicobacter Pylori Selective Supplement

Firstly 19,5 gram Columbia Blood Agar Base was balanced and dissolved in 500 ml ddH_2O. Flask was put on a heater with magnetic stirrer and boiled until dissolve completely, sterilized by autoclaving 15 min at 121 °C, than cooled to 50 °C.

In parallel, 1 vial of Helicobacter Pylori Selective Supplement Dent was opened aseptically, reconstituted by adding 2 ml sterile ddH_2O and mixed by pipeting. When Colubia Blood Agar Base's temperature reached to 50 °C, reconstituted Helicobacter Selective Supplement Dent was added. Subsequently 35 ml defibrinated horse blood was added, mixed under aseptic condition and poured into sterile Petri dishes. Petri dishes were cooled in order to solidify under UV light for 1 hour at room temperature and then sealed with stretch film and store at 4 °C.

2.4.5.2 Isolation of Bacteria from Patient with Gastric Disorder, Primary Culture, and Sub-Culture of Helicobacter Pylori

Gastric biopsy specimens were collected from patient with gastric disorder from Cerrahpasa Medical School/Istanbul University, Istanbul. Biopsy taken out from patient and immediately is placed in sterile 40 µl, 0,09 % saline solution. Than biopsy specimen was transferred to laboratory at room temperature within 2-4 hours. When its reached the microbiology laboratory, immediately 200 µl sterile saline solutions was added to biopsy specimen and homogenized by homogenizer.

Homogenized tissue solution was transferred to Columbia Based blood agar which is containing 5 % horse blood. Transferred tissue solution was spread by steril loop on agar plate. Inoculated agar plates were incubated in humidified CO_2 incubator at 37 °C. Residual homogenized tissue solution was stored at -80 °C in Brucella Broth containing 30 % sterile glycerol for DNA extraction directly or as a bacteria stock when it's necessary.

When primary cultured bacteria colonies were observed, 5 different single colonies were inoculated in different new Petri dish via a sterile loop.

2.4.5.3 Clo (Campylobacter Like Organism) (Rapid Urease) Test Preparation

Clo (rapid urease) test was prepared as detailed below:

Solution 1:

- 0,75 gram bacto agar was balanced
- Dissoleved in 45 ml water
- Sterilized by autoclaving 15 min at 121 °C.

Solution 2:

- 1,45 gram Urea Agar base and 1 gram Urea was balanced.
- Put in a small beaker
- Mixed to ecah other and dissolved completely in 5 ml dH_2O water at room temparature.
- Sterilized with filter.

After finishing autoclave, solution 1 is cooled to 55 °C in a water bath. Solution 2 was added to solution 1 through a 0,2 µm pore sized filter and mixed until completely homogenized. Mixture was put in microcentrifuge tubes and stored at 4 °C for long time.

2.4.5.4 Characterization of Bacteria According to CagA and VacA Genes

Cultured bacteria were harvested with a wetted cotton swap and inoculate into 1 ml PBS solution in a microcentrifuge tube. After that bacteria were centrifuged at 10.000 rpm for 5 min. to obtain a bacteria pellet. Following this DNA extraction was performed as describe MN-Nucleospin Tissue Kit manual as describe below:

- Cell pellet was resuspend the in 180 µl Buffer T1 by pipetting up and down, 25 µl Proteinase K was added. Vortexed vigorously and incubated at 56°C until complete lysis was obtained (at least 1-3 h). During incubation tubes was vortexed occasionally or shaking incubator was used.
- Samples were vortex and 200 µl Buffer B3 were added. Tubes were incubated incubate at 70°C for 10 min after vortexing vigorously.
- After that step, DNA extraction procedure is performed as described in section 2.4.3.2.

Subsequently obtaining highly pure and concentrated DNA, CagA and VacA gene profile of bacteria was illustrated by multiplex PCR reaction as detailed below.

The primers used for the amplification of vacA alleles and cagA genes were listed in Table 2.5 and multiplex PCR reaction mixture content was prepared as shown in Table 2.4. Following preparation of the reaction mixture tubes were placed on thermal cycler. In order to amplification, 4 min at 95 °C for initial denaturation, a 35 cycle of 30 seconds denaturation at 95 °C, 45 seconds annealing at 55 °C, and 45 seconds extension at 72 °C is performed. The reaction is ended with 5 min final extension at 72 °C and then cooled to 4 °C for storage.

Table 2.4 Multiplex PCR reaction mixture content.

Reagent	Initial Concentration	Final Concentration	Final Volume
Taq Buffer	10X	1X	2,5 µl
dNTP	2mM	0,2uM	2,5 µl
$MgCl_2$	25mM	2mM	2 µl
Primers (x6)	25pmol/µl	25pmol	1 µl x 6 = 6 µl
ddH_2O	-	-	6,8 µl
Taq DNA Polymerase	5 U/µl	1 U	0,2 µl
Template DNA			5 µl
Total Reaction Volume			25 µl

Table 2.5 Primers used for the amplification of vacA alleles and cagA

Genotype	Primer	Primer Sequences	Size (bp)
vacA s1s2	VAI-F VAI-R	5'-ATGGAAATACAACAAACACAC-3' 5'-CTGCTTGAATGCGCCAAAC-3'	259/286
vacA m1m2	VAG-F VAG-R	5'-CAATCTGTCCAATCAAGCGAG-3' 5'-GCGTCAAAATAATTCCAAGG-3'	567/642
cagA	cag5c-F cag3c-R	5'-GTTGATAACGCTGTCGCTTC-3' 5'-GGGTTGTATGATATTTTCCATAA-3'	350

2.4.6 Infection of Cell Lines With Helicobacter Pylori

- Helicobacter Pylori was sub-cultured and incubated for 3 days in CO_2 incubator.
- 750 µl sterile PBS solution was added to a sterile ependorf tube for harvesting of bacteria.
- Bacteria were harvested with sterile loop or cotton swap which are in exponential phase and transferred to sterile PBS solution in microcentrifuge tube. This harvesting step was performed for many times to increase to collected bacteria mass.
- Bacteria which had been transferred to sterile PBS solution were homogenized with micropipet by pipetting and centrifuged in 7000rpm for 10 min.
- After centrifuge, supernatant was discarded and 750 µl sterile PBS was added and pellet re-homogenized.
- Resuspended pellet was centrifueged with 7500 rpm for 10 min.
- This washing step was repeated for 3 times.
- OD value of bacteria was measured in 590 nm with microplate reader and density of bacteria was determined.
- Approximate bacteria number was calculated according to OD value by using OD590= 1, Bacteria number = $2. 10^8$ formula.
- To determine the viable bacteria number columbia based blood agar was prepared.
- 1000 bacteria were inoculated into two agar plates and 100 bacteria were inoculated for other two plates.
- Inoculated plates were incubated in CO_2 incubator for 5 days.
- After colony formation of bacteria, colonies were counted and CFU (colony forming unit) value was determined.
- MOI (multiciplity of infection) value was calculated by considering CFU value (100 bacteria per cell).

- According to MOI value desired bacteria number was calculated by considering c ells number which are overnight incubated and seeded in 10% FBS containing, antibiotic free DMEM in culture flask.
- Then desired bacteria volume is directly transferred to cell culture flask and incubated 24 and 48 hours.

2.4.7 Gene Expression Profiling of Frizzled and Wnt Genes in Tissues and Cell Lines

2.4.7.1 RNA Isolation From Tissue and Cell Lines

- 30 mg of tissue or 5 x 10^6 eukaryotic cultured cells was obtained.
- 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol were added to the cell pellet or to tissue and vortexed vigorously. Tissue samples were homogenized by homogenizator or by freezing with liquid nitrogen in a mortar. Cultured cells were homogenized by vortexing and pipetting following adding of RA1 solution. But during this procedure freezed tissues or cells should be avoid from thawing to avoid RNase activity.
- In order to reduce the viscosity and clear the lysate filtration was performed. NucleoSpin® Filter Columns was placed in a Collection Tube, loaded the mixture, and centrifuged for 1 min at 11,000 x *g*.
- NucleoSpin® F ilter Column was discarded and 350 µl ethanol (70%) was added to the homogenized lysate and mixed by pipetting.
- For each preparation, one NucleoSpin® RNA II Column was placed in a Collection Tube and loaded the lysate to the column, centrifuged for 30 sec at 11,000 x *g*. After centrifugation collection tube is discarded and column was placed in a new collection tube.

- 350 µl MDB (Membrane Desalting Buffer) was added and centrifuged at 11,000 x *g* for 1 min.

- DNase reaction mixture was prepared in a sterile 1.5 ml microcentrifuge tube: for each isolation, 10 µl reconstituted rDNase was added to 90 µl Reaction Buffer for rDNase.

  95 µl DNase reaction mixture was directly applied onto the center of the silica membrane of the column and incubated at room temperature for 15 min.

- 200 µl Buffer RA2 was added to the NucleoSpin® RNA II Column. Centrifuge for 30 sec at 11,000 x *g* after tat column was placed into a new Collection Tube.

- For washing 600 µl Buffer RA3 was added to the NucleoSpin® RNA II Column. Centrifuge for 30 sec at 11,000 x *g*. Following centrifugation, flow-through was discarded and column was put back into same collection tube.

- In order to second step of washing 250 µl Buffer RA3 was added to the NucleoSpin® RNA II Column and centrifuged for 2 min at 11,000 x *g* to dry the membrane completely.

- To obtain highly pure RNA, column was put in a RNase, DNase free microcentrifuge tube. Following that, 60 µl RNase-free water was added to center of the membrane and centrifuged at 11,000 x *g* for 1 min.

2.4.7.2 cDNA Synthesis

cDNA synthesis was carry out with RevertAidTM M-MuLV cDNA syhthesis Kit (Fermentas) according to manufacturers' recommendation as describe below:

- Reaction mixture was prepared as explained below on ice:

Total RNA	0,1 to 5 ug
Primer (Random Hexamer 0,2 ug/µl)	1 µl
DEPC treated water	to 12 µl

- Mixture was mixed gently and spinned down for 3-5 second by a microcentrifuge.
- Mixture was incubated for 5 min at 70 °C, and then placed on ice.
- Following components were added to reaction mixture as given order:

Table 2.6 Reactive materials for cDNA synthesis

5X Reaction Buffer	4 µl
RiboLockTm Ribonuclease Inhibitor (20 U/µl)	1 µl
10 mM dNTP mix	2 µl

- Tubes were mixed gently and spinned down for 3-5 second by a microcentrifuge.
- Mixture was incubated for 5 min at 25 °C, and then placed on ice.
- 1 µl (200 U) RevertAid TM M-MuLV Reverse Trasncriptase (200U/µl) was added to final volume 20 µl.
- Mixture was incubated at 25 C for 10 min and then at 42 °C for 60 min.
- Reaction was stopped the by heating at 70 °C for 10 min.
- Chilled on ice and kept for long period at -20 °C.

2.4.7.3 cDNA Normalization and Cycle Threshold Determination

After synthesis of cDNA, cDNA was normalized and cycle threshold was determined by amplifying housekeeping gene named Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Amplification reaction mixture was prepared as illustrated at section 2.4.5.3. During reaction to determine the exponential phase of amplification (cycle threshold), reaction was stopped at 25^{th} to 35^{th} cycle step by step. All samples

were loaded to agarose gel and cycle threshold was determined according to band intensity.

Following cycle threshold determination cDNA quality was determined by amplifying all samples with GAPDH gene primers for number of cycle threshold.

2.4.7.4 RT-PCR Analysis

For the amplification of target sequneces, Frizzled and Wnt genes, a reaction mixture containing 1 u Tag Polymerase, 2 mM $MgCl_2$, 0,2mM dNTP, 10 pmol forward and reverse primers, 2,5 µl from 10X buffer, and DNA template is prepared and completed to 25 µl with molecular biolgy grade ddH_2O. 4 min at 94 °C initial denaturation, a 30 cycles of 30 second denaturation at 94 °C, 45 second annealing according to Table 3.7 and 3.8, 45 second extension at 72 °C is performed. The reaction is ended with a final extension 5 min at 72 °C and cooled to 4 °C for storage.

2.4.7.5 Agarose Gel Electrophoresis

Preparation of the gel:

A 0,8 to 2% agarose gel was used to detect PCR products and genomic DNAs, prepared as explained below:

1. 0.5 g of agarose (Sigma, St. Louis, USA) was mixed with 50 ml of 0.5X Tris-borate EDTA (TBE) buffer.

2. Then it was heated until boiling.

3. The gel was cooled to 50°C and 3.5 µl Ethidium bromide was added. The gel was then poured and a comb was placed in the gel.

Loading:

1. 10 µl PCR product was mixed with 2 µl bromophenol blue as a tracking dye.

2. 10 µl PCR product was then put in each slot.

3. 1 µl of a 100 bp DNA Ladder (MBI Fermentas, Hanover, MD, USA) was mixed with 1 µl deionized water and 1 µl bromophenol blue. Then 5 µl of this mix was put into the side slot as a molecular marker.

4. The gel was run at 95 V in 0.5X TBE buffer for 50 min.

5. The gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the bands were detected under UV transilluminator.

Table 2.7 Primers used for the amplification of Frizzled genes.

Name	Primer Sequences	Amplicon Size	Temp °C	Ref
FZD1	Forward 5 - CTCGAGGTTTCCTCACTAGACAA -3 Reverse 5 – AATGGTTAAACCGCCCTAAATAA – 3	282bp	59°C	150
FZD2	Forward 5-TACCCAGAGCGGCCTATCATTTTT-3 Reverse 5-ACGAAGCCGGCCAGGAGGAAGGAC-3	485 bp	59°C	151
FZD3	Forward 5-AAGGCTTCCACAGTGACACAAGG-3 Reverse 5-AGAGGAGAGAAACCCCAACTACCAC-3	330 bp	55°C	151
FZD4	Forward 5-GGAAATGGTTGGGTGAAGCCTG-3 Reverse 5-TTTTTGATGCTGGGGTCGGG-3	215 bp	56°C	151
FZD7	Forward 5-ACAGACTTAGCCACAGCAGCAAGG-3 Reverse 5-TTTCCAAATCACCCCTCGCC-3	214 bp	57°C	151
FZD8	Forward 5-TGGAGTGGGGTTACCTGTTG-3 Reverse 5-AGCGGCTTCTTGTAGTCCTC-3	310 bp	58°C	151
FZD10	Forward 5-TATCGGGCTCTTCTCTGTGC-3 Reverse 5-GACTGGGCAGGGATCTCATA-3	402 bp	59°C	151

Table 2.8 Primers used for the amplification of Wnt genes, β- Catenin, and GAPDH.

Name	Primer Sequences	Size	Temp °C	Ref
Wnt1	Forward 5CACGACCTCGTCTACTTCGAG-3' Reverse 5'-ACAGACACTCGTGCAGTACGC-3'	253 bp	60°C	151
Wnt 2b	Forward 5'-AAGATGGTGCCAACTTCACCG-3' Reverse 5'-CTGCCTTCTTGGGGGCTTTGC-3'	321 bp	60°C	151
Wnt 3	Forward 5-TGAACAAGCACAACAACGAG-3 Reverse 5-CAGTGGCATTTTTCCTTCC-3	439 bp	56°C	151
Wnt 4	Forward 5-CCTTCTCACAGTCGTTTG-3 Reverse 5-CACAGCCGTCGATGGCCTT-3	429 bp	57°C	151
Wnt 5a	Forward 5-GGGAGGTTGGCTTGAACATA-3 Reverse 5-GAATGGCACGCAATTACCTT-3	141 bp	56°C	151
Wnt 5b	Forward 5-AGATCGTGGACCAGTACATCTG-3 Reverse 5-TTACGGAACCCATCTACATTCTG-3	505 bp	56°C	151
wnt 9a	Forward 5-TGGAGTGCAGGCAGTGCACG-3 Reverse 5-AAAAGCCTCTCAGTCCTTGCAG-3	575 bp	57°C	151
Wnt6	Forward 5- AGTCTTCAGCGGCTTGTATCTTGT -3 Reverse 5- GCTCCGTCCGCTTTCACCTCT -3	561 bp	61 C	151
β- Catenin	Forward 5- CAGAAGCTATTGAAGCTGAGG-3 Reverse 5- TTCCATCATGGGGTCCATAC-3	326 bp	60 C	151
GAPDH	Forward 5- GACCTGCCGTCTAGAAAAAC-3' Reverse 5- TTGAAGTCAGAGGAGACCAC-3'	126 bp	60 C	151

CHAPTER 3

RESULTS

The relation between gastric cancer and wnt pathway was reported before but underlying molecular mechanism was not well understood. In order to explain the underlying molecular mechanism, in vitro experiment were performed using gastric cancer cell lines such as MKN45 and 23132/87, and frozen tissue samples that are collected from gastric cancer patient.

This study can be considered as two different parts. Firstly we aimed to determine the activity of wnt pathway in gastric cancer patients and cell lines, and secondly to analyze the response of cell lines against *H. pylori* infection.

3.1 Analysis of Wnt Pathway Activity in Gastric Cancer Patient

We collected 61 pairs of normal and cancerous tissues from patients during gastric surgery. The activity of the wnt pathway was determined via expression profiling of wnt pathway components such as wnt ligands, frizzled receptors, and as a downstream and pivotal protein β-Catenin by semi-quantitative RT-PCR method. Expression profiles of FZD genes were compared with β-catenin expression in order to understand whether there is any correlation between their expressions or not. If any correlation is determined, this data will be associated with localization of β-Catenin.

3.1.1 Frizzled Genes Expression Analysis

In this study, 7 members of the frizzled (FZD) genes expression were analyzed in normal and cancerous tissues. FZD1, 2,3,4,7,8,10 genes, GAPDH as an internal control and β-catenin genes were amplified by RT-PCR and visualized in 2% agarose gel under UV light. The amplified fragment size was illustrated in Table 2.8. Frizzled family proteins consist of 11 different members but in this study we did not tested all member of the family, only 7 members of receptors were decided to be analyzed after literature review. Whilst FZD1, FZD3, FZD4, GAPDH, and β-catenin were analyzed in 61 pair tissue, FZD2, FZD8, and FZD10 in 57, FZD7 in 28 pair of tissue were analyzed.

Semi-quantitative RT-PCR expression analysis of Frizzled and β-catenin genes of normal and tumor tissue samples were demonstrated in Fig. 3.1. Results showed that FZD1 was expressed in 25 normal (41 %) and 29 tumor (48 %), FZD2 was expressed in 19 normal (35 %) and 24 tumor (44 %), FZD3 was expressed in 3 normal (5 %) and 8 tumor (13 %), FZD4 was expressed in 21 normal (34 %) and 30 tumor (49 %), FZD7 was expressed in 6 normal (21 %) and 8 tumor (29 %), FZD8 was expressed in 22 normal (40 %) and 25 tumor (45 %), FZD10 was expressed in 18 normal (33 %) and 28 tumor (51 %), and β-catenin was expressed in 27 normal (44 %) and 42 tumor (69 %) (Figure 3.2a). When we compare the coexpression of *Frizzled* genes and *β-catenin* in the tumor tissues, *β-catenin* was coexpresed with *Fzd1* gene in 19 (31%), *Fzd3* in 4 (7 %), *Fzd4* in 20 (33%) out of 61 sample, *Fzd2* gene in 27 (19%), *Fzd8* gene in 29 (40%), *Fzd10* gene in 31 (20%) out of 55 samples, *Fzd7* in 5 (33.3%) out of 28 samples (Fig. 3.2b).

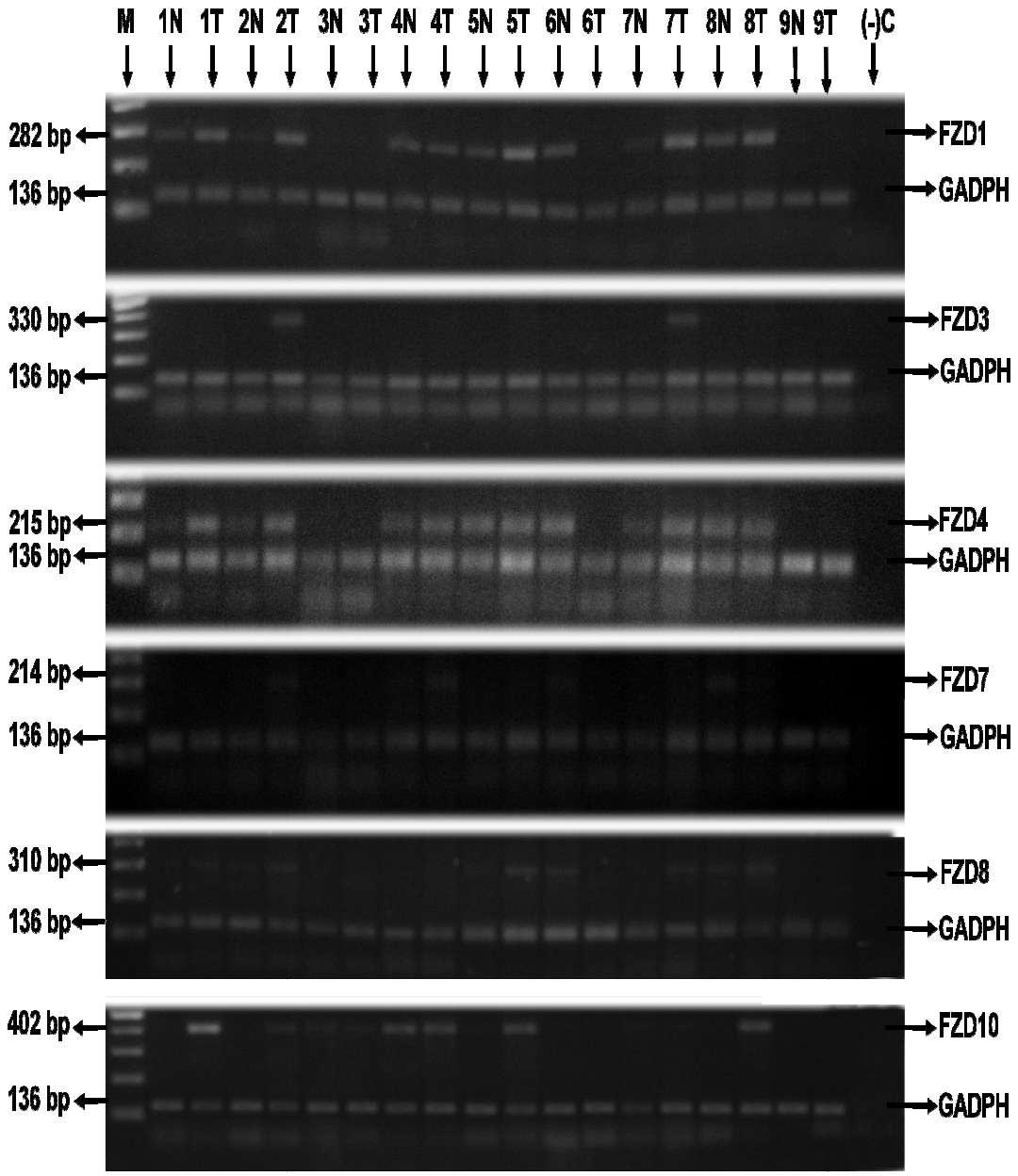


Figure 3.1 Representative semi-quantitative RT-PCR analyses of mRNA expressions for FZD1, FZD2, FZD3, FZD4, FZD7, FZD8, and FZD10 genes for 9 gastric cancer patients.

N and T, matched samples from normal and tumor tissues, respectively; M, Molecular weight marker (100 bp) ; GAPDH, internal control; (-)C, negative control.

.

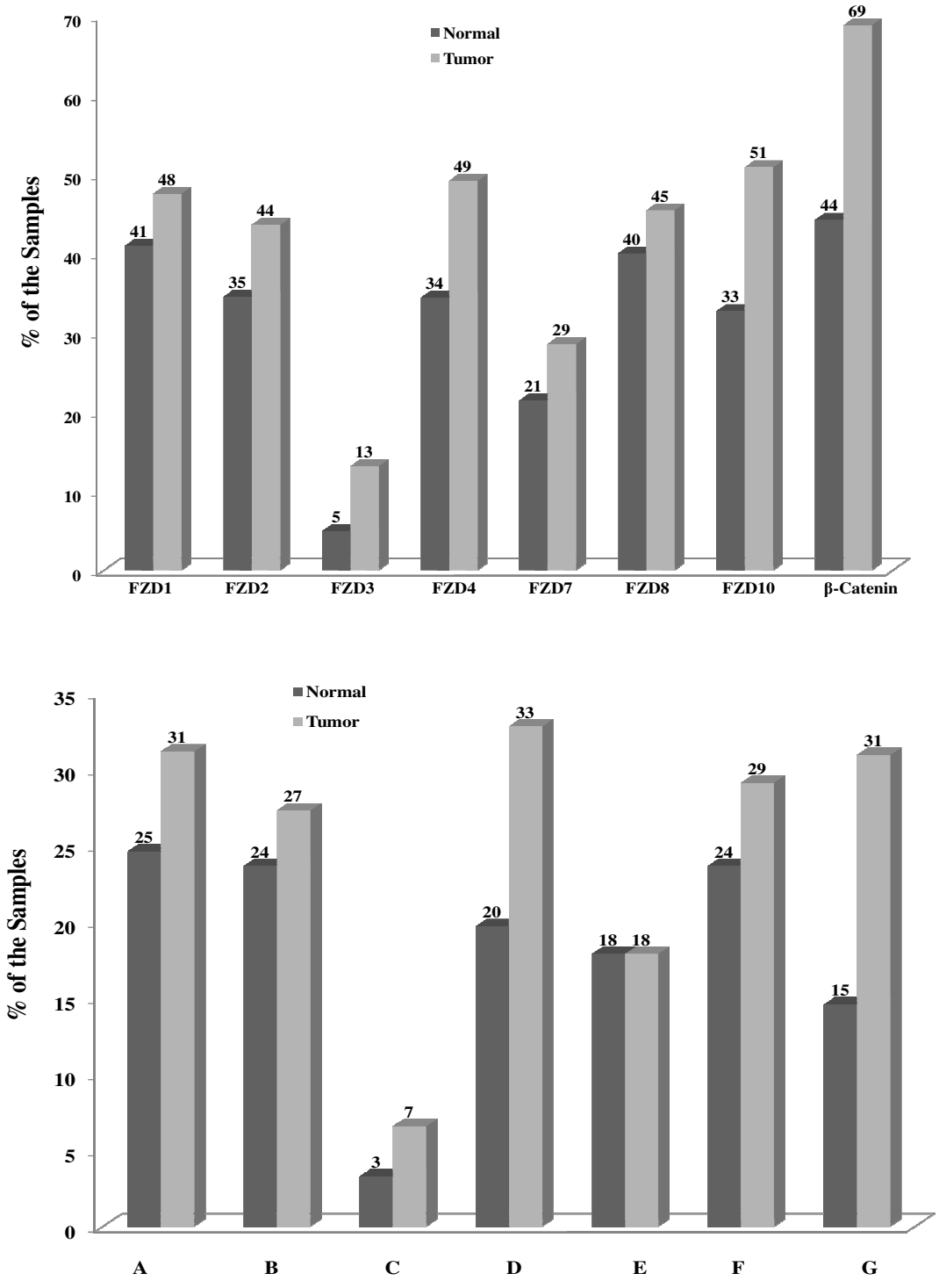


Figure 3.2 Frizzled genes expression analysis in tissues.

A) FZD1, FZD2, FZD3, FZD4, FZD7, FZD8, and FZD10 expressions in normal and tumor tissues; B) A, B,……..F, G illustrate the coexpressions of β-catenin with FZD1, FZD2, FZD3, FZD4, FZD7, FZD8, FZD10 respectively in normal and tumor tissues respectively.

3.1.2 Wnt Genes Expression Analysis

In this study, 8 members of the Wnt genes expression were analyzed in normal and cancerous tissues. Wnt 1, 2b, 3, 4, 5a, 5b, 6, 9a genes, GAPDH as an internal control and β-catenin genes were amplified by RT-PCR and visualized in 2% agarose gel under UV light. Amplified fragment size of target genes were shown in Table 2.9. All genes were analyzed in 51 pair of tissue.

Semi-quantitative RT-PCR expression analysis of Wnt and β-catenin genes of normal and tumor tissue samples was demonstrated in Figure 3.3. Results showed that Wnt1 was expressed in 13 normal (25 %) and 22 tumor (43 %), Wnt2b was expressed in 19 normal (37 %) and 24 tumor (47 %), Wnt3 was expressed in 9 normal (18 %) and 19 tumor (37 %), Wnt4 was expressed in 21 normal (41 %) and 26 tumor (51 %), Wnt5a was expressed in 24 normal (47 %) and 37 tumor (73 %), Wnt5b was expressed in 20 normal (39 %) and 26 tumor (51 %), Wnt6 was expressed in 10 normal (20 %) and 8 tumor (16 %), Wnt9a was expressed in 16 normal (31 %) and 21 tumor (41 %), and β-catenin was expressed in 24 normal (47 %) and 34 tumor (67 %) (Fig. 3.4a). When we compare the coexpression of *Wnt* genes and *β-catenin* in the tumor tissues, *β-catenin* was coexpresed with *Wnt1* gene in 14 (47 %), *Wnt2b* in 16 (31 %), *Wnt3* in 11 (22 %), *Wnt4* gene in 19 (37 %), *Wnt5a* gene in 26 (51%), *Wnt5b* gene in 18 (35%), *Wnt6* in 7 (14 %), *Wnt9a* in 12 (24 %) out of 51 samples (Fig. 3.4b).

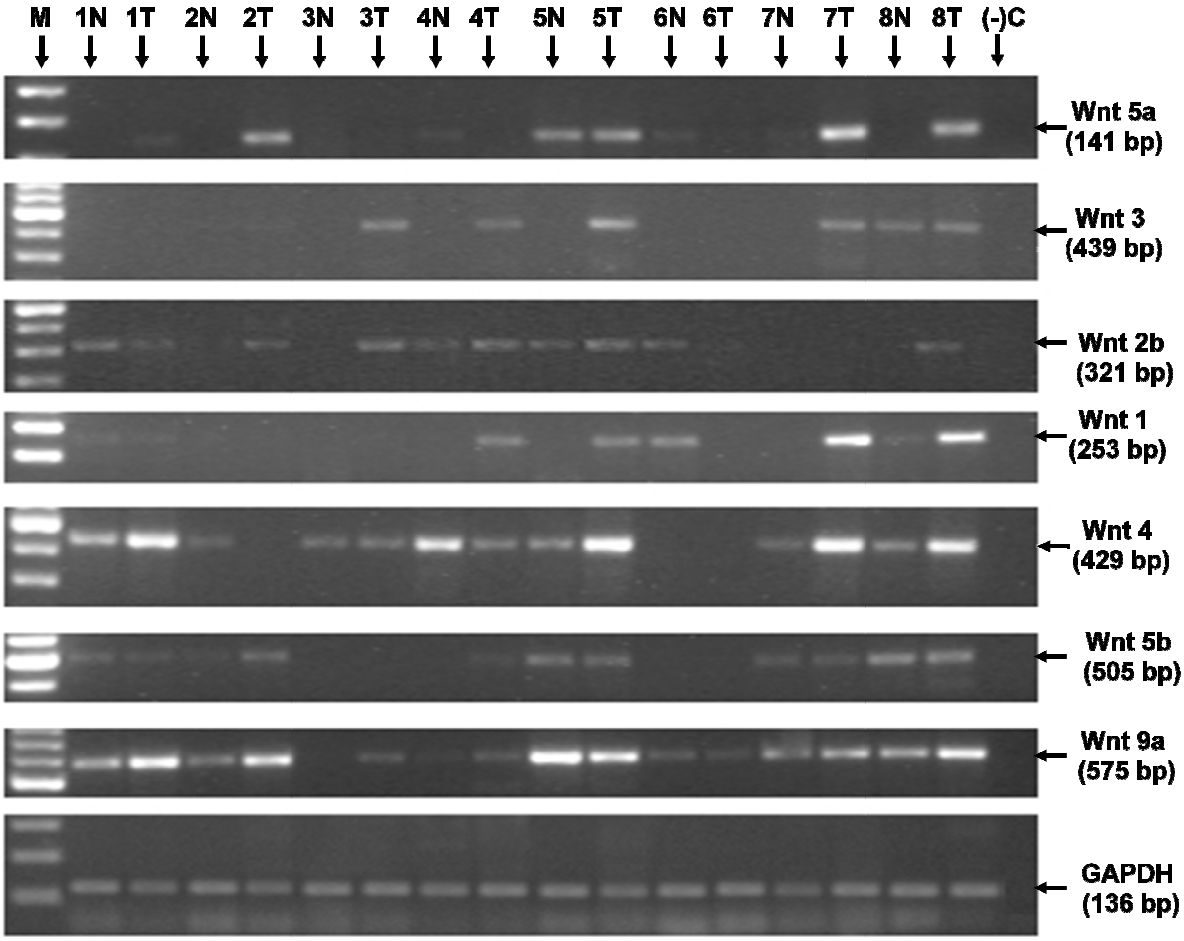


Figure 3.3 Representative semi-quantitative RT-PCR analysis of mRNA expressions for Wnt5a, Wnt3, Wnt2b, Wnt1, Wnt4, Wnt5b, Wnt4 and Wnt9a genes for nine gastric cancer patients.

N and T, matched samples from normal and tumor tissues, respectively; M, Molecular weight marker (100 bp) ; GAPDH, internal control; (-)C, negative control.

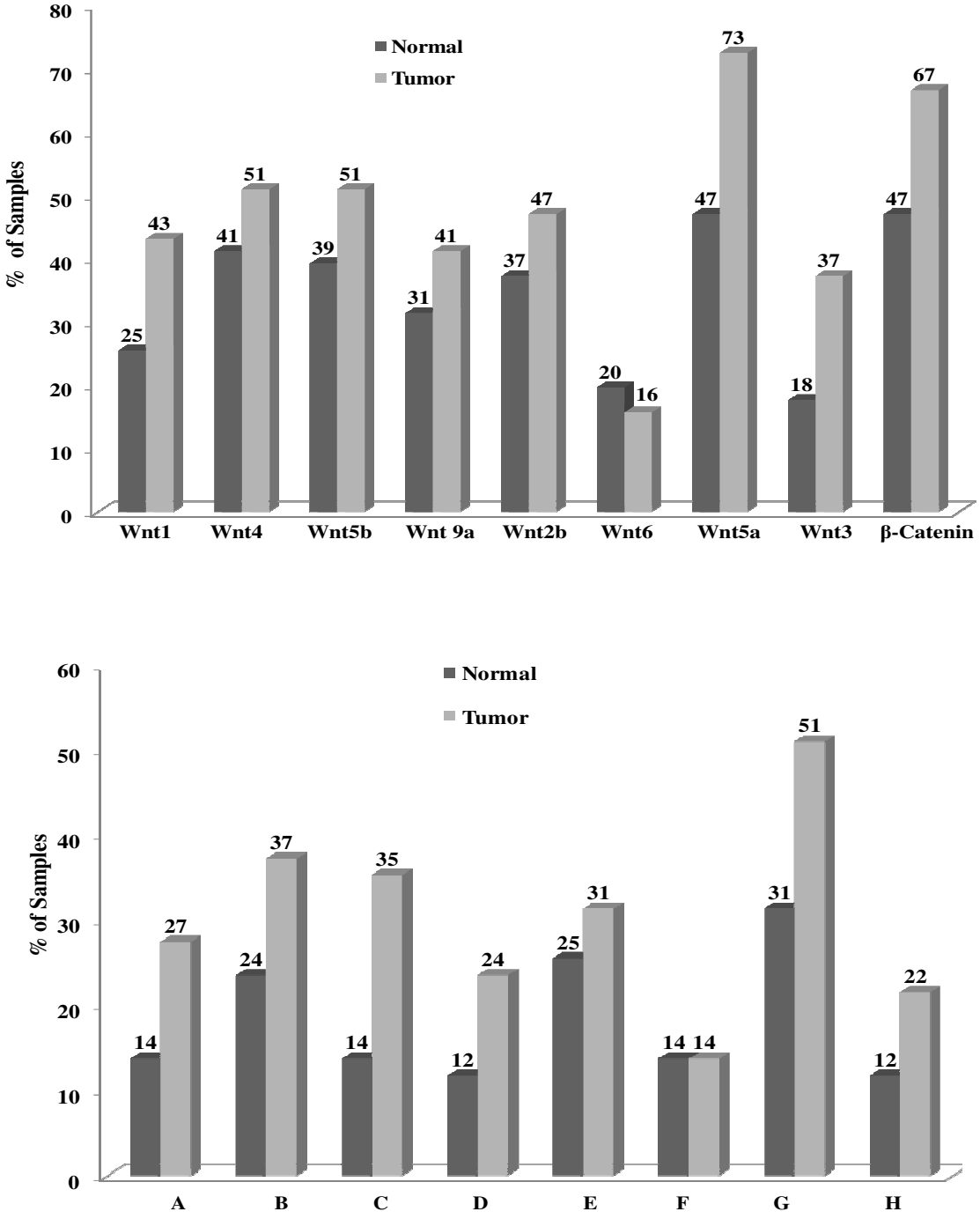


Figure 3.4 Expression analysis of Wnt ligands in tissues.

A) Wnt5a, Wnt3, Wnt2b, Wnt1, Wnt4, Wnt5b, Wnt4 and Wnt9a expressions in normal and tumor tissues; B) A, B,…….. G, H illustrate the coexpressions of β-catenin with Wnt1, Wnt4, Wnt5b, Wnt9a, Wnt2b, Wnt6, Wnt5a and Wnt3 respectively in normal and tumor tissues.

3.2 Helicobacter Pylori Genotyping Results

Gastric cancer cell lines were infected with two different strains of *H. pylori* in order to understand the effect of *H. pylori* on gastric cancer cells and to observe their response. To find out that, gastric cancer biopsies were collected from patients with gastric disorder and *H. pylori* were cultured.

Firstly, pathogen characteristic related genes of *H. pylori* were amplified by multiplex PCR method and the amplified bands were classified according to size as demonstrated at Table 2.1. Results reveal that two different strains were obtained as desired. One of the strains genotyped as $cagA^{(+)}$ and $vacA^{m1s1}$ which has toxic characteristics and other strain genotyped as $cagA^{(-)}$ and $vacA^{m2s2}$ which has non toxic characteristics.

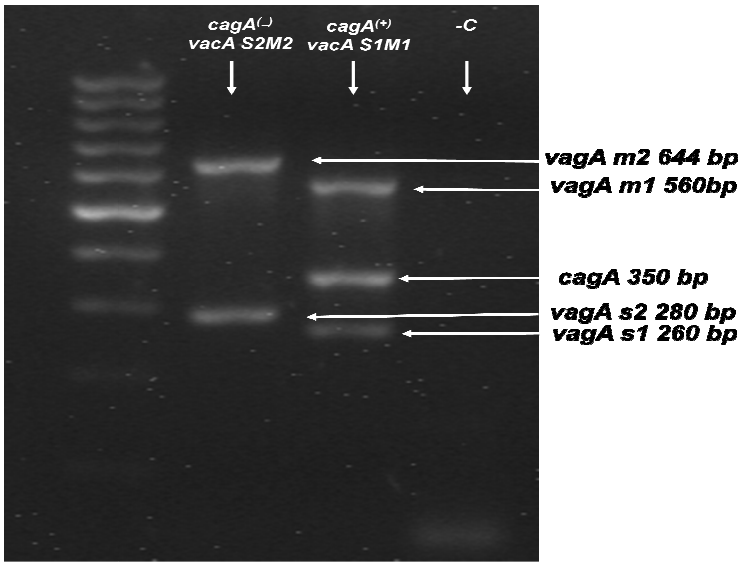


Figure 3.5 Genotyping results of pathogenecity related genes that are *cag and cagA* of *H. pylori*.

Arrows show *cagA* and *vacA* gene amplifications of *H. pylori* isolated from biopsy samples of patients. Genotyping results of pathogenecity related genes of *H. pylori*. (-)C negative control.

3.3 Morphological Changes After *H. pylori* Infection

After *H. pylori* infection, firstly we aimed to determine whether there are any changes in morphological structure. Cells were plated and *H. pylori* that are in exponential phase were added to culture medium after 3 times washing with PBS.

Behavior of cell and bacteria were investigated with periods of 2 hours. After 1 hour, bacteria began to attach to cells and colonization formation began around the cells. Following hours, cells started to response against infection that can be observable under inverted microscope. Firstly their membrane structures, cell adhesions were disrupted and then cell scattering started which is resulted as “Humming Bird phenotype”. Lose of membrane structure and humming bird phenotype can be observable in Figure 3.6 after 24 and 48 hours.

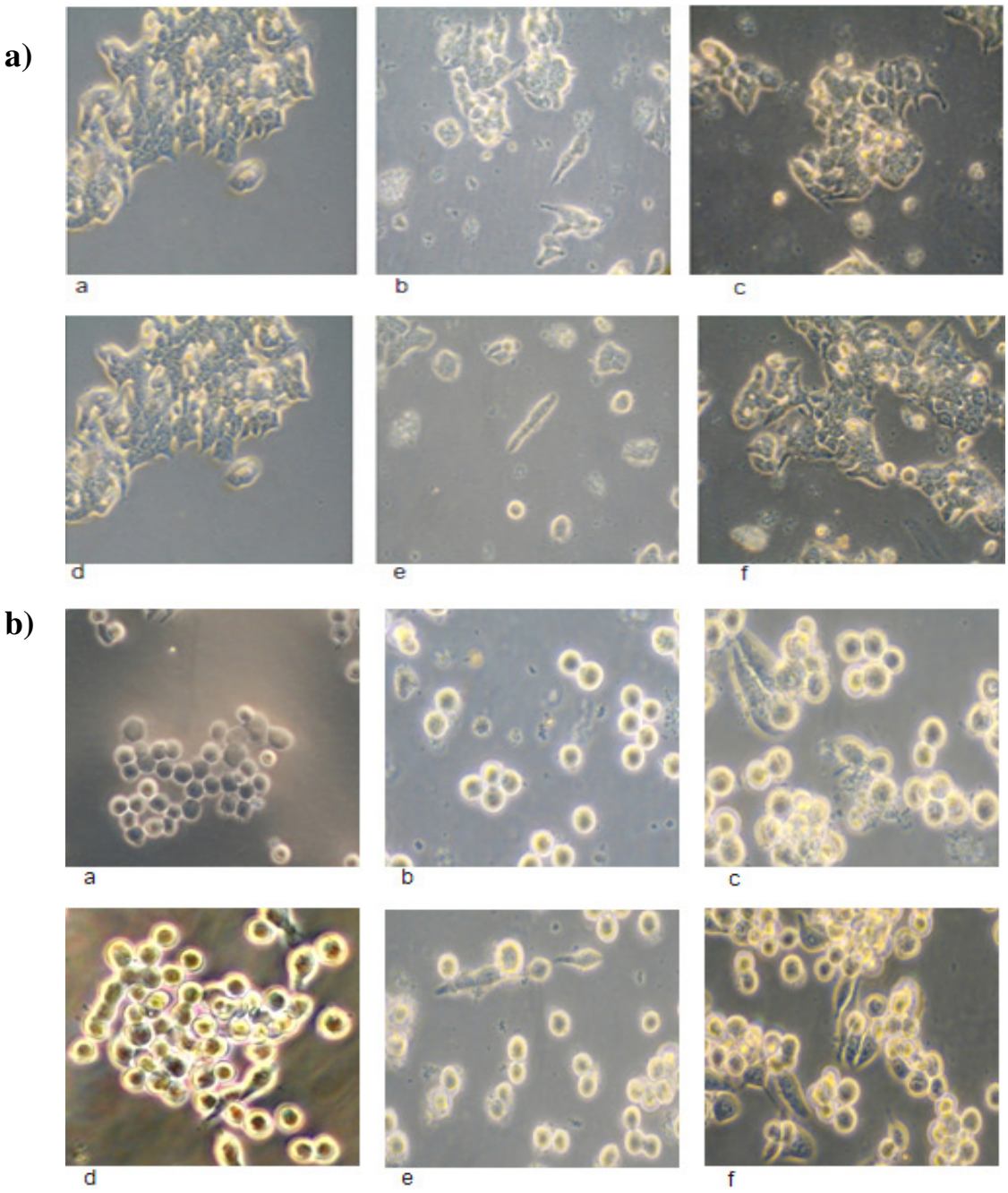


Figure 3.6 Morfological changes after 24 and 48 hours *H. pylori* infection.

A) Morphological changes of MKN45 cells against *H. pylori* infection for 24 and 48 hours. B) Morphological changes of 23132/87 cells against *H. pylori* infection for 24 and 48 hours. (a: with no infection for 24 hours, b) infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori* for 24 hours c) 24 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori*, f)) 48 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*).

3.4 Apoptosis Assay Results Regarding Helicobacter Pylori Infection

Following *H. pylori* infection determine the fate of cell whether it is going under apoptosis or not. In order to determine apoptosis, four experiments were performed. As described before, in apoptotic cell DNA fragmentation, caspases activity and single or double stranded DNA breaks can be observed. We tested this apoptosis by DNA fragmentation assay, Hoechst staining, caspase 3 activity assay and TUNEL assay. Thus, all results were confirmed.

3.4.1 Hoechst Staining Analysis

Hoechst is a kind of fluorescent dye which is bind to double stranded DNA. During apoptosis DNA is fragmented and degraded. Apoptotic cell's nucleuses are smaller than normal cells and their intensity is lower as well. Thus apoptotic cells were determined.

Cells were seeded on glass slides in six well plates and infected with *H. pylori*. Response of cells were against *H. pylori* is analyzed by nuclear Hoechst Staining after 24 and 48 hours infection. Hoechst staining results indicate that both MKN45 and 23132/87 cell lines were going under apoptosis following infection with $cagA^{(+)}$ and $vacA^{s1m1}$ *H. pylori* strain for 24 and 48 hours. However in both MKN45 and 23132/87 cell lines following infection with $cagA^{(-)}$ and $vacA^{s2m2}$ *H. pylori* strain, apoptotic nucleus formation is not observed in control cells as shown Fig. 3.7.

3.4.2 DNA Fragmentation Assay

DNA fragmentation is one of the most important indicators of apoptosis. Moreover it can be observed in necrosis as well. In order to confirm the Hoechst staining results, DNA fragmentation assay was performed as a preliminary study.

MKN45 and 23132/87 cells were infected with both *H. pylori* strains for 24 and 48 hours. Consequently, DNA was extracted from uninfected cells and *H. pylori* infected cells, separated on 2% agarose gel, and visualized under UV light.

Agarose gel results reveal that, both MKN45 and 23132/87 cell lines were going under apoptosis following infection with $cagA^{(+)}$ and $vacA^{s1m1}$ *H. pylori* strain for 24 and 48 hours. However in both MKN45 and 23132/87 cell lines following infection with $cagA^{(-)}$ and $vacA^{s2m2}$ *H. pylori* strain, apoptotic nucleus formation is not observed in control cells , as shown Figure 8.

3.4.3 TUNEL Assay Results

In general, cells undergoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal length DNA fragments after activation of a calcium-dependent endogenous endonuclease.

Cleavage of genomic DNA during apoptosis may yield doublestranded, low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks (“nicks“) in high molecular weight DNA. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase,

an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

TUNEL assay is performed MKN45 and 23132/87 cell lines which are infected with only in *cagA*$^{(+)}$ and *vacA*^{s1m1} strain in order to confirm the positive results of Hoechst and DNA fragmentation study due to specificity of TUNEL assay.

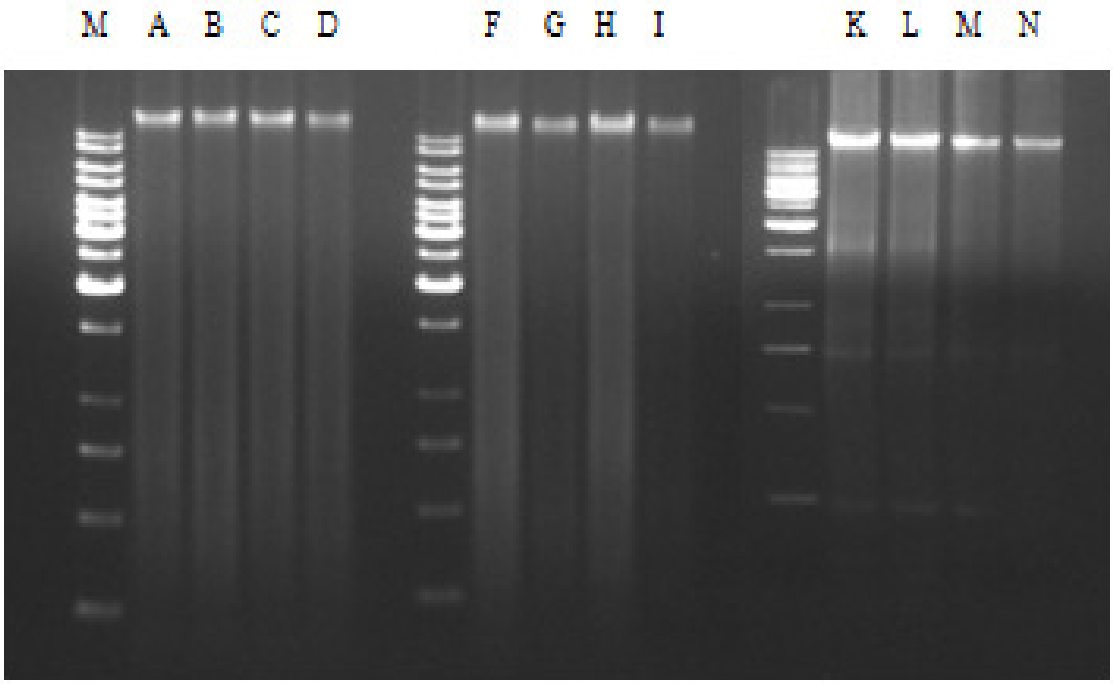


Figure 3.7 DNA fragmentation formation of MKN45 and 23132/87 cells following infection with both strains of *H. pylori*. K, L, M, and N lanes show the fragmented DNAs which is an indicator of apoptosis.

(A: uninfected MKN45 after 24 hours, B: uninfected 23132/87 after 24 hours, C: uninfected MKN45 after 48 hours, D) uninfected 23132/87 after 48 hours, F: *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori* infection of MKN45 after 24 hours, G: *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori* of MKN45, H: 24 hours infection *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori* of 23132/87 after 48 hours infection, I: *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori* 23132/87, K: 24 hours infection *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori* MKN45 after 48 hours infection, L: *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori* MKN45, M: 24 hours infection *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori* 23132/87 after 48 hours infection, N: *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori* 23132/87 after 48 hours infection).

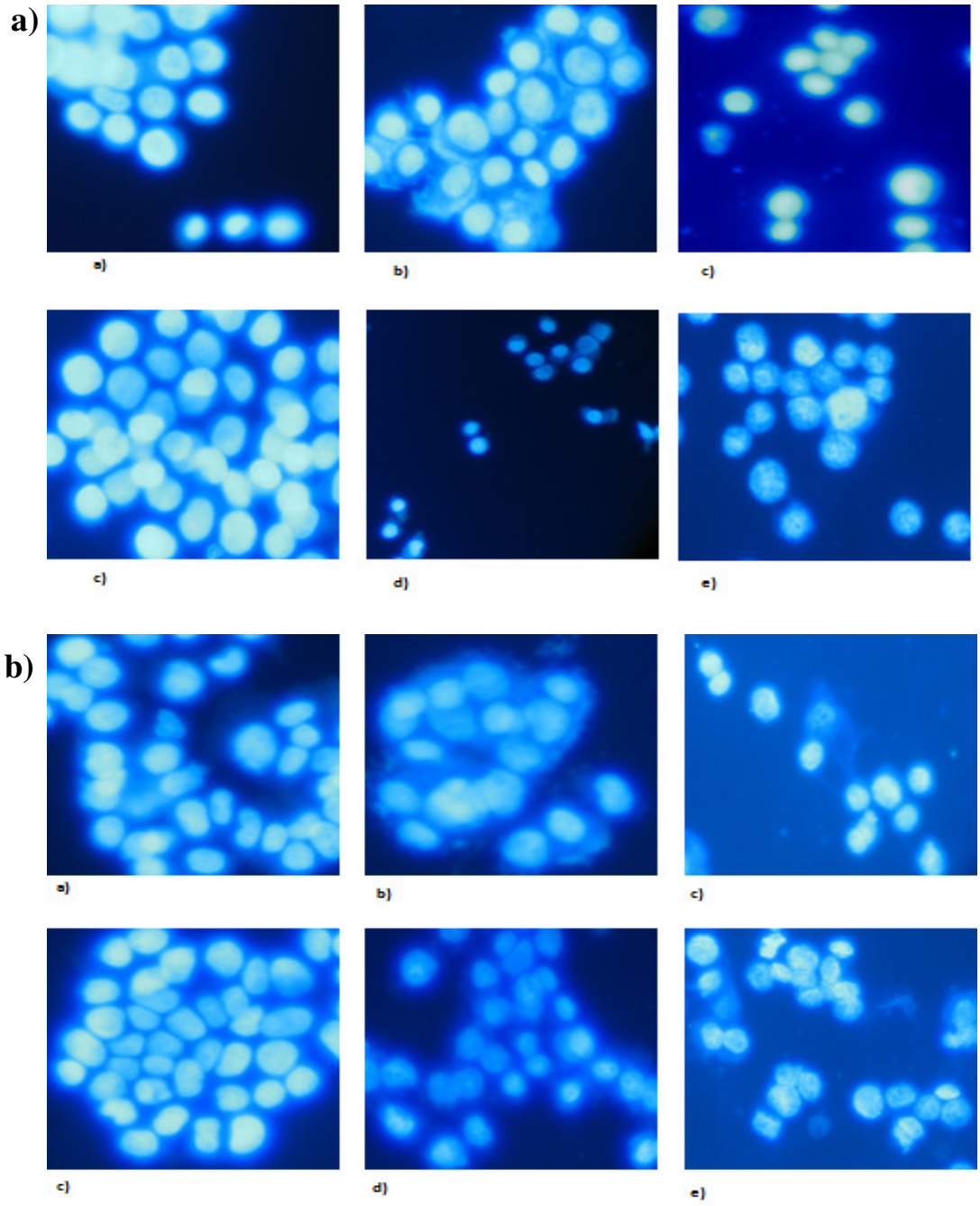


Figure 3.8 Nucleus formation of MKN45 and 23132/87 cells after 24 and 48 hours *H. pylori* infection.

A) Nuclear formation changes of MKN45 cells against *H. pylori* infection for 24 and 48 hours. B) Nuclear formation changes of 23132/87 cells against *H. pylori* infection for 24 and 48 hours. (a: with no infection for 24 hours, b) 24 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, c) 24 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, f)) 48 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*).

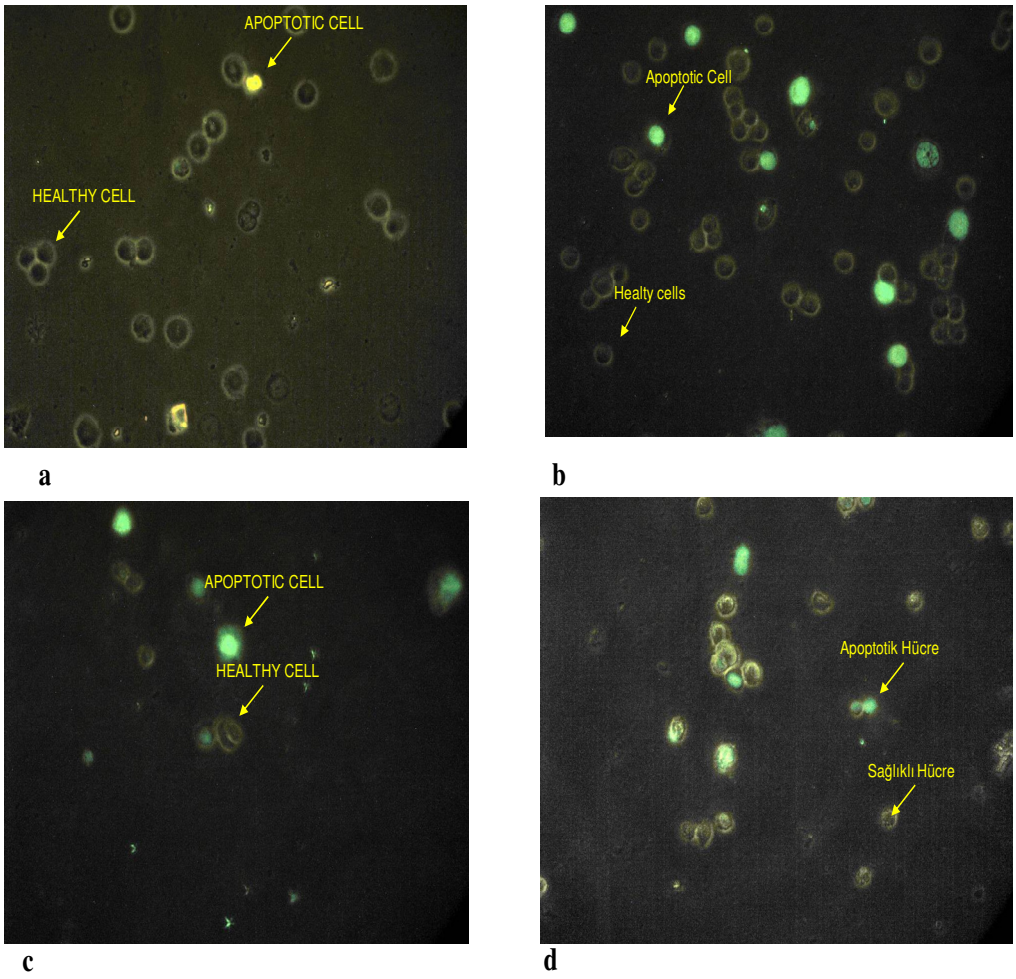


Figure 3.9 TUNEL assay results of MKN45 and 23132/87 cell following infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*. Arrow show the healthy and apoptotic cells.

(a: 24 hours infection of MKN45, b: 48 hours infection of MKN45, c: 24 hours infection of 23132/87, d) 48 hours infection of 23132/87).

3.4.4 Caspase 3 Assay Results

Occasionally, TUNEL assay can give false positive results as well due to DNA damage related to oxidative stress.

Caspases (Cysteine-requiring Aspartate protease) are a family of proteases that mediate cell death and are important to the process of apoptosis. Caspase 3 (also referred to as CPP32, Yama, and apopain) is a member of the CED-3 subfamily of caspases and is one of the critical enzymes of apoptosis. As explained in section 3.4.1, 3.4.2, and 3.4.3 apoptosis process has many indicators but all these parameters can be effected by other factors such as oxidative stress or necrosis. Therefore, in order to confirm all previous results and caspase 3 enzyme activity that is certain indicator of apoptosis was performed.

H. pylori infected and control cells were collected, lyzed, and cell debris was removed as explain in section 2.4.3.3. Finally, cell lysate was tested in 96 well plate with plate reader.

Obtained results ilustrated that both MKN45 and 23132/87 cell lines were going under apoptosis following infection with *cagA*$^{(+)}$ and *vacA*^{s1m1} *H. pylori* strain for 24 and 48 hours. However in both MKN45 and 23132/87 cell lines following infection with *cagA*$^{(-)}$ and *vacA*^{s2m2} *H. pylori* strain, apoptotic nucleus formation is not observed as control as shown Figure 3.10.

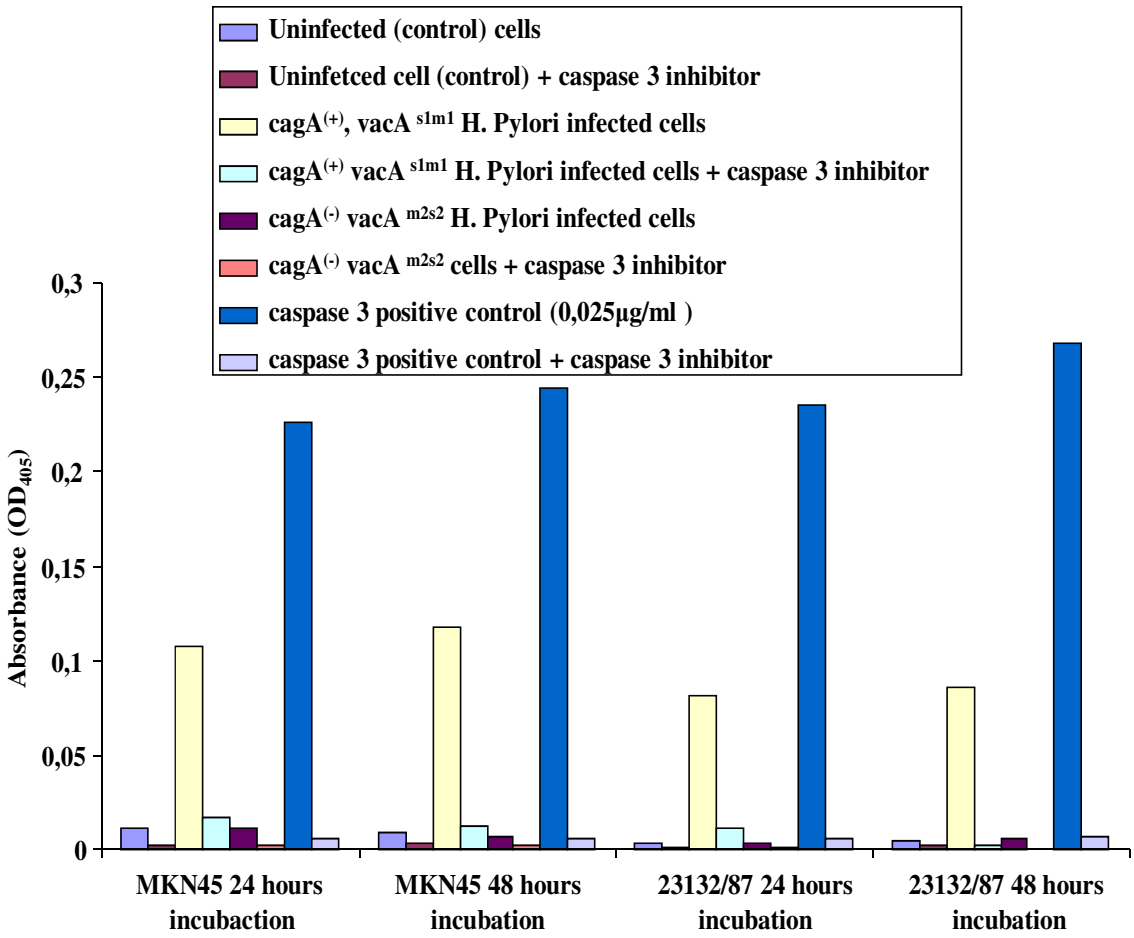


Figure 3.10 Absorbance value of caspase 3 enzyme activity in cells which are infected with both *H. pylori* strains for 24 and 48 hours.

3.5 Proliferation analysis of cell lines

3.5.1 Ki67 Staining Results

Ki-67 is a nuclear protein that is expressed in proliferating cells and may be required for maintaining cell proliferation. Ki-67 has been used as a marker for cell proliferation of solid tumors and some hematological malignancies. A correlation has been demonstrated between Ki-67 index and the histopathological grade of neoplasms. Assessment of Ki-67 expression in renal and ureter tumors shows a correlation between tumor proliferation and disease progression, thus making it possible to differentiate high-risk patients. Ki-67 expression may also prove to be important for distinguishing between malignant and benign peripheral nerve sheath tumors.

In order to determine whether MKN45 and 23132/87 cell lines going under proliferation, Ki67 staining was performed after *H. pylori* infection by immunocytochemistry method. As shown in figure 11, while level of Ki67 very high in control cells, when the cells were infected it's level is decreasing in both cell lines for 24 and 48 hours. Data suggests that cells are losing their proliferation capacity after infection. We found results which are contrary to our expectations and literature.

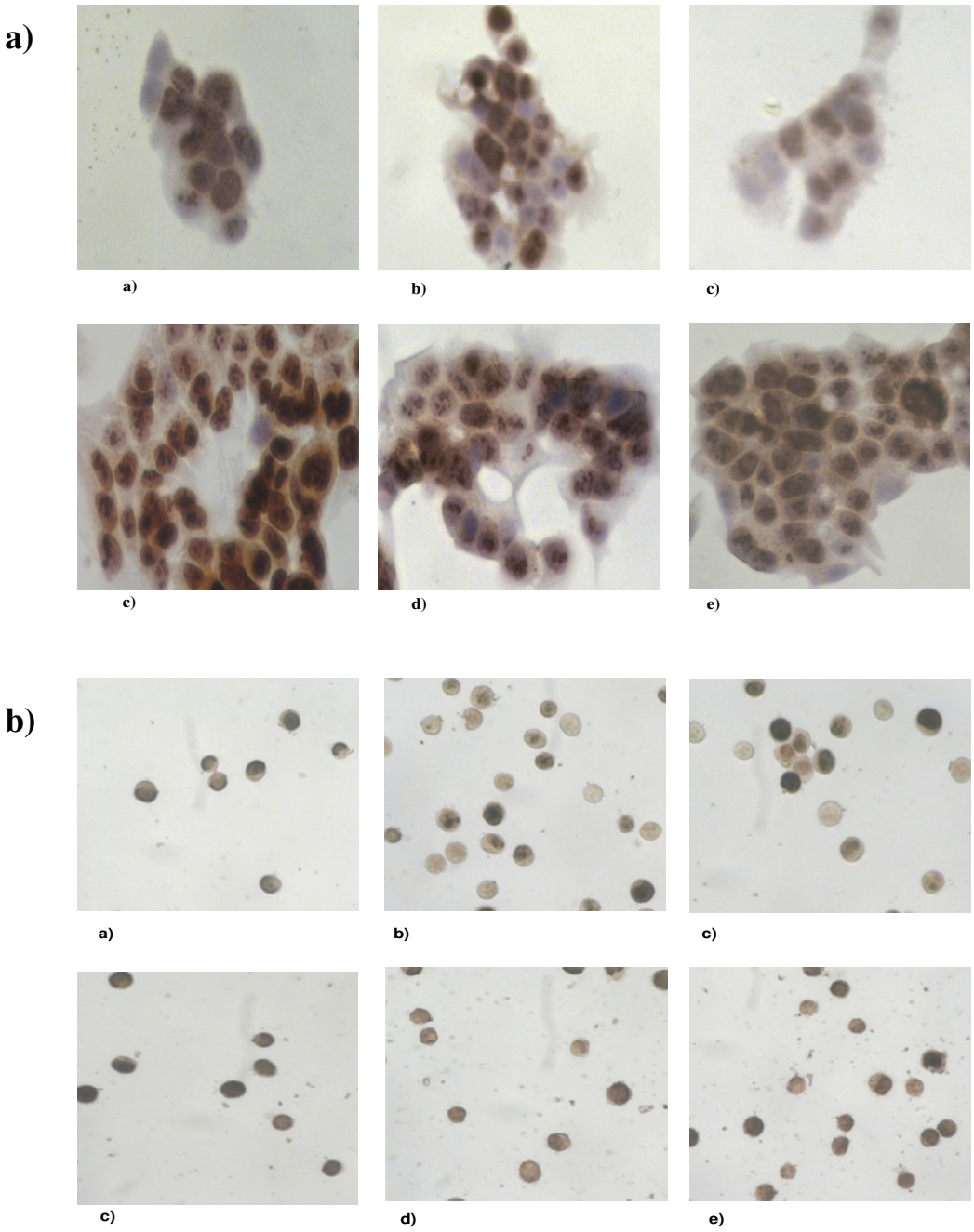


Figure 3.11 Qualitative illustration of Ki67 level in MKN45 and 23132/87 cells following 24 and 48 hours *H. pylori* infection.

A) Ki67 level of 23132/87 cells following *H. pylori* infection for 24 and 48 hours, B) Ki67 level of MKN45 cells following *H. pylori* infection for 24 and 48 hours (a: with no infection for 24 hours, b) 24 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, c) 24 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, f)) 48 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*).

3.6 Oxidative Stress Analysis

3.6.1 DCFHDA Staining

Dichlorofluorescin diacetate (DCFH-DA) has been used as a substrate for measuring intracellular oxidant production in neutrophils. DCFH-DA is hydrolyzed by esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. DCFH-DA cannot be appreciably oxidized to a fluorescent state without prior hydrolysis.

As reported in previous studies, *H. pylori* infection lead cell to oxidative stress. Oxidative stress may change cell homeostasis so cell characteristics may be change. In order to find out whether our strains cause oxidative stress, we performed DCFH-DA staining.

We infected the cell with both *cagA*$^{(+)}$, vac*A*^{s1m1} and *cagA*$^{(-)}$, *vacA*^{s2m2} strains for 24 and 48. Following infection cells were stained with DCFH-DA and fluorescent excited cells were counted under inverted microscope all experiment were performed for 3 times to decrease the experimental error. Results indicate that both H. pylori strains lead to cells to apoptosis. both *cagA*$^{(+)}$, vac*A*^{s1m1} genotyped H. Pylori cause lead to 50% of cells to oxidative stress after 24 hours, and as expected after 48 hours, oxidatively stressed cell number increased to 75 % of cells that show the oxidative stress increases in time dependent manner. However, 23132/87 cell line also undergo oxidative stress in time dependent manner as shown in Figure 3.12. Interestingly, control cells already oxidatively stress that are not infected with H. pylori. When we compare the effect of *cagA*$^{(+)}$, vac*A*^{s1m1} and *cagA*$^{(-)}$, *vacA*^{s2m2} H. pylori strains on oxidative stress, we observed that *cagA*$^{(+)}$, vac*A*^{s1m1} strains cause to more stress than the *cagA*$^{(-)}$, *vacA*^{s2m2} strains that is related with pathogen characteristics of bacteria.

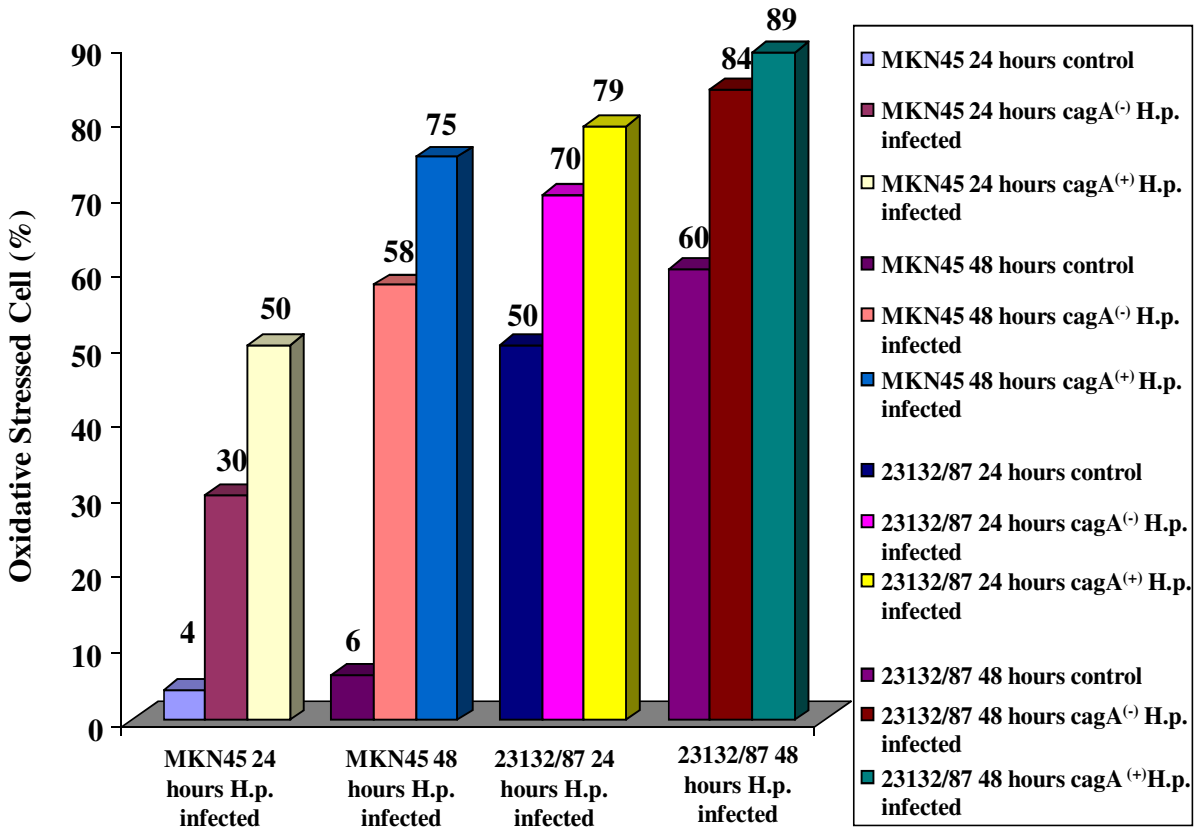


Figure 3.12 Percentage of fluorescent excited cell number according to non fluorescent cells that shows the live oxidative stressed cells following infection with both *H. pylori* strains for 24 and 48 hours.

3.6.2. 8-dOHG Formation Detection In MKN45 and 23132/87 Cell Lines

The DNA base-modified product 8-hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most commonly used markers for the evaluation of oxidative DNA damage. A monoclonal antibody specific for 8-OHdG was characterized and applied in quantitative and semi-quantitative immunohistochemistry.

In order to show the effect of H. pylori effect on gastric cancer cell lines, firstly cells were plated on glass slide in six well plate, after overnight incubation H. pylori infection was performed. Cells were exposure to bacterial infection for 24 and 48 hours. Then 8-OHdG staining was carried out.

Semi-quantitative 8-OHdG staining results showed that both toxic and less toxic H. pylori strain causes to oxidative stress in time dependent manner as shown in Figure 3.13 that confirm the DCFH-DA staining results as well.

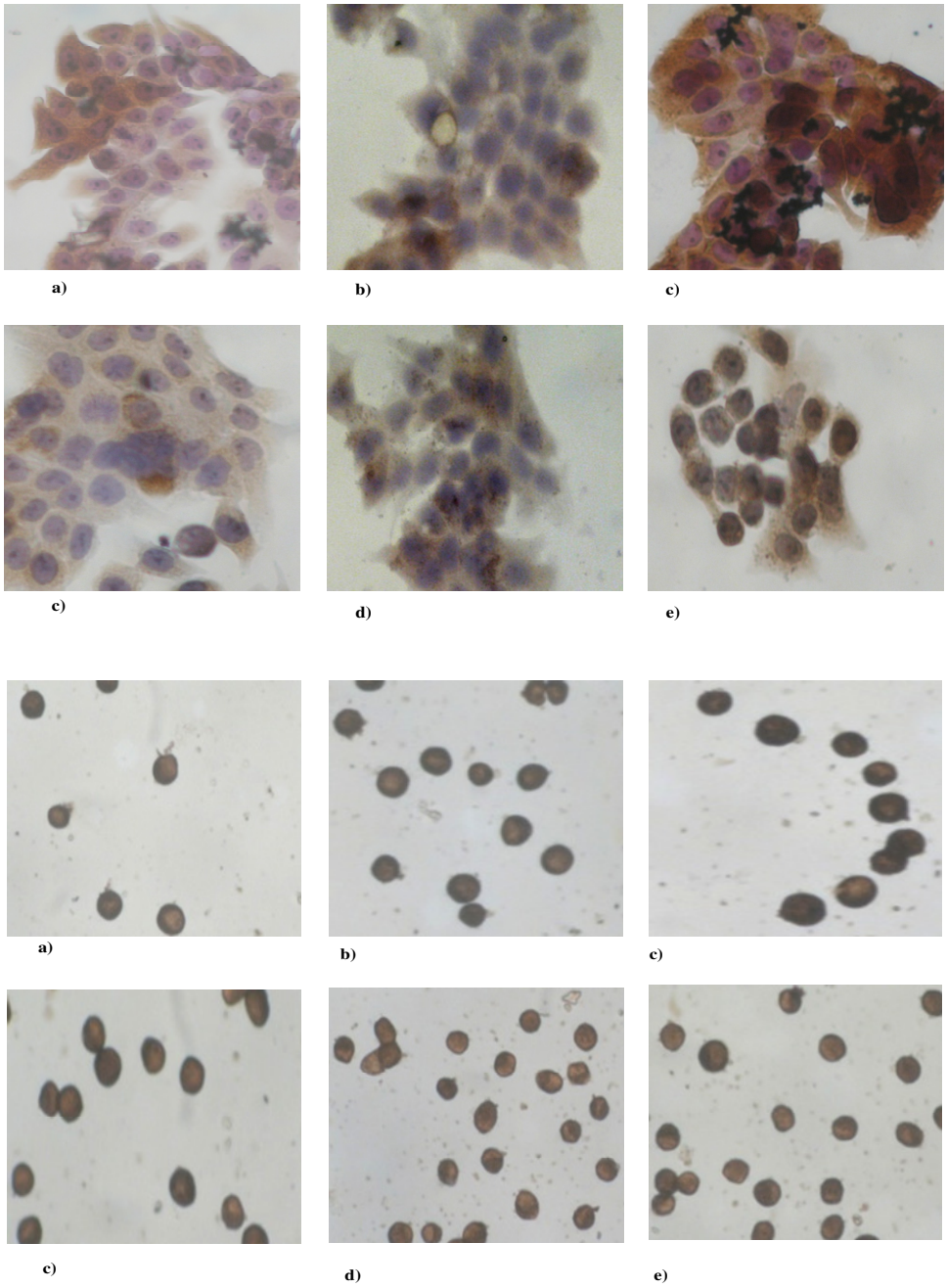


Figure 3.13 Qualitative illustration of 8-dOHG level in MKN45 and 23132/87 cells following 24 and 48 hours *H. pylori* infection.

A) 8-dOHG level of 23132/87 cells following *H. pylori* infection for 24 and 48 hours, B) 8-dOHG level of MKN45 cells following *H. pylori* infection for 24 and 48 hours (a: with no infection for 24 hours, b) 24 hours infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori*, c) 24 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori*, f)) 48 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*).

3.7 Immunocytochemistry

3.7.1 β-Catenin Staining Results

β-Catenin is a protein which has a very important and central role in wnt pathway. Localization of β-Catenin is significant to determine whether wnt pathway is active or not. In order to determine the activity of the wnt pathway, β-Catenin localization is determined by immunocytohemistry method via staining nucleus with hemotoxylen. Thus, we understand that protein localized in nucleus or cytoplasm.

MKN45 and 23132/87 cells were seeded in glass slide in six well plate and infected with *H. pylori* for 24 and 48 hours after overnight incubation. Semi-quantitative immunocytochemistry results indicate that in MKN45 and 23132/87 cells have no significant changes following *H. pylori* infection. However, β-Catenin localization of MKN45 cell line was difficult to be determined due to cell shape, structure and characteristic. When nucleus of the cell stained with hematoxylen, cells completely seems purple due to 90% of the cells are composed of nucleus. Therefore it is impossible to find out protein localization. To solve this problem, cells were not stained with hemotoxylen and protein localization was determined estimatedly. Probable results showed that there is no significant correlation between β-Catenin localization and *H. pylori* infection as shown in Figure 3.14.

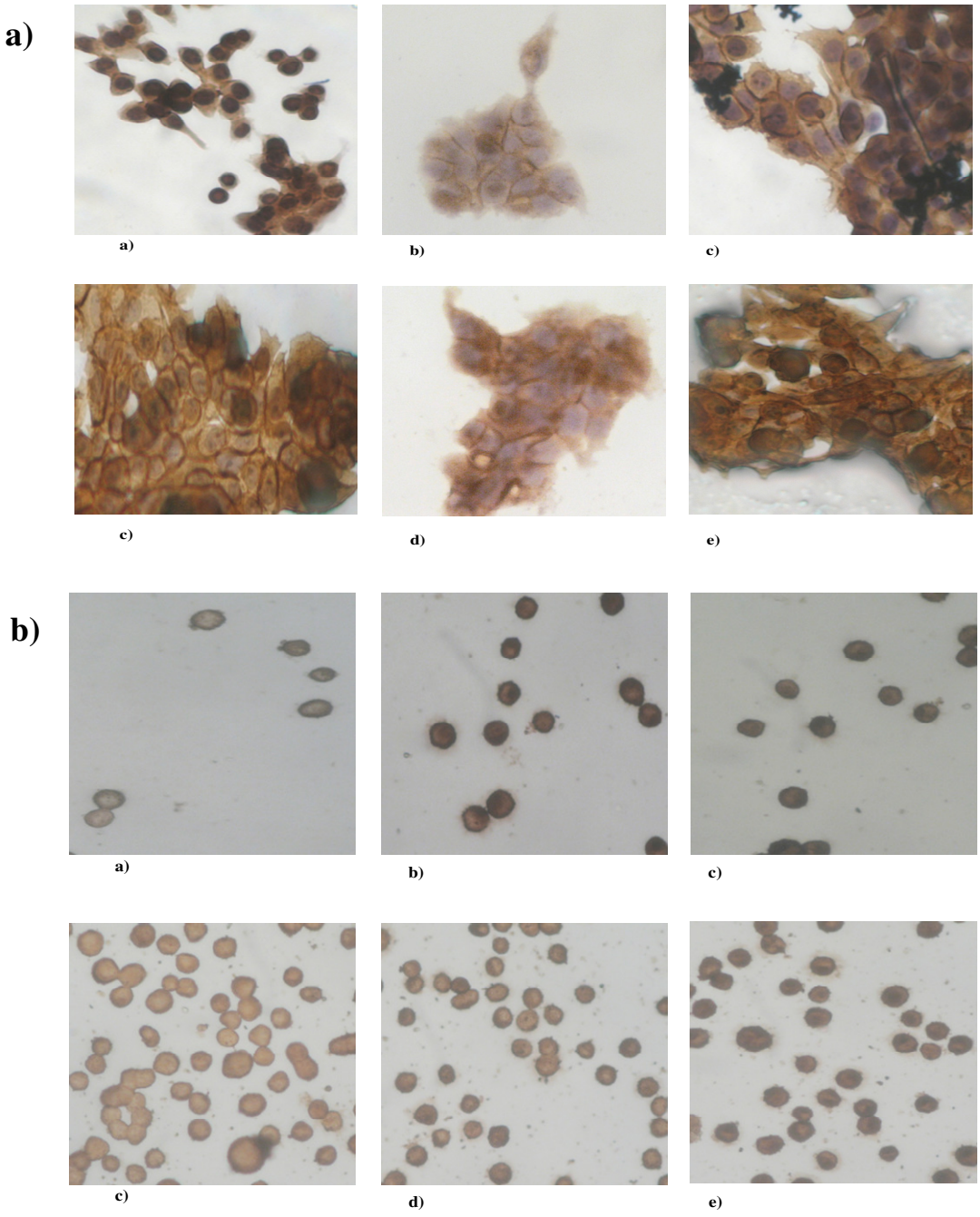


Figure 3.14 Illustration of β-Catenin level and localization in MKN45 and 23132/87 cells following 24 and 48 hours *H. pylori* infection.

A) β-Catenin level and localization in 23132/87 cells following *H. pylori* infection for 24 and 48 hours, B) β-Catenin level and localization in MKN45 cells following *H. pylori* infection for 24 and 48 hours (a: with no infection for 24 hours, b) 24 hours infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori*, c) 24 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with $cagA^{(-)}$, $vacA^{s2m2}$ *H. pylori*, f)) 48 hours infection with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori*).

3.7.2 CyclinD1 Staining Results

CyclinD1 is a protein which has a role in transition from G1 to S phase of the cell cycle of which expression is regulated by TCF/ β-Catenin transcription factor in the nucleus. Nuclear localization of the β-Catenin is resulted in increased expression of cyclinD1. Its increased expression may give idea about activity of the wnt pathway.

Therefore, we analyzed its protein level and localization by immunocytohemistry method following *H. pylori* infection in order to understand the activity of the wnt pathway and whether these cells are proliferative or not. As shown in Figure 3.15 in both 23132/87 and MKN45 cells, following *H. pylori* strain infection there is no considerable changes in protein level and its localization in time dependent manner.

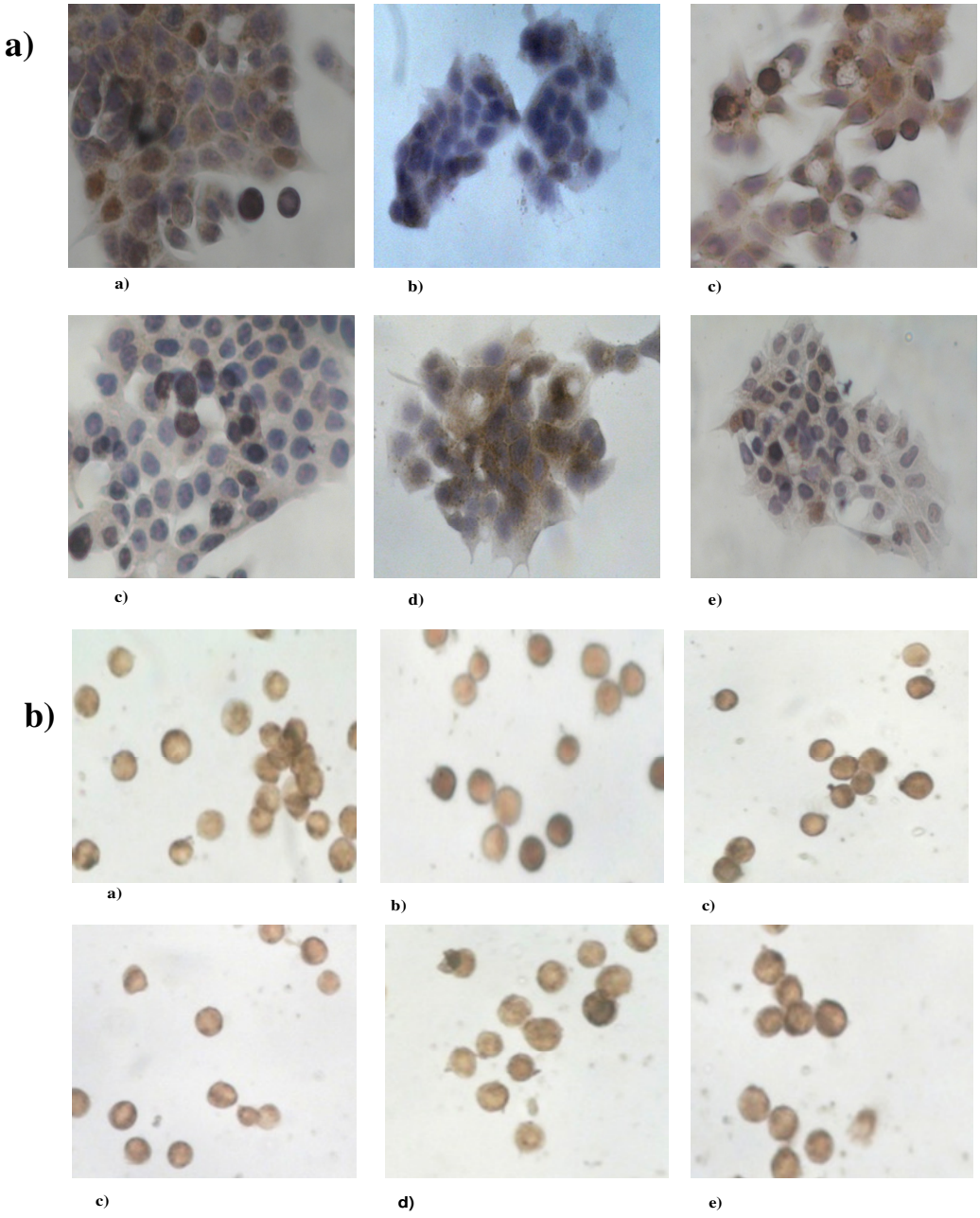


Figure 3.15 Illustration of CyclinD1 level in MKN45 and 23132/87 cells following 24 and 48 hours *H. pylori* infection.

A) CyclinD1 level of 23132/87 cells following *H. pylori* infection for 24 and 48 hours, B) CyclinD1 level of MKN45 cells following *H. pylori* infection for 24 and 48 hours (a: with no infection for 24 hours, b) 24 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, c) 24 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*, d) with no infection for 48 hours, e) 48 hours infection with *cagA*$^{(-)}$, *vacA*^{s2m2} *H. pylori*, f)) 48 hours infection with *cagA*$^{(+)}$, *vacA*^{s1m1} *H. pylori*).

3.8 Semi-Quantitaive RT-PCR Results of Helicobacter Pylori Infected Cell Lines

3.8.1 Frizled and Wnt Genes Expression

Frizlleds receptors are families of protein which have a role initiating the proliferation signal into cell. Their high level of expression may increase the initiating of the wnt signal due to rising possibility of binding to Wnt ligand. In order to show how cells response to *H. pylori* infection, the level of gene expression was tested by semi-quantitative RT-PCR method.

FZD1, FZD2, FZD3, FZD8 and FZD10 expression levels were tested following infection with both *H. pylori* strain for 24 and 48 hours. In MKN45 cells following infection for 48 hours FZD1 genes expression is decreasing following $cagA^{(+)}$, $vacA^{s1m1}$ strain, but there is no changes in FZD1 gene expression following $cagA^{(-)}$ and $vacA^{s2m2}$ strain. In 23132/87 cells we observed decreased level of expression following 24 hour $cagA^{(-)}$, $vacA^{s2m2}$ strain (Figure 16).

For FZD2 gene, in both MKN45 and 23132/87 cells, there is no signification expression changes following with both *H. pylori* strain in time dependent manner as shown in Figure 3.16.2. FZD3 and FZD8 genes expressions almost disappeared when each MKN45 and 23132/87 cells were infected with $cagA^{(+)}$, $vacA^{s1m1}$ *H. pylori* but when they were infected with *H. pylori* $cagA^{(-)}$, $vacA^{s2m2}$, we observed considerable amount of decrease in gene expression as seen on Figure 3.16.3-4. FZD10 results was not shown due to have no expression in normal and *H. pylori* infected MKN45 and 23132/87 cells. İn addition to Frizzled receptor we analyze the 2 member of Wnt genes that are Wnt2b and Wnt5b in both cell line. Results indicate that, there is no signification expression changes following with both *H. pylori* strain in time dependent manner as shown in Figure 3.16-21.

.

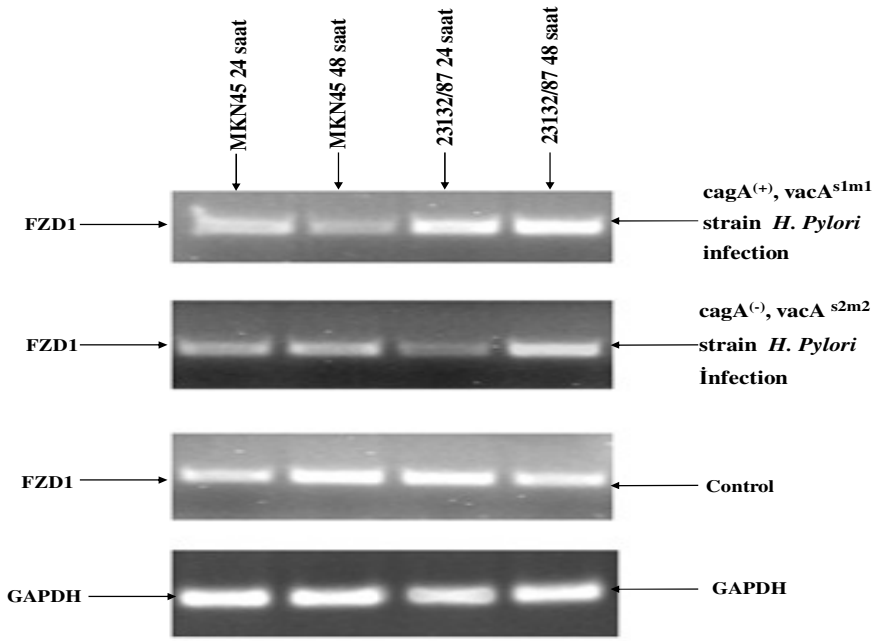


Figure 3.16 Frizzled 1 gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

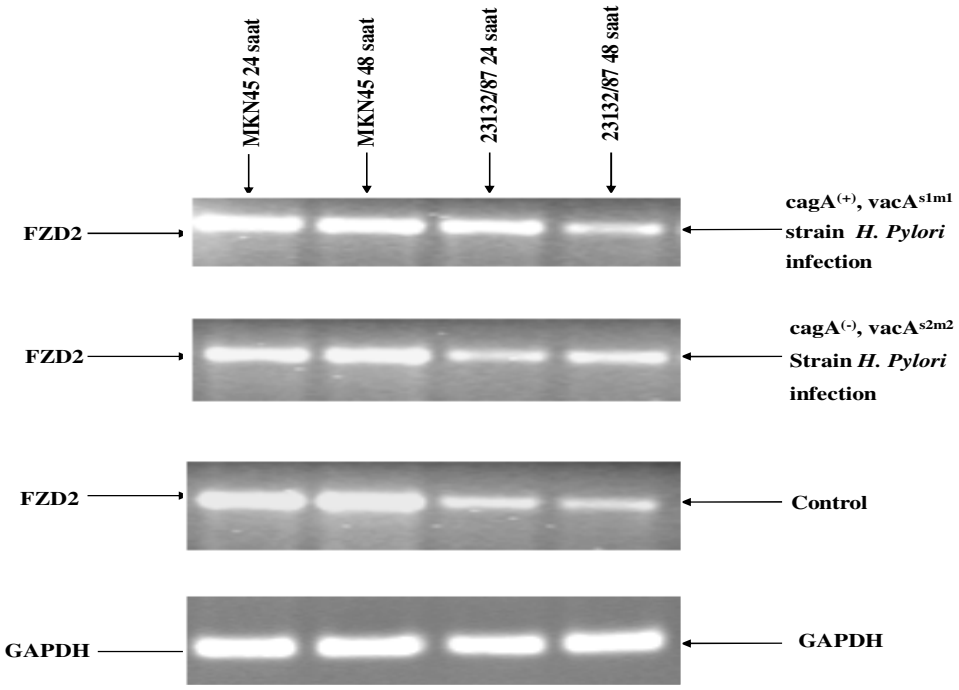


Figure 3.17 Frizzled 2 gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

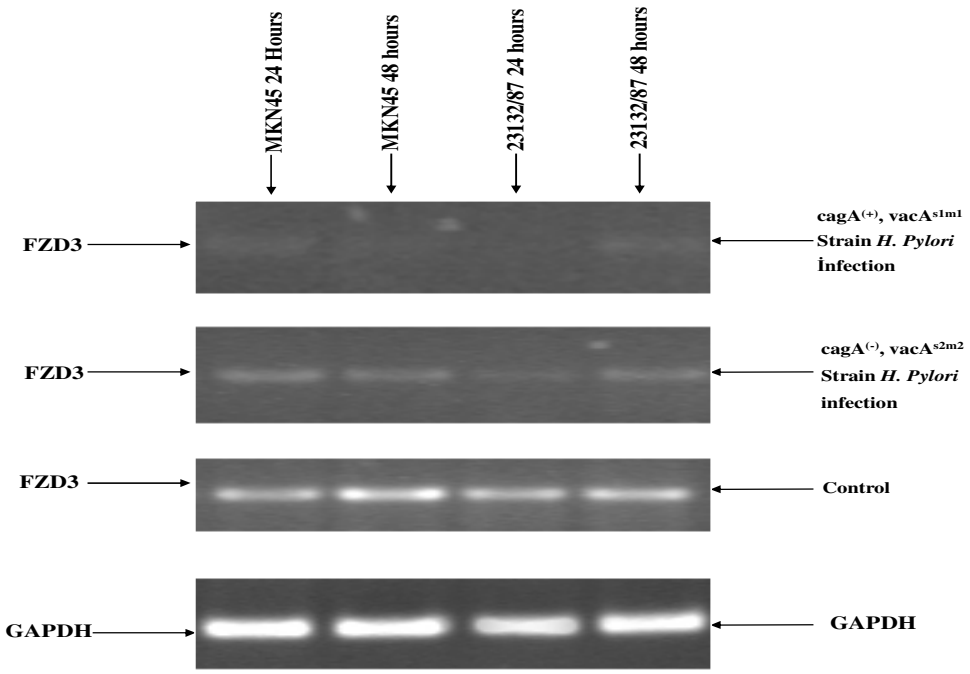


Figure 3.18 Frizzled 3 gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

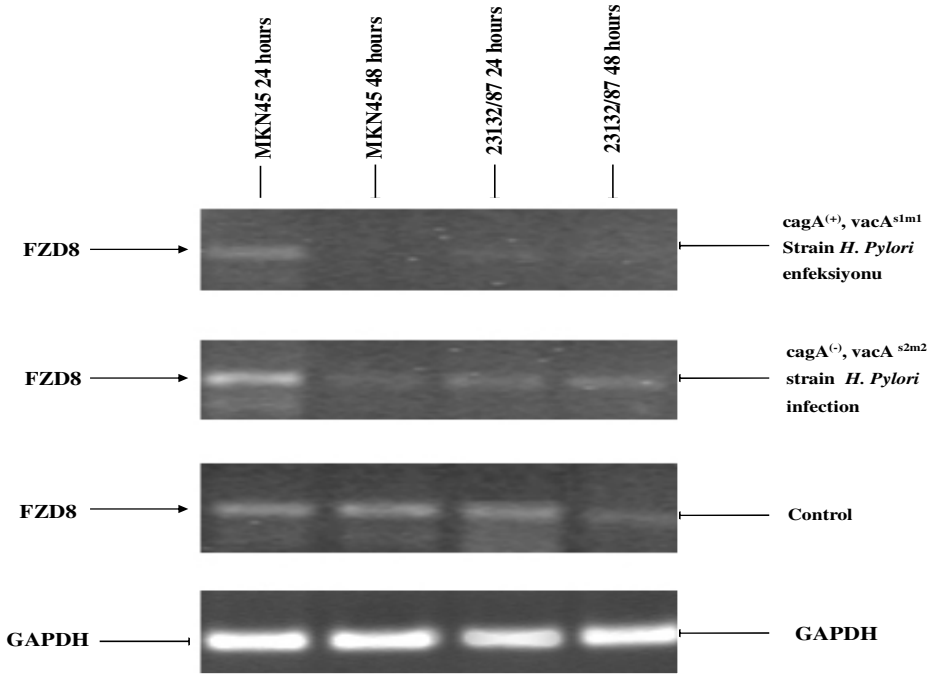


Figure 3.19 Frizzled4 gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

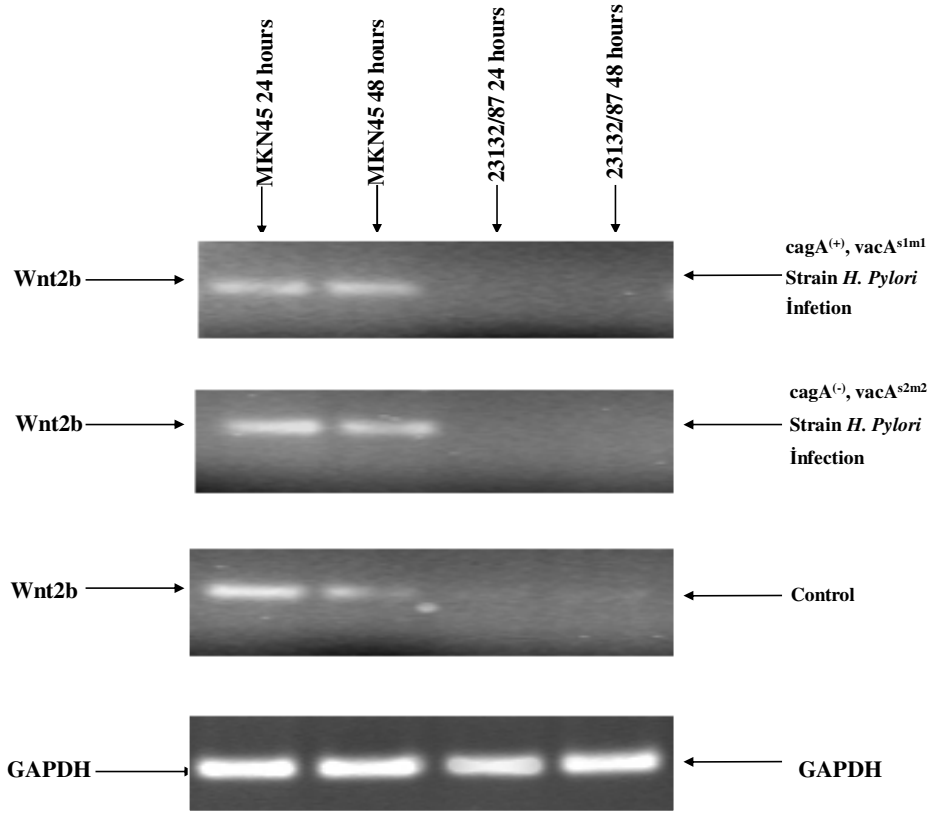


Figure 3.20 Wnt2b gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

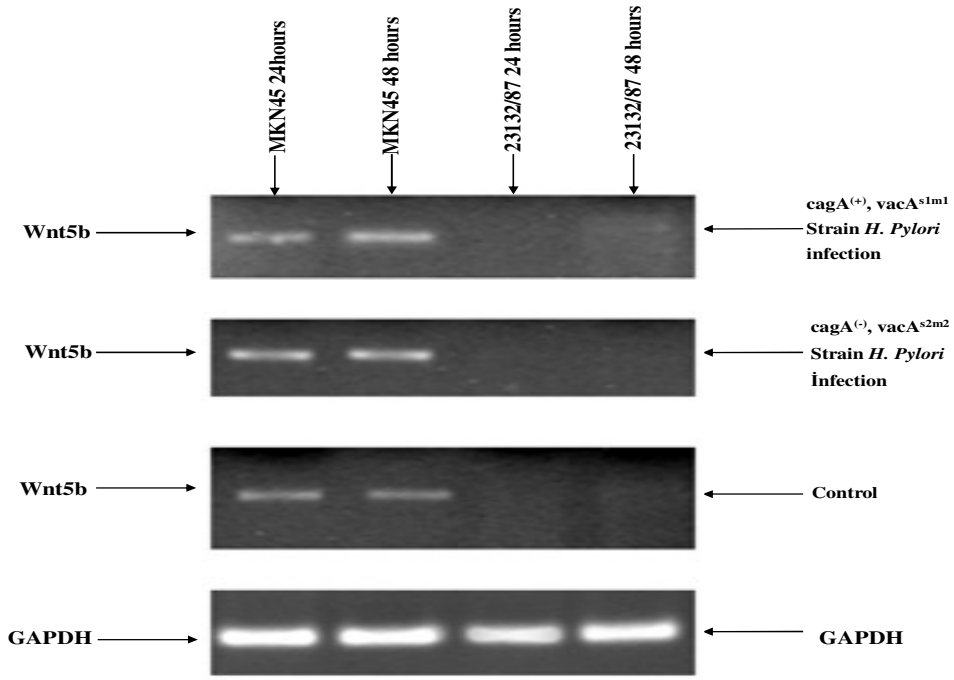


Figure 3.21 Wnt5b gene expression profile after H.pylori infection for 24 and 48 hours in MKN45 and 23132/87.

CHAPTER 4

DISCUSSION

The activity of the wnt pathway is observed many gastrointestinal tract cancers such as esophageal, gastric, intestinal, and colonic. However there is no strong evidence that activity of the wnt pathway in gastric cancer especially Wnt genes and Frizzled receptor expression levels. All previous studies about wnt pathway in gastric cancer are related to *β-Catenin* which is most important member of the pathway.

Activity analysis of the wnt pathway in gastric adenocarcinoma tissue was performed in 2002 at Division of Surgical Oncology in University of Cincinnati. In that study 311 gastric adenocarcinoma tissues was collected and activity of the wnt pathway is established by immunohistrochemical staining of β-Catenin and also β-Catenin mutation was analyzed. According to their results in 90 gastric cancers (29%), β-catenin nuclear staining was present. In the remaining 221 tumors (71%), β-catenin localized to the cell membrane only [149].

Considering this study we want to analyze Wnt ligands and Frizzled receptor expression in order to understand the activity of the wnt pathway. Because wnt pathway is activated by binding of wnt ligand to frizzled receptor that mean expression of these two genes will be activate the signaling pathway.

Activity of the wnt pathway is determined by the localization of β-catenin in the nucleus. Before localization in nucleus the nucleus it should be expressed and accumulate in cytoplasm. As a preliminary study expression of the Wnt, Frizzled, and β-catenin genes profiled and their correlation was analyzed. Results indicate that, as expected, there is strong correlation between Wnt, Frizzled, and β-catenin genes expression due to non-specific binding capacity of ligand to receptor. This is meant that, any member of the Wnt ligand can bind to any Frizzled receptor non specifically by their binding domain which contain a conserved CRD domain.

The big handicap of this study is regulation of the β-catenin expression and localization. Firstly, β-catenin localization is not investigated that give exact result about the activity of the wnt pathway. Secondly, complexity of regulation steps of the Wnt pathway. Because, theoretically, when Frizzled and Wnt genes expressed at the same time in a tissue, that will be resulted in Wnt pathway activation. However Wnt pathways have many regulators in extracellular and intracellular area. For example in extracellular area, there are many regulators such as Wnt inhibiting factor (WIF), Frizzled related proteins (FRPs), Dickoff. All these inhibitors compete with Wnt ligands and Frizzled receptors. For instance, if the FRP level is very high, it will be bind to wnt ligands in extracellular area and will be resulted in loss of binding capability of the Wnt ligands to Frizzled receptor that will lead to inactivation of wnt pathway.

As many biological system, there is a dynamic stability in Wnt signaling pathway regulation as well. If Wnt pathway is inactivated this is not mean that the wnt pathway components such as Wnt and FZDs were not expressed. In that circumstance, most probably, wnt pathway activator and inhibitors are expressed and their level balance to each other so wnt pathway was not activated.

In addition to that, there are two different wnt pathway; canonical and noncanonical. While canonical Wnt pathway has a role in development, survival, and many physiological, noncanonical Wnt pathway has role in actin cytoskeleton regulation, cell adhesion, and cell movement. Noncanonical Wnt pathway activators are

not known exactly however Wnt5a and FZD5 are the mostly known activators. But it is thought that, almost certainly other receptors and ligands may activate the noncanonical wnt pathway as well.

On the other hand, wnt receptors and frizzled genes are not specific to each other. That mean, any member of wnt and frizzled may bind to each other and activate the signaling pathway due to homology and conserved structure of their binding domain which contain cysteine rich domain (CRD).

Finally, Wnt pathway is not activated in all patients even if Wnt ligands, Frizlled receptors, and β-catenin is expressed due to regulation in many different step. As we explain before even if wnt ligands and frizzled receptors are expressed, regulatory proteins may be expressed as well that can be lead to inactivation of the wnt pathway.

In other part of the study, we aimed to investigate the effect of H. pylori on MKN45 and 23132/87 gastric cancer cells and response of cells against toxic and non-toxic bacterial infection. H. pylori is a bacteria which is classified as a Class I Carcinogen by World Health Organization (WHO). H. pylori is a variable bacteria so it's effect is also variable on the cells that mean host response can be variable according to infected bacteria sub-species H. pylori has a cytotoxicity associated pathogenecity island (cagPAI) that region is variable among bacteria and determine its pathogenecity.

In order to study the effect of different bacteria strains on the cells, bacteria strains were cultured from tissues which are collected from patient with gastric disorder. Firstly these obtained strains were genotyped by multiplex PCR and results indicate that one strain was $cagA^{(+)}$, $vacA^{s1m1}$ which mean has both toxic genes and other strain was $cagA^{(-)}$, $vacA^{s2m2}$ which mean has no toxic genes. After characterization of the bacteria, we infect the cells and firstly morphological changes were observed. It is monitored that the cells have lost the cell adhesion, membrane structure, cell shape. In addition to that cells began to scattering which called "Humming Bird Phenotype" as explained before.

MKN45 cells have ability to survive as clumps in the culture medium. Because of that tendency, after H. pylori infection MKN45 cells detached from culture flask. Even though, especially after $cagA^{(+)}$, $vacA^{s1m1}$ strain infection, in most attached MKN45 cells were monitored "Humming Bird" phenotype formation. In 23132/87 cell lines also "Humming Bird" phenotype formation monitored as well that shows the effect H. pylori infection effect on gastric tissue.

Considering this data we decided to analyze whether cells going under apoptosis or not following infection with both strain. Results revealed that, $cagA^{(+)}$, $vacA^{s1m1}$ strain cause apoptosis but $cagA^{(-)}$, $vacA^{s2m2}$ strain is not. As explained in the literature vacA gene causes apoptosis in the host cells via interacting with mitochondria. So this data also confirm our results. However, these results cause big confusing challenge for us. Because H. pylori cause cancer due to activating MAPK signaling pathway that lead to cells to proliferation that also resulted in suppression of apoptosis. But our results were not compatible with our expectations. Because, when we infect the cells by , $cagA^{(+)}$, $vacA^{s1m1}$ H. pylori strain, cells go under apoptosis but in literature this is shown that , $cagA^{(+)}$ gene of H. pylori lead to cells to proliferation.

Considering these confusing results we tried to determine the cells whether proliferative or not. To facilitate that we determine the Ki67 protein level qualitatively by immunocytohemistry method. Results showed that both H. pylori strain decreasing the proliferation capacity of the cells. But as explained before cagA protein should increase the proliferation but our results were not compatible with our expectations. We conducted those results with properties of the cells that related to be a member of innate immune system. MKN45 and 23132/87 cells were epidermal that are a member of innate immune system. Those cells have Toll like receptors (TLRs) that initiate the immune response via activating a signal system that resulted in producing of Interleukin 8 and other cytokines. The production of cytokines may change the homeostasis of the cells due to activating several signaling pathway. Therefore our results we found were not the way we expected to find.

H. pylori is a cancerous microorganism however it is a pathogenic bacteria that recognized by immune system cells by their lypopolysaccharide (LPS), flagella, or membranous proteins. Hence, seemingly this bacteria is activating other signaling patwhway such as NFKB that lead to cells to survival.

In these cells oxidative stress was studied as well with the aim of determination of whether oxidative stress leads to cells to proliferation or apoptosis. Results showed, oxidative stress is increasing following H. pylori infection however 23132/87 cells have already stressed cells even if lacking of H. pylori infection. Thus it was very hard to determine changes in apoptosis or proliferation which are whether related with oxidative stress or not.

Following determination of changes in apoptosis and proliferation we wonder the activity of the Wnt signaling pathway. To find out the activity of the pathway, β-Catenin was stained in normal and H. pylori infected cells then localization of β-Catenin and its production level was compared qualitatively. Results reveal that, there are no significant changes in β-Catenin localization or protein level in the cell. In this experiment, β-Catenin localization was tried to establish by immunocytohemsitry method. But the real challenges in that experiment was MKN45 cells structure. MKN45 cells are small and has very big nucleus almost including 85% of the cytoplasm. Thus, when the nucleus of the cells stained with hematoxylen, all cells color turn to purple that makes us hard to see β-Catenin localization. Therefore, hematoxylen staining was not performed but in that situation correct localization of β-Catenin could not be determined. However qualitative localization was established however results were obvious that there are no significant changes in localization of β-Catenin before and after infection. This result led us to think about the interaction mechanism of cagA gene of H. pylori. CagA protein of H. pylori is effect the signaling protein via binding their SH2 domain that is commonly found in MAPK signaling pathway protein such as SRC. But in Wnt pathway, signaling molecules does not have SH2 domain that allow to

binding of cagA protein. Therefore cagA protein might not be effect the Wnt singaling pathway.

Moreover, CyclinD1 protein was studied that its expression is regulated by β-Catenin, is an indicator of Wnt pathway. CyclinD1 is a protein that has a role in cell cycle check points. Its promoter is working dependent of β-Catenin/TCF4 transcription factor, thus its protein level giving idea about activity of the Wnt pathway. Its protein level was qualitatively analyzed by immunocytochemistry method. Although there are no significant changes in cyclinD1 protein level, in some cases, increased level of cyclinD1 is observed but this is not giving exact idea about activity of wnt pathway related to H. pylori infection. Because in that experiment, the big handicap was transcription factors that regulate CyclinD1 gene expression. Normally, CyclinD1 promoter is regulated by β-Catenin/TCF4 transcription factor but in addition that jun and fos transcription factor that are activated by MAPK pathway may also regulate the activity of CyclinD1 gene. Hence, determination of CyclinD1 does not give exact idea about activity of the Wnt pathway.

Considering Wnt pathway activation results, we decided to determine the expression profile of other components of the wnt pathway, such as Wnt ligands and Frizzed receptors. Results indicate that H. pylori infection is decreasing the expression level of Wnt ligands and Frizzled receptors. Theoretically, that decreased expression of ligands and receptors cause inactivation of the Wnt pathway, consequently will be resulted in localization of β-Catenin in cytoplasm or membrane and its level will be decreased. However our results showed that β-Catenin level is not changing. This data led us to thought about the idea of another regulatory step. That is also known that in cells several signaling pathway has a crosstalk between them, due to that reason, Wnt pathway might be effected by other pathway especially, PIP3/AKT pathway that are have a shared protein which have a very important role in both pathway, named GSK3.

CHAPTER 5

CONCLUSION

Gastric cancer is the one of most dangerous cancer type which reasons to second most cancer related death after lung cancer. As in all cancer type in many signaling pathway is deregulated in gastric cancer. Wnt is one of the signaling pathway that has a role in cancer. In this study we aimed to analyze the activity of the Wnt pathway in gastric cancer cells and tissues.

In order to determine that firstly 61 pair tissue samples were collected from the gastric cancer patient and in these tissue wnt ligands, frizzled receptors, β-Catenin gene, and as an internal control GAPDH gene were amplified by RT-PCR method and Wnt, Frizzled and β-Catenin expression profiles were compared. Results indicate that there is highly coreation between these 3 genes. However this data cannot give the exact idea to determine the whether Wnt pathway is activated or not, since the presence of many regulatory protein in the different step. Therefore, this data might not be sufficient to show activity of the Wnt pathway in tissues. In order to obtain exact result about the activity of the Wnt patwhway activation, β-Catenin protein localization should be determined in formalin fixed paraffin embedded tissues.

Moreover, effect of one of the most important risk factor of the gastric cancer is H. pylori was aimed to analyze. It is tested according to 4 parameter, those are apoptosis, proliferation, Wnt pathway activation, and changes in gene expression. But in these experiment we had obtain unexpected results due to complexity of the signaling

pathway that mean H. pylori effect could not be understand exactly. However, as an extension of this study, the real effect of Cag Pathogenecity Island on gastric cancer progression was planned to determine via producing of Cag Pathogenecity Island coded protein by gene recombination method.

CHAPTER 6

REFERANCES

[1] Garcia M, Jemal A, Ward EM, Center MM, Hao Y, Siegel RL, Thun MJ. Global Cancer Facts & Figures 2007. Atlanta, GA: American Cancer Society, 2007.

[2] Hanahan D and Weinberg R. A. The Hallmarks of Cancer, Cell, Vol. 100, 57–70, January 7, 2000.

[3] Zandi R. et al. Mechanisms for oncogenic activation of the epidermal growth factor receptor, Cellular Signalling 19 (2007) 2013–2023.

[4] T. Holbro, G. Civenni, N.E. Hynes, Exp. Cell Res. 284 (2003) 99.

[5] J. Schlessinger, Cell 110 (2002) 669.

[6] Allen, P. D., Bustin, S. A. & Newland, A. C. (1993) Blood Reviews 7, 63–73.

[7] Cohen, J. J. & Duke, R. C. (1992) Annu. Rev. Immunol. 10, 267–293.

[8] Clarke, P. G. H. (1990) Anat. Embryol. 181, 195–213.

[9] Johnson, E. M. & Deckwerth, T. L. (1993) Annu. Rev. Neurosci. 16, 31–46.

[10] Batistatou, A. & Greene, L. A. (1993) J. Cell Biol. 122, 523–532.

[11] Strange, R. et al. (1992) Development 115, 49–58.

[12] Carson, D. A. & Ribeiro, J. M. (1993) Lancet 341, 1251–1254.

[13] Edgington, S. M. (1993) Biotechnology 11, 787–792.

[14] Gougeon. M.-L. & Montagnier, L. (1993) Science 260, 1269–1270.

[15] Christina Falschlehner, Christoph H. Emmerich, Bjorn Gerlach, Henning Walczak, TRAIL signalling: Decisions between life and death, *The International Journal of Biochemistry & Cell Biology* 39 (2007) 1462–1475.

[16] Hayflick L Mortality and immortality at the cellular level. A review. Biochemistry (Mosc). 1997 Nov;62(11):1180-90

[17] Engelhardt M, Martens UM. The implication of telomerase activity and telomere stability for replicative aging and cellular immortality. Oncol Rep. 1998 Sep-Oct;5(5):1043-52

[18] Shay JW, Wright WE Telomeres and telomerase: implications for cancer and aging. Radiat Res. 2001 Jan;155(1 Pt 2):188-193.

[19] Ferrara, N., 2000. VEGF: an update on biological and therapeutic aspects. Curr. Opin. Biotechnol. 11, 617–624.

[20] Bohle, A. S. and Kalthoff, H. (1999) Molecular mechanisms of tumor metastasis and angiogenesis. Langenbecks Arch. Surg. 384, 133-140.

[21] Nash, G. F., Turner, L. F., Scully, M. F. and Kakkar, A. K. (2002) Platelets and cancer. Lancet Oncol. 3, 425-430.

[22] Westermark, J. and Kahari, V. M. (1999) Regulation of matrix metalloproteinase expression in tumor invasion. FASEB J. 13, 781-792.

[23] Chang, C. and Werb, Z. (2001) The many faces of metalloproteases: cell growth, invasion, angiogenesis and metastasis. Trends Cell. Biol. 11, S37-S43.

[24] Johnsen, M., Lund, L. R., Romer, J., Almholt, K. and Dano, K. (1998) Cancer invasion and tissue remodeling: common themes in proteolytic matrix degradation. Curr. Opin. Cell Biol. 10, 667-671.

[25] Liabakk, N. B., Talbot, I., Smith, R. A., Wilkinson, K. and Balkwill, F. (1996) Matrix metalloprotease 2 (MMP-2) and matrix metalloprotease 9 (MMP-9) type IV collagenases in colorectal cancer. Cancer Res. 56, 190-196.

[26] Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. Int J Cancer 1993;54:594–606

[27] Pisani P, Parkin DM, Ferlay J. Estimates of worldwide mortality from eighteen major cancers in 1985. Implications for prevention, and projections of future burden. Int J Cancer 1993;54:891–903.

[28] Anna D. Panani Cytogenetic and molecular aspects of gastric cancer: Clinical implications Cancer Letters 266 (2008) 99–115

[29] Piet A. van den Brandt Nutrition in the prevention of gastrointestinal cancer. Best Practice & Research Clinical Gastroenterology Vol. 20, No. 3, pp. 589–603, 2006

[30] Jon R. Kelley , John M. Duggan Gastric cancer epidemiology and risk factors Journal of Clinical Epidemiology 56 (2003) 1–9

[31] 1. Salaspuro M. Microbial metabolism of ethanol and acetaldehyde and clinical consequences. Addiction Biology 1997; 2: 35–46.

[32] Homann N. Alcohol and upper gastrointestinal tract cancer: the role of local acetaldehyde production. Addiction Biology 2001; 6: 309–323.

[33] Salaspuro M. Acetaldehyde, microbes and cancer of the digestive tract. Critical Reviews in Clinical and Laboratory Sciences 2003;.

[34] Charnley G, Tannenbaum SR. Flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat. Cancer Res 1985; 45: 5608–16.

[35] Kono S, Hirohata T. Nutition and stomach cancer. Cancer Causes Control 1996;7:41–55.

[36] Blot WJ, Li JY, Taylor PR, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality inthe general population. J Natl Cancer Inst 1993;85:1483–91.

[37] Piet A. van den Brandt, Nutrition in the prevention of gastrointestinal cancer, Best Practice & Research Clinical Gastroenterology, Vol. 20, No. 3, pp. 589–603, 2006.

[38] Neugut AI, Hayek M, Howe G. Epidemiology of gastric cancer. Semin Oncol 1996;23:281–91.

[39] Blot WJ, Li JY, Taylor PR, et al. Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality inthe general population. J Natl Cancer Inst 1993;85:1483–91.

[40] Correa P, Fontham ET, Bravo JC et al. Chemoprevention of gastric dysplasia: randomized trial of antioxidant supplements and anti-helicobacter pylori therapy. J Natl Cancer Inst 2000; 92: 1881e1888.

[41] Pobel D, Riboli E, Cornee J et al. Nitrosamine, nitrate and nitrite in relation to gastric cancer: a casecontrol study in Marseille, France. Eur J Epidemiol 1995; 11(1): 67–73.

[42] Sander J, Burkle G, Schweinsberg F. Induction of tumors by nitrite and secondary amines or amides. In: Nakahara W, Takayama S, Sugimura T, Odashima S. *Topics in chemical carcinogenesis*. Baltimore: University Park Press, 1972: 292–312.

[43] Ruddel WSJ, Bone ES, Hill MJ, *et al*. Gastric juice nitrate: a risk factor for cancer in the hypochlorhydric stomach. *Lancet* 1976; **2:** 1037–39

[44] Knight TM, Forman D, Al Dabbagh SA et al. Estimation of dietary intake of nitrate and nitrate in Great Britain. Food Chem Toxicol 1987; 25: 277e285

[45] Parkin DM, Whelan SL, Ferlay J, et al. Cancer incidence in fivecontinents, vol VII. Lyon: International Agency for Research on Cancer; 1997. p. 922–4.

[46] Crabb DW, Edenberg HJ, Bosron WF & Li T-K. Genotypes for aldehyde dehydrogenase deficiency and alcohol sensitivity. The inactive ALDH2*2 allele is dominant. Journal of Clinical Investigation 1989; 83: 314–316.

[47] Goedde HW, Agarwal DP, Fritze G et al. Distribution of ADH2 and ALDH2 genotypes in different populations. Human Genetics 1992; 88: 344–346.

[48] Harada S, Agarwal DP & Goedde HW. Aldehyde dehydrogenase deficiency as cause of facial flushing reaction to alcohol in Japanese. Lancet 1981; 2: 982.

[49] Peng G-S,Wang M-F, Chen C-Yet al. Involvement of acetaldehyde for full protection against alcoholism by homozygosity of the variant allele of mitochondrial aldehyde dehydrogenase gene in Asians. Pharmacogenetics 1999; 9: 463–476.

[50] Chen CC, Lu RB, Chen YC et al. Interaction between the functional polymorphism of the alcoholmetabolism genes in protection against alcoholism. American Journal of Human Genetics 1999; 65: 795–807.

[51] Yokoyama A, Muramatsu T, Ohmori T et al. Alcohol-related cancers and aldehyde dehydrogenase-2 in Japanese alcoholics. Carcinogenesis 1998; 19: 1383–1387.

[52] Murata M, Tagawa M,Watanabe S et al. Genotype difference of aldehyde dehydrogenase 2 gene in alcohol drinkers influences the incidence of Japanese colorectal cancer patients. Japanese Journal of Cancer Research 1999; 90: 711–719.

[53] Bosron WF & Li T-K. Genetic polymorphism of human liver alcohol and aldehyde dehydrogenases, and their relationship to alcohol metabolism and alcoholism. Hepatology 1986; 6: 502–510.

[54] Seitz HK. Alcohol effects on drug-nutrient interaction. Drug and Nutrition Interaction 1985; 4: 143–146.

[55] Lieber CS. Microsomal ethanol oxidizing system (MEOS): the first 30 years (1968–1998)—a review Alcoholism, Clinical and Experimental Research 1999; 23: 991–1007.

[56] Seitz HK, Po¨ schl G & Simanowski UA. Alcohol and cancer. In Galanter M Recent Developments in Alcoholism, vol. 14, pp 67–95, New York: Plenum Press, 1998.

[57] Feldman RA, Eccersley AJ, Hardie JM. Epidemiology of *Hpylori*: acquisition, transmission, population prevalence and disease-to-infection ratio. *Br Med Bull* 1998; 54: 39-53

[58] Dooley CP, Cohen H, Fitzgibbons PL, Bauer M, Appleman MD, Perez-Perez GI, Blaser MJ. Prevalence of *Helicobacter pylori* infection and histologic gastritis in asymptomatic persons. *N Engl J Med* 1989; 321: 1562-1566

[59] Asaka M, Kimura T, Kudo M, Takeda H, Mitani S, Miyazaki T, Miki K, Graham DY. Relationship of *H pylori* to serum pepsinogens in an asymptomatic Japanese population.*Gastroenterology* 1992; 102: 760-766

[60] Youn HS, Ko GH, Chung MH, Lee WK, Cho MJ, Rhee KH.Pathogenesis and prevention of stomach cancer. *J Korean Med Sci* 1996; 11: 373-385

[61] Pentti Sipponen, Hanna Vauhkonen, Matti Vauhkonen, Pathology and molecular biologyof gastric cancer, Best Practice & Research Clinical Gastroenterology, Vol. 20, No. 4, pp. 651e674, 2006

[62] IARK Working Group on the evaluation of carcinogenic risks to humans,1994. Schistosomes, liver flukes and Helicobacter pylori. Lyon, June 7–14, 1994. IARC Monogr. Eval. Carcinog. Risks Hum. 61, 1– 241.

[63] Stoicov C. et. al. Molecular biology of gastric cancer: Helicobacter infection and gastric adenocarcinoma: bacterial and host factors responsiblefor altered growth signaling, Gene 341 (2004) 1 –17

[64] Michael Naumann and Jean E. Crabtree, Helicobacter pylori-induced epithelial cell signalling in gastric carcinogenesis TRENDS in Microbiology Vol.12 No.1 ,2004

[65] Peek, R.M. and Blaser, M.J. (2002) Helicobacter pylori and gastrointestinaltract adenocarcinomas. Nat. Rev. Cancer 2, 28–37

[66] El-Omar, E.M. et al. (2003) Increased risk of noncardia gastric cancer associated with proinflammatory cytokine gene polymophisms. Gastroenterology 124, 1193–1201

[67] Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. Gastroenterology 2008;134:306–23.

[68] Scott DR,Weeks D, Hong C, Postius S, Melchers K, Sachs G. The role of internal urease in acid resistance of *Helicobacter pylori*. Gastroenterology 1998;114:58–70.

[69] Weeks DL, Eskandari S, Scott DR, Sachs G. A H+-gated urea channel:the link between *Helicobacter pylori* urease and gastric colonization.Science 2000;287:482–5.

[70] Censini S, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A. 1996. Cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc Natl Acad Sci. 93, 14648-14653.

[71] Akopyants NS, Clifton SW, Kersulyte D, Crabtree JE, Youree BE, Reece CA, Bukanov NO, Drazek ES, Roe BA, Berg DE. 1998. Analyses of the cag pathogenicity island of *Helicobacter pylori*. Mol. Microbiol. 28, 37–53.

[72] Yamazaki S, Yamakawa A, Okuda T, Ohtani M, Suto H, Ito Y, Yamazaki Y, Keida Y,.Higashi H, Hatakeyama M, Azuma T. 2005. Distinct diversity of vacA, cagA, and cagE genes of *Helicobacter pylori* associated with peptic ulcer in Japan. Clin Microbiol. 43, 3906-3916.

[73] Keates S, Keates AC, Warny M, Peek Jr RM, Murray PG, Kelly CP.Differential activation of mitogen-activated protein kinases inAGS gastric epithelial cells by cag+ and cag− *Helicobacter pylori*. J Immunol 1999;163:5552–9.

[74] Asahi M, Azuma T, Ito S, Ito Y, Suto H, Nagai Y, et al. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. J Exp Med 2000;191:593–602.

[75] Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, et al. SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. Science 2002;295:683–6.

[76] Tsutsumi R, Higashi H, Higuchi M, Okada M, Hatakeyama M. Attenuation of *Helicobacter pylori* CagA×SHP-2 signaling by interaction between CagA and C-terminal Src kinase. J Biol Chem 2003;278:3664–70.

[77] Naito Y, Yoshikawa T. Molecular and cellular mechanisms involved in *Helicobacter pylori*-induced inflammation and oxidative stress. Free Radic Biol Med 2002;33:323–36.

[78] H. Higashi, R. Tsutsumi, S. Muto, T. Sugiyama, T. Azuma, M. Asaka, M. Hatakeyama, SHP-2 tyrosine phosphatase as an intracellular target of Helicobacter pylori CagA protein, Science 295 (2002) 683–686.

[79] R. Tsutsumi, H. Higashi, M. Higuchi, M. Okada, M. Hatakeyama, Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase, J. Biol. Chem. 278 (2003) 3664–3670.

[80] M. Suzuki, H. Mimuro, T. Suzuki, M. Park, T. Yamamoto, C. Sasakawa Interaction of CagA with Crk plays an important role in Helicobacter pylori-induced loss of gastric epithelial cell adhesion, J. Exp. Med. 202 (2005) 1235–1247.

[81] M. Gerhard, N. Lehn, N. Neumayer, T. Boren, R. Rad, W. Schepp, S.Miehlke, M. Classen, C. Prinz, Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin, Proc. Natl. Acad. Sci. USA 96 (1999) 12778–12783.

[82] J. Parsonnet, G.D. Friedman, N. Orentreich, H. Vogelman, Risk for gastric cancer in people with CagA positive or CagA negative Helicobacter pylori infection, Gut 40 (1997) 297–301.

[83] Peek Jr., R.M., Blaser, M.J., 2002. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat. Rev., Cancer 2, 28– 37.

[84] Peek Jr., R.M., Moss, S.F., Tham, K.T., Perez-Perez, G.I., Wang, S., Miller, G.G., Atherton, J.C., Holt, P.R., Blaser, M.J., 1997. Helicobacter pylori cagA+ strains and dissociation of gastric epithelial cell proliferation from apoptosis. J. Natl. Cancer Inst. 89, 863– 868.

[85] Stein, M., Rappuoli, R., Covacci, A., 2000. Tyrosine phosphorylation of the Helicobacter pylori CagA antigen after cag-driven host cell translocation. Proc. Natl. Acad. Sci. U. S. A. 97, 1263–1268.

[86] Kuck D, Kolmerer B, Iking-Konert C, Krammer PH, Stremmel W, Rudi J. Vacuolating cytotoxin of *Helicobacter pylori* induces apoptosis in the human gastric epithelial cell line AGS. Infect Immunol 2001;69:5080–7.

[87] Yuan JP, Li T, Chen HB, Li ZH, Yang GZ, Hu BY, et al. Analysis of gene expression profile in gastric cancer cells stimulated with *Helicobacter pylori* isogenic strains. J Med Microbiol 2004;53:965–74.

[88] Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL. 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*: Association of specific vacA types with cytotoxin production and peptic ulceration. J. Biol. Chem. 270, 17771–17777.

[89] Xiang Z, Censini S, Bsyeli PF, Telford JL, Figura N, Rappuoli R, Covacci A. 1995. Analysis of expression of cagA and vacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that cagA is not necessary for expression of the vacuolating cytotoxin. Infect. Immun. 63, 94–98.

[90] Van Doorn LJ, Figueiredo C,. Sanna R, Plaisier A, Schneeberger P, de Boer W, Quint W. 1998. Clinical relevance of cagA, vacA, and iceA status of *Helicobacter pylori*. Gastroenterol. 115, 58–66.

[91] Martins LC, Corvelo TC, Demachki S, Araujo MT, Assumpcao MB, Vilar SC, Freitas FB, Barbosa HP, Fecury AA, do Amaral RK, Dos Santos SE. 2005. Clinical and pathological importance of vacA allele heterogeneity and cagA status in peptic ulcer disease in patients from North Brazil. Mem Inst Oswaldo Cruz. 100, 875-881.

[92] Pan Z, Berg DE, van der Hulst RWM, Su W, Raudonikiene A, Xiao S, Dankert J, Tytgat GNJ, van der Ende A. 1998. Prevalence of vacuolating cytotoxin production and distribution of distinct vacA alleles in *Helicobacter pylori* from China. J. Infect. Dis. 178, 220–226.

[93] Mukhopadhyay AK, Kersulyte D, Jeong J, Datta S, Ito Y, Chowdhury A, Chowdhury S, Santra A, Bhattacharya SK, Azuma T, Nair B, Berg DE. 2000. Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. J. Bacteriol. 182, 3219–3227.

[94] Willhite CD, Blanke RS. 2004. *Helicobacter pylori* vacuolating cytotoxin enters cells, localizes to the mitochondria, and induces mitochondrial membrane permeability changes correlated to toxin channel activity. Cell Microbiol. 6, 143-154.

[95] Handa O, Naito Y, Ishii T, Tsuboi H, Adachi S, Takagi T, et al. Helicobacter pylori Cag A induces mitochondriadependent production of reactive oxygen species in gastric epithelial cell. Gastroenterology 2006;130.

[96] Handa O, Naito Y, Ishii T, Tsuboi H, Adachi S, Takagi T, et al. Cytotoxin-associated gene product A of Helicobacter pylori induced cellular response in rat gastric epithelial cell. Digestion 2006;73(Suppl 2):69.

[97] Wei YH, Lu CY, Lee HC, Pang CY, Ma YS. Oxidative damage and mutation to mitochondrial DNA and age-dependent decline of mitochondrial respiratory function. Ann N Y Acad Sci 1998;854:155–70.

[98] Dobson AW, Xu Y, Kelley MR, LeDoux SP, Wilson GL. Enhanced mitochondrial DNA repair and cellular survival after oxidative stress by targeting the human 8-oxoguanine glycosylase repair enzyme to mitochondria. J Biol Chem 2000;275:37518–23.

[99] Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, et al. Gastric cancer originating from bone marrow-derived cells. Science 2004;306:1568–71.

[100] Ernst P. Review article: the role of inflammation in the pathogenesis of gastric cancer. Aliment Pharmacol Therapeut 1999;13(Suppl 1):13–8.

[101] Farinati F, Cardin R, Russo VM, Busatto G, Franco M, Rugge M. Helicobacter pylori CagA status, mucosal oxidative damage and gastritis phenotype: a potential pathway to cancer? Helicobacter 2003;8:227–34.

[102] Maaroos HI, Vorobjova T, Sipponen P, Tammur R, Uibo R, Wadstrom T, et al. An 18-year follow-up study of chronic gastritis and Helicobacter pylori association of CagA positivity with development of atrophy and activity of gastritis. Scand J Gastroenterol 1999;34:864–9.

[103] Ferrero R.L., Innate immune recognition of the extracellular mucosal pathogen,*Helicobacter pylori* Molecular Immunology 42 (2005) 879–885

[104] Blaser, M.J., Perez-Perez, G.I., Kleanthous, H., Cover, T.L., Peek, R.M., Chyou, P.H., Stemmermann, G.N., Nomura, A., 1995. Infection with *Helicobacter pylori* strains possessing cagA is associated with an increased

risk of developing adenocarcinoma of the stomach. Cancer Res. 55, 2111–2115.

[105] Peek Jr., R.M., Miller, G.G., Tham, K.T., P´erez-P´erez, G.I., Zhao, X.,Atherton, J.C., Blaser, M.J., 1995. Heightened inflammatory response and cytokine expression in vivo to *cagA+ Helicobacter pylori* strains. Lab. Invest. 71, 760–770.

[106] Wu, A.H., Crabtree, J.E., Bernstein, L., Hawtin, P., Cockburn, M., Tseng, C.C., Forman, D., 2003. Role of *Helicobacter pylori* CagA+ strains and risk of adenocarcinoma of the stomach and esophagus. Int. J. Cancer 103, 815–821.

[107] S. Wen, S.F. Moss, Helicobacter pylori virulence factors in gastric carcinogenesis, Cancer Lett. (2008)

[108] Richter, C., Godvadze, V., Laffranchi, R., Schlapbach, R., Schweizer, M., Suter, M., Walter-Royal, J. A., and Ischiropoulus, H. (1993). Evalution of 2'-7'-dichlorodihydrofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch. Biochem. Biophys. 302, 348-355

[109] Polakis P. The many ways of Wnt in cancer, Current Opinion in Genetics & Development 2007, 17:45–51

[110] He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW: Identification of c-MYC as a target of the APC pathway. Science 1998;281: 1509–1512.

[111] Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, Ben-Ze'ev A: The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 1999; 96:5522–5527.

[112] BHANOT, P., BRINK, M., SAMOS, C.H., HSIEH, J.C., WANG, Y., MACKE, J.P., ANDREW, D., NATHANS, J. and NUSSE, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature 382: 225-230.

[113] TAMAI, K., SEMENOV, M., KATO, Y., SPOKONY, R., LIU, C., KATSUYAMA, Y., HESS, F., SAINT-JEANNET, J.P. and HE, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature 407: 530-535.

[114] WEHRLI, M., DOUGAN, S.T., CALDWELL, K., O'KEEFE, L., SCHWARTZ, S.,VAIZEL-OHAYON, D., SCHEJTER, E., TOMLINSON,

A. and DINARDO, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature 407: 527-530.

[115] Nelson, W. J. & Nusse, R. Convergence of Wnt, β-catenin, and cadherin pathways. *Science* **303**, 1483–1487 (2004).

[116] Bienz M, Clevers H: Linking colorectal cancer to Wnt signaling. Cell 2000;103:311–320.

[117] Fodde R, Smits R, Clevers H: APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 2001;1:55–67.

[118] Fearon ER, Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990;61:759–767.

[119] Renan MJ: How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinog 1993;7:139–146.

[120] Polakis P: Wnt signaling and cancer. Genes Dev 2000;14:1837–1851.

[121] Pisani P, Parkin DM, Bray F, Ferlay J: Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999;83:18–29.

[122] CAPELLUTO, D.G., KUTATELADZE, T.G., HABAS, R., FINKIELSTEIN, C.V., E, X. and OVERDUIN, M. (2002). The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. Nature 419: 726-729.

[123] ROUSSET, R., MACK, J.A., WHARTON, K.A., JR., AXELROD, J.D., CADIGAN, K.M., FISH, M.P., NUSSE, R. and SCOTT, M.P. (2001). Naked cuticle targets dishevelled to antagonize Wnt signal transduction. Genes Dev 15: 658-671.

[124] SUN, T.Q., LU, B., FENG, J.J., REINHARD, C., JAN, Y.N., FANTL, W.J. and WILLIAMS, L.T. (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. Nat Cell Biol 3: 628-636.

[125] CHEYETTE, B.N., WAXMAN, J.S., MILLER, J.R., TAKEMARU, K., SHELDAHL, L.C., KHLEBTSOVA, N., FOX, E.P., EARNEST, T. and MOON, R.T. (2002). Dapper, a Dishevelled-associated antagonist of beta-catenin and JNK signaling, is required for notochord formation. Dev Cell 2: 449-461.

[126] SEIDENSTICKER, M.J. and BEHRENS, J. (2000). Biochemical interactions in the wnt pathway. Biochim Biophys Acta 1495: 168-182.

[127] ZENG, L., FAGOTTO, F., ZHANG, T., HSU, W., VASICEK, T.J., PERRY, W.L., 3RD, LEE, J.J., TILGHMAN, S.M., GUMBINER, B.M. and COSTANTINI, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell 90: 181-192.

[128] BEHRENS, J., JERCHOW, B.A., WURTELE, M., GRIMM, J., ASBRAND, C., WIRTZ, R., KUHL, M., WEDLICH, D. and BIRCHMEIER, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280: 596-599.

[129] HART, M.J., DE LOS SANTOS, R., ALBERT, I.N., RUBINFELD, B. and POLAKIS, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr Biol 8: 573-581.

[130] IKEDA, S., KISHIDA, S., YAMAMOTO, H., MURAI, H., KOYAMA, S. and KIKUCHI, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. EMBO J 17: 1371-1384.

[131] KISHIDA, S., YAMAMOTO, H., IKEDA, S., KISHIDA, M., SAKAMOTO, I., KOYAMA, S. and KIKUCHI, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem 273: 10823-10826.

[132] LIU, C., LI, Y., SEMENOV, M., HAN, C., BAEG, G.H., TAN, Y., ZHANG, Z., LIN, X. and HE, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell 108: 837-847.

[133] AMIT, S., HATZUBAI, A., BIRMAN, Y., ANDERSEN, J.S., BEN-SHUSHAN, E., MANN, M., BEN-NERIAH, Y. and ALKALAY, I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev 16: 1066-1076.

[134] Kolligs FT, Nieman MT, Winer I, Hu G, Van Mater D, Feng Y, Smith IM, Wu R, Zhai Y, Cho KR, Fearon ER: ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with beta-catenin defects and promotes neoplastic transformation. Cancer Cell 2002;1:145–155.

[135] Rhee CS, Sen M, Lu DS, et al. Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas. Oncogene 2002, 21(43), 6598–6605.

[136] Kirikoshi H, Sekihara H, Katoh M. Molecular cloning and characterization of human WNT11. Int J Mol Med 2001, 8(6), 651–656.

[137] Holcombe RF, Marsh JL, Waterman ML, et al. Expression of Wnt ligands and Frizzled receptors in colonic mucosa and in colon carcinoma. J Clin Pathol – Mol Pathol 2002, 55(4), 220–226.

[138] Wehrli M, Dougan ST, Caldwell K, et al. Arrow encodes an LDL-receptor-related protein essential for wingless signalling.Nature 2000, 407(6803), 527–530.

[139] Schweizer L, Varmus H. Wnt/Wingless signaling through beta catenin requires the function of both LRP/arrow and frizzled classes of receptors. BMC Cell Biol 2003, 4(1), 4.

[140] Tamai K, Semenov M, Kato Y, et al. LDL-receptor-related proteins in Wnt signal transduction. Nature 2000, 407(6803), 530–535.

[141] Pinson KI, Brennan J, Monkley S, et al. An LDL-receptorrelated protein mediates Wnt signalling in mice. Nature 2000, 407(6803), 535–538.

[142] Terasaki H, Saitoh T, Shiokawa K, et al. Frizzled-10, upregulated in primary colorectal cancer, is a positive regulator of the WNT – beta-catenin – TCF signaling pathway. Int J Mol Med 2002, 9(2), 107–112.

[143] Kirikoshi H, Sekihara H, Katoh M. Expression profiles of 10 members of Frizzled gene family in human gastric cancer. Int J Oncol 2001, 19(4), 767–771.

[144] Kirikoshi H, Sekihara H, Katoh M. Up-regulation of Frizzled-7 (FZD7) in human gastric cancer. Int J Oncol 2001, 19(1), 111–115.

[145] To KF, Chan MW, Leung WK, et al. Alterations of frizzled (FzE3) and secreted frizzled related protein (hsFRP) expression in gastric cancer. Life Sci 2001, 70(4), 483–489.

[146] Kawasaki Y, Senda T, Ishidate T, Koyama R, Morishita T, Iwayama Y, Higuchi O, Akiyama T: Asef, a link between the tumor suppressor APC and G-protein signaling. Science 2000, 289:1194-1197.

[147] Kawasaki Y, Sato R, Akiyama T: Mutated APC and Asef are involved in themigration of colorectal tumour cells. Nat Cell Biol 2003, 5:211-215.

[148] Habas R, Dawid IB, He X: Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. Genes Dev 2003, 17:295-309.

[149] Clements W. M, Wang J., Sarnaik A., Kim O. J., MacDonald J., Preiser C. F., Groden J., and Lowy A. J. β-Catenin Mutation Is a Frequent Cause of Wnt Pathway Activation in Gastric Cancer CANCER RESEARCH 62, 3503–3506, June 15, 2002

[150] Cinzia F. Sala, Elisa Formenti, Georg C. Terstappen, and Andrea Caricasole Identification, Gene Structure, and Expression of Human Frizzled-3 (*FZD3*) Biochemical and Biophysical Research Communications **273,** 27–34 (2000)

[151] S. Sonderegger, H. Husslein, C. Leisser, M. Knofler Complex Expression Pattern of Wnt Ligands and Frizzled Receptors in Human Placenta and its Trophoblast Subtypes Placenta 28, Supplement A, Trophoblast Research, Vol. 21 (2007) S97eS102