SEPTEMBER 2018

M.Sc. in Biology

IBRAHIM AL-DOORI

UNIVERSITY OF GAZİANTEP

GRADUATE SCHOOL OF

NATURAL & APPLIED SCIENCES

INVESTIGATION OF SP1 AND SP3 GENE EXPRESSION LEVELS IN COLORECTAL CANCER

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IBRAHIM AL-DOORI

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Investigation of Sp1 and Sp3 Gene Expression Levels in Colorectal Cancer

M.Sc. Thesis

in

Biology

University of Gaziantep

Supervisor

Assist. Prof. Dr. Türkan GÜRER

by

Ibrahim AL-DOORI

September 2018

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Ibrahim AL-DOORI

ABSTRACT

INVESTIGATION OF SP1 AND SP3 GENE EXPRESSION LEVELS IN COLORECTAL CANCER

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M.Sc. in Biology
Supervisor: Assist. Prof. Dr. Türkan GÜRER
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Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. Specificity protein 1 (Sp1) is a well-known member of a family of transcription factors that also includes (Sp3). Sp1 and Sp3 proteins can enhance or repress gene expression by binding to similar, if not the same, DNA tracts and compete for binding. Sp1 activates the transcription of many cellular genes that contain putative CG-rich Sp-binding sites in their promoters. Evidence exists that the Sp-family of proteins regulates the expression of genes that play pivotal roles in cell proliferation and metastasis of various tumors. Also, they are implicated in a great variety of the essential biological processes and have been proven valuable in cell growth, differentiation, apoptosis, and carcinogenesis. We collected cancerous tissues and normal adjacent tissues from the 41 patients who were diagnosed with CRC clinically. We detected the levels of mRNA for both Sp1 and Sp3 by using real-time PCR in both cancerous and tumor-adjacent normal tissues of the samples. We analyzed the results of real-time PCR by using (IBM SPSS Statistics version 22) software. In the results of this study, there were no significant differences in the expression levels of both Sp1 and Sp3 in tumor tissues compared to normal tissues ($p > 0.05$), as well as no association with clinicopathological factors. Sp1 expression was found highly correlated to the expression of Sp3 ($r = 0.827$). In conclusion, our results suggest that Sp1 and Sp3 may not present an important role in CRC carcinogenesis.

Key Words: colorectal cancer, specificity proteins, Sp1, Sp3, gene expression, real-time PCR.

ÖZET

KOLOREKTAL KANSERDE SP1 VE SP3 GEN EKSPRESYON DÜZEYLERİNİN ARAŞTIRILMASI

AL-DOORI, Ibrahim
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72 sayfa

Kolorektal kanser (KRK), dünya çapında kansere bağlı ölümlerin önde gelen nedenlerinden biridir. Spesifiklik proteinleri olarak bilinen Sp1 ve Sp3, transkripsiyon faktör ailesinin iyi bilinen birer üyeleridir. Sp1 ve Sp3 proteinleri, aynı DNA bağlanma bölgelerine ya da benzer bölgelere bağlanarak; bağlanmaya rekabet ederek gen ekspresyonunu arttırabilir veya baskılayabilir. Sp1, birçok hücresel genin GC açısından zengin Sp bağlanma bölgelerine bağlanarak ilgili genlerin transkripsiyonunu aktive eder. Sp-protein ailesinin, hücre proliferasyonunda ve çeşitli tümörlerin metastazında önemli roller oynayan genlerin ekspresyonunu düzenlediğine dair kanıtlar vardır. Ayrıca, önemli biyolojik süreçlerin çeşitliliğininin birçoğu ile ilişkili olduğu ve hücre büyümesi, farklılaşması, apoptoz ve karsinojenezde de oldukça önemli olduğu kanıtlanmıştır. Bu tez çalışmasında, KRK tanısı konmuş 41 hastadan kanserli ve komşu normal dokular toplanmıştır. Toplanan örneklerin kanserli ve normal dokularında Real-Time PCR kullanarak, Sp1 ve Sp3 için mRNA ekspresyon seviyeleri analiz edilmiştir. Real-Time PCR sonuçları SPSS programı kullanılarak analiz edilmiştir. Çalışma sonuçlarına göre, tümör ve normal dokular arasında Sp1 ve Sp3 ekspresyonları açısından anlamlı bir fark bulunmamıştır ($p>0.05$). Sp1 ekspresyonu ve Sp3 ekspresyonu arasında oldukça ilişkili bir korelasyon bulunmuştur. Bu çalışmanın sonucunda, Sp1 ve Sp3'ün KRK karsinogenezinde herhangi bir rol oynamadığı düşünülmektedir.

Anahtar Kelimeler: kolorektal kanser, spesifiklik proteinler, SP1, SP3, gen ekspresyonu, Real-time PCR

To My Family

ACKNOWLEDGEMENTS

First of all, I would like to present my real, honest, and countless thanks to the one and only God, creator and sustainer of the universe '**Allah**'.

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LIST OF ABBREVIATION

AD	Activation domains
ADP-Ribose	Adenosine diphosphate ribose
AJCC	American Joint Committee on Cancer
AP2	Human adipocyte fatty acid-binding protein
APC	Adenomatous polyposis coli
APRT	Adenine phosphoribosyltransferase
BMP3	Bone morphogenic protein 3
Btd	Buttonhead
BTEB	Basal transcription element binding proteins
C2H2	Cys2His2
C3H10T1/2	Mouse embryo fibroblast
Caco-2 cells	Heterogeneous human epithelial colorectal adenocarcinoma cells
CD151	Cluster of Differentiation 151
cDNA	Complementary DNA
CHIP	Chromatin immunoprecipitation
CRC	Colorectal Cancer
Ct	Cycle threshold
CTα	Phosphocholine Cytidylyltransferase alpha
dH_2O	Distilled water
Dhfr	Dihydrofolate reductase gene
DNA	Deoxyribonucleic acid
DNMT1	DNA cytosine methyltransferase
ERα	Estrogen receptor alpha
g	g-force
GAL4	Galactose-induced gene 4
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HD	Huntington's disease

HDAC1	Histone deacetylase 1
HDAC2	Histone deacetylase 2
HSC-2	Human oral cancer cell lines
ID	Inhibitory domain
IE gene	Immediate Early gene
KLFs	Krüppel-like factors
KRAS	Kirsten at sarcoma viral oncogene homolog
mDhfr	Mouse dihydrofolate reductase gene
MECB	Methanol extracts of *C. bursa ‑pastoris*
MECO	Methanol extracts *C. officinale* Makino
mg	Milligram
ml	Milliliters
mm	Millimeters
MSI	Microsatellite instability
N	Normal
n	Number of samples
NDRG4	N-Myc downstream-regulated gene 4
ng	Nano gram
NIS	Sodium/iodide symporter
nm	Nanometer
NMR	Nuclear magnetic resonance
NTC	no-template controls
OA	Oncogene addiction
O.D.	Optical Density
p	p value
PCR	Polymerase chain reaction
PIAS1	Protein Inhibitor of Activated STAT 1
pol II	DNA polymerase II
PTMs	Post-translational modifications
qPCR	Quantitative polymerase chain reaction
PARP	Poly (ADP-ribose) polymerase
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse Transcription

siRNA	Small interfering RNA
SL2	Schneider's Drosophila Line 2
Sp	Specificity protein
Sp1	Specificity Protein 1
Sp2	Specificity protein 2
Sp3	Specificity Protein 3
Sp4	Specificity protein 4
SSP	Sessile serrated polyps
STAT1	Signal transducer and activator of transcription 1
SUMOs	Small ubiquitin-related modifiers
SV40	Polyomavirus simian virus
SW613-S	Human colon carcinoma cell line
T	Tumor
TAFs	TATA-box-binding protein-associated factors
TGFβ	Transforming growth factor β
TIEG	TGFβ-inducible early gene
TNM	Tumor-node-metastasis
VEGF	Vascular endothelial growth factor
℃	Celsius degree
µL	Microliter
µM	Micro molar
ΔCt	Delta cycle threshold value
ΔΔCt	Delta-delta cycle threshold value
χ^2	Chi Square

CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a genetic disease that arises from the accumulation of mutations in critical genes. It is defined as malignant neoplasia (tumor), characterized by abnormal growth and unregulated proliferation of cells that tend to invade surrounding tissues and metastasize to distant sites. The word "cancer" means too many different types of tumors which are categorized depending on their original cellular types. In all tumor kinds, there are a reciprocal set of hallmarks including some biological capabilities acquired in the course of the multistage development of human tumors. These common traits include sustained proliferative signaling, evasion of growth suppressors, resistance to apoptotic signals, replicative immortality, sustained angiogenesis, reprogrammed energy metabolism, evasion of immune destruction, genomic instability and increased mutagenesis, inflammation, invasiveness, and ability to form metastasis (Hanahan and Weinberg, 2011). Cancer comes right after cardiovascular diseases in the order of death causes in Turkey (Tatar and Tatar, 2010).

1.2 Colorectal Cancer

Colorectal cancer (CRC) is in the third place of the most familiar cancers in men and in the second place for women internationally. Around 861,663 CRC death cases were occurred in 2018. CRC is thought to be the fourth deadly cancer as it forms 9.68% of all cancer-linked death reasons universally (The Global Cancer Observatory, 2018). Whereas, in the United States, CRC was given the third place among the leading causes of cancer death because of its 135,430 new incidences besides 50,260 deaths in 2017 (Siegel et al., 2017). In 2014, CRC ranked third on the incidence of Turkey in both genders (Hacıkamiloğlu et al., 2017).

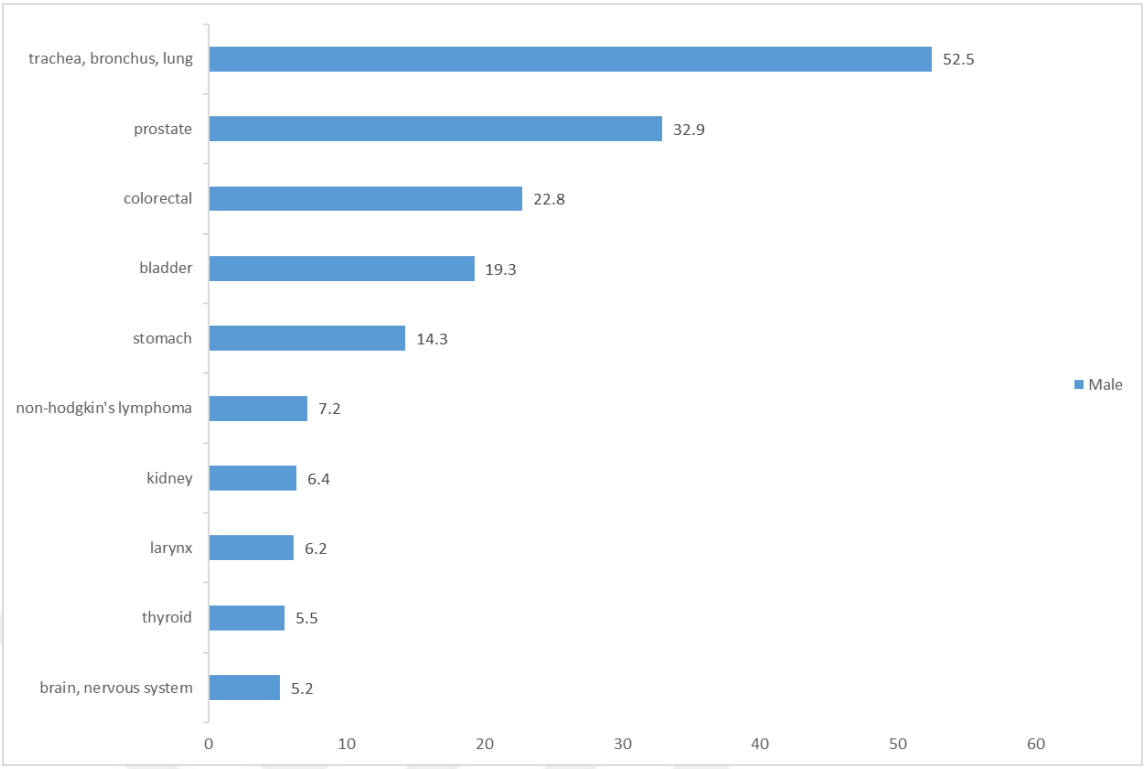

Figure 1.1 Estimated age-standardized rates of incidence cases of CRC in males in Turkey (World Standard Population, 100,000 people) (Hacıkamiloğlu et al., 2017).

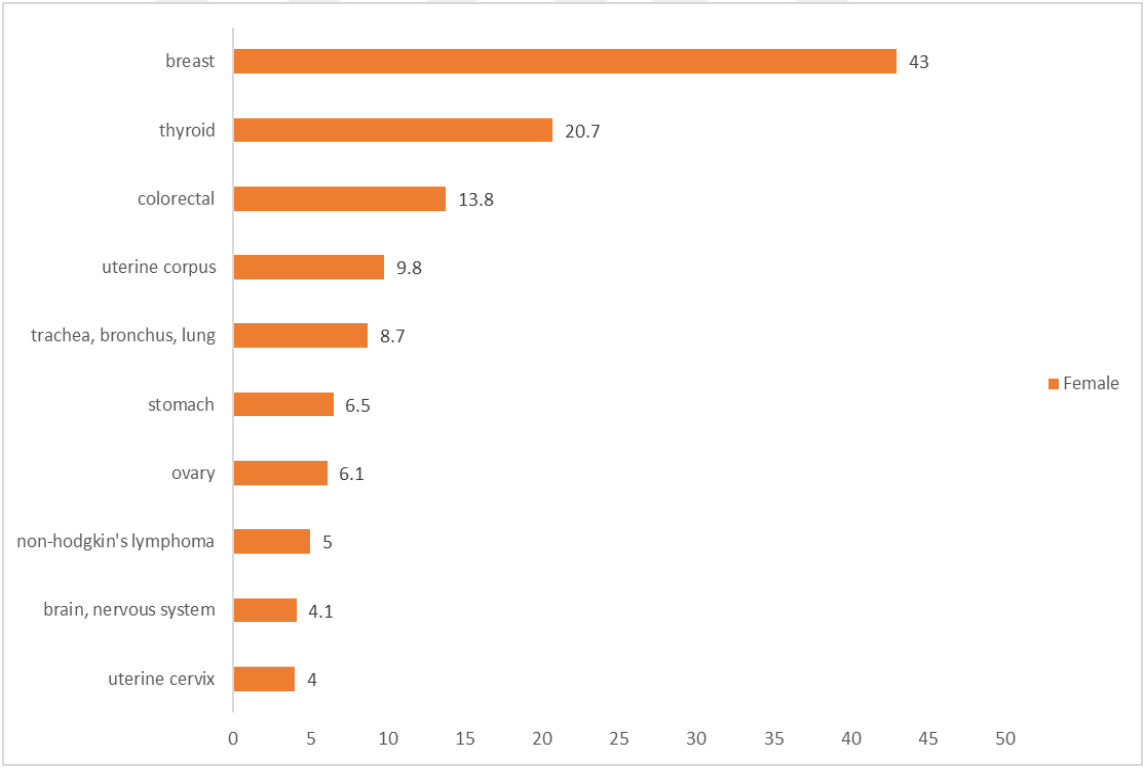

Figure 1.2 Estimated age-standardized rates of incidence cases of CRC in females in Turkey (World Standard Population, 100,000 people) (Hacıkamiloğlu et al., 2017).

The previous data spotlight on the CRC extraordinary impact on the human society, hence pushing towards searching for novel tools of diagnosing and treatment for this cancer.

More than 90% of all CRCs are adenocarcinomas derived from epithelial crypts (Fleming et al., 2012). The rest 5-10% of CRC forms the other malignancies such as adenosquamous carcinoma, undifferentiated carcinoma, mucinous tumors, and signet ring tumors (Jessurun et al., 1999). Usually, no symptoms are seen till CRC advances in stage. Even when it is present, symptoms can be generic and unclear such as a changing bowel behavior, weight loss, spasmodic pain in abdomen, hematochezia, tiredness, and melena (Benedix et al., 2010). Patient survival has improved by virtue of the newly witnessed CRC diagnosis and treatment strategies during the last decade. Treatment decisions are taken according to specific foundation depending on stage of disease, differentiation, type of cancer, general health, age of patient, and further medical circumstances. CRC patients can be treated by surgical operation, radiation, and chemotherapy separately or by combining them. Mostly surgery is most common therapy for different CRC stages plus it is potentially curative treatment exclusively (L. Lee et al., 2007). Chemotherapy is extensively used for treating stage III CRC post-surgically to prevent the recurrence of the disease. Sometimes, when surgery in not applicable chemotherapy can be the first therapy to be used (Shead et al., 2017a, 2017b). The adjuvant chemotherapy used in stage II of CRC is still unclear and controversial (André et al., 2004).

Even after a successful surgery, recurrence in CRC patients stays a significant clinical problem. It has been made more difficult to agree on the finest therapeutic strategy because the way to define early and late recurrence differs from one study to another. While some studies stated that early recurrence is the first two years after surgery, others have defined it as the first year or the first three years after curative surgery (Ryuk et al., 2014). These challenges in dealing with CRC underline the value of genetic research in particular in order to improve outcomes in patients with CRC, for example, identifying biomarkers for further stratifying patients and as targets for evolving innovative therapeutic strategies.

1.3 Epidemiology of Colorectal Cancer

Cancer is foremost among one of the significant public health problems in Turkey. Scientists have predicted that cancer deaths will be the highest number of deaths in males and females by the year 2030 (Tatar and Tatar, 2010). Generally, in Turkey and

the developed countries, CRC is considered as one of the main reasons that involve in mortality and morbidity (Parkin et al., 2005). As a common cause for cancer death in Turkey, CRC is the third cause in men following the lung and the prostate cancers, as well as in women following the breast and the thyroid cancers (Hcıkamiloğlu et al., 2017). In 2014, about 9.2%, and 8.6% of total cancer incidences in males and females respectively were diagnosed in Turkey with over 50 years' age-group (Hacıkamiloğlu et al., 2017). In the same year, according to percentage distribution of the histological types of CRC in Turkey, it was apparent that local, regional, and remote organ involvement, had 35.3%, 23.0%, and 41.7% in order (Hacıkamiloğlu et al., 2017).

1.4 Etiology of Colorectal Cancer

Even though the precise basis of CRC is not hitherto fully understood, the environmental and genetic issues together perform important roles in developing these tumors. Although CRC is developable in anyone, some factors are linked with a risk increase of the disease. Some risk factors are adjustable, for example, diet, obesity, physical activity, tobacco usage, and liquor usage range. On the other hand, dietary fiber intakes, fresh vegetables, folate, and calcium have been stated to be protective factors against the CRC development. The previously mentioned factors' modification may lead to a decrease in CRC risk.

There are non-modifiable factors, such as CRC familial history, Lynch syndrome, history of bowel inflammatory disease, racial and ethnic backgrounds, and the existence of type 2 diabetes.

Despite that, CRC can take place in early- to mid-adulthood, principally in certain genetic biases people, age is considered to be the best significant risk factor in this kind of people, as the majority of cancers happen in individuals considered as an average risk. The CRC occurrence opportunity rises obviously in patients above 50 years' old to 90% of new incidences and 94% of CRC-linked death cases (Simon, 2016).

1.5 Anatomy of the Large Intestines

The large intestine is a tubular tract which elongates from the small intestine for five feet approximately and ends at the anus. The large intestine consists of the caecum with appendix extension, the ascending colon, transverse colon, descending colon, and the rectum. The large intestine encircles the small intestine forming the shape of the horseshoe. the large intestine's primary physiological function is the water absorption

from remainings of undigested food and temporal storage for wastes. Also, the large intestine shows essential roles in the immunological defense, water homeostasis, enteroendocrine signaling, and other functions (Rogers, 2010; and Vdoviaková et al., 2016).

The cross-section of the intestinal wall shows the composition of four layers, which are the serosa, muscularis, submucosa, and mucosa from outside to inside (intestinal lumen). Both tissues and functions differ from layer to another. The serosa layer consists of connective tissue and serve as a sheath for the digestive tract. The muscularis layer surrounds the submucosa and is liable for intestinal peristalsis. With an obvious abundance in vessels like arteries, veins, lymphatics, and nerves, the submucosa layer supplies the intestine with blood and is contains various non-epithelial cells that comprise the intestinal stroma such as fibroblasts, cells of the innate and adaptive immune systems. Finally, mucosa is the layer that forms the lumen of the intestinal tube which is made by lines of absorptive and secretive epithelial cells. The epithelium is a simple columnar epithelial layer with millions of invaginations or finger-shaped structures known as “crypts of Lieberkühn” (Figure 1.3) (Fleming et al., 2012).

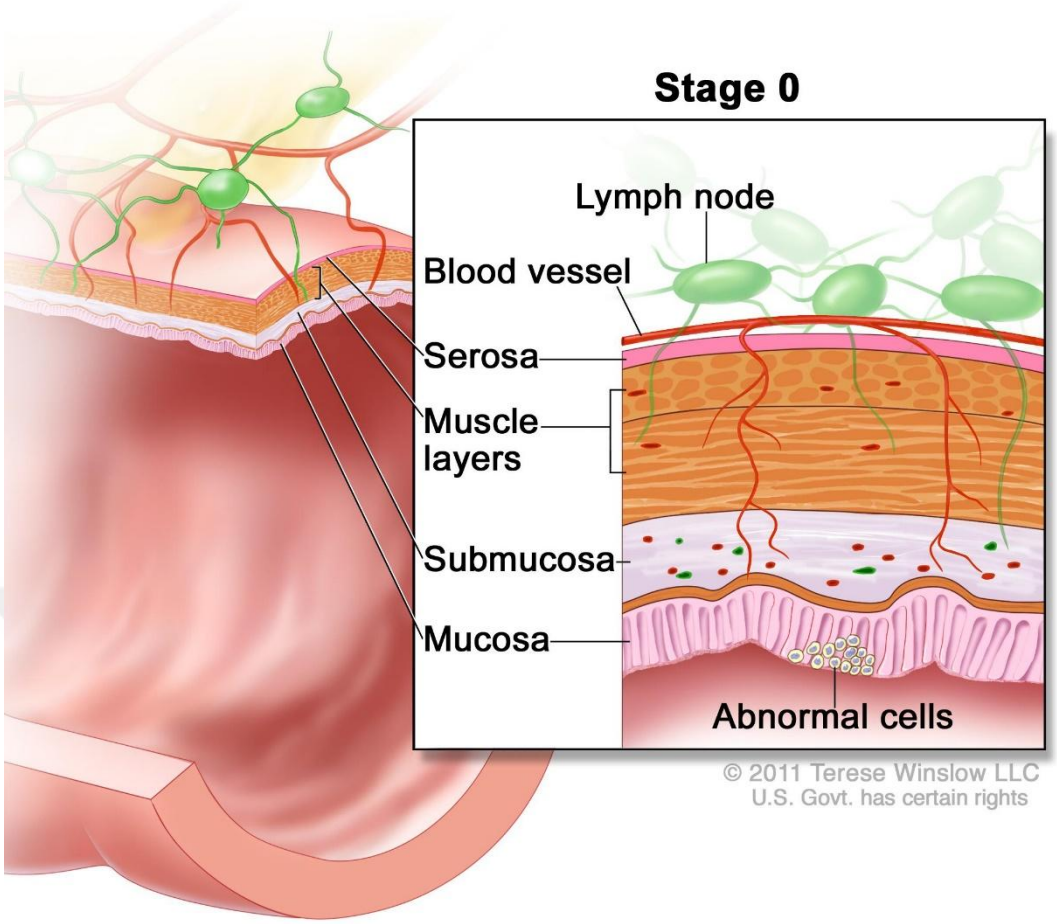

Figure 1.3 Anatomy of the intestinal tract (Winslow, 2011).

1.6 Staging of Colorectal Cancer

The staging system for CRC was produced by the American Joint Committee on Cancer (AJCC). The system is basing on the situation of three essential characters; tumorous invasion (how deeply the tumor has invaded intestinal tissue layers), lymph nodal metastasis (number of positive lymph nodes observed), and distant metastasis (presence or absence of metastatic tumor to further organs) and known as TNM staging system. TNM staging system offers intrinsic information about patients' prognosis (Wolpin and Mayer, 2008; Edge and Compton, 2010). (Table 1.1) represents the TNM classification system.

Table 1.1 TNM Staging System for CRC (Wolpin and Mayer, 2008).

Primary tumor (T)		
T_x	Primary tumor cannot be assessed Tis Carcinoma in situ	
T_1	Tumor invades submucosa	
T_2	Tumor invades muscularis propria	
T_3	Tumor invades through the muscularis propria into the subserosa	
T_4	Tumor directly invades other organs or structures or perforates visceral peritoneum	
Regional lymph nodes (N)		
N_x	Regional lymph nodes cannot be assessed	
N_0	No regional lymph node metastases	
N_1	Metastases in 1–3 regional lymph nodes	
N_2	Metastases in ≥4 regional lymph nodes	
Distant metastases (M)		
M_x	Presence or absence of distant metastases cannot be determined	
M_0	No distant metastases detected	
M_1	Distant metastases detected	
Stage grouping and 5-year survival		
Stage	TNM classification	5-year survival
I	T_{1-2}, N_0, M_0	>90%
IIA	T_3, N_0, M_0	80%–85%
IIB	T_4, N_0, M_0	70%–80%
IIIA	T_{1-2}, N_1, M_0	65%–80%
IIIB	T_{3-4}, N_1, M_0	50%–65%
IIIC	T_{1-4}, N_2, M_0	25%–50%
IV	T_{1-4}, N_{0-2}, M_1	5%–8%

1.7 Colorectal Cancer Development

Usually, growth of CRC is slow, and without showing up symptoms till it's arrival to a critical size, that might cause blockage to food passage leading to abdominal pain, constant bleeding with bowel movement, or rare dark stool.

The CRC progression is ordinarily passing through a process of multistep implicating an overtime accumulated different modifications as in morphology, histology, and genetic (Fearon and Vogelstein, 1990; Simon, 2016).

The different stages of CRC evolution, together with their escorting histological, morphological, and genetic alterations, are outlined in Figure 1.4.

Typically, CRCs are developed from pivotal modifications in non-malignant polyps. Polyps result from accumulation of confined abnormal cells inside the intestinal epithelial layer that bulge into the lumen of intestines. Routinely, polyps might be either sessile or pedunculated. Little by little, the divided cells of polyps possibly will accumulate abundant genetic deviations by which they obtain the capacity to invade the wall of the intestine, and in time may turn into more transformed and expand to near lymph nodes and lastly to distant metastatic locations. Luckily, merely a minor proportion of polyps gain malignant characteristics, and even though for polyps that do, the whole progression from polyps to cancer commonly takes more than a few years or even a decade (Simon, 2016).

Polyp size increase and genetic mutations and epigenetic alterations may commence gathering through its proliferation. Over time, these polyps may be able to enter neighboring tissue and develop into the colonic and rectal wall. This growth may turn out to be neovascularized, hence will gain an easy entrance to the lymphatic system and thus stimulating cancerous cells' extent to further organs. The earlier detecting and resecting precancerous polyps, the easier to object and preclude the adenoma-carcinoma transformation and the CRC from developing and spreading to other organs. Histologically developing from polyp to CRC is resulted from cumulating hereditary and epigenetic adjustments. Although hereditary gene mutations MLH1, MSH2, PMS2, and the APC are related to CRC, they are infrequent and make up to ~5% of CRCs. But studying these hereditary mutations, along with periodically taking place APC and DNA mismatch repair mutations, has offered key understandings for the stepwise genetic advancement from premalignant polyps to cancer. CRC is usually developed from two polyp types; adenomas and sessile serrated polyps (SSP). Genetically, there are two CRC developing pathways correspond with these polyp types. Adenomas are commonly related to the pathway 'chromosomal instabilities'.

The pathway of chromosomal instability is witnessed in around 70% of sporadic cancers and is described by a cascade of mutations accumulation. Characteristically, the first to develop is the APC gene mutation that affects chromosome discrimination

throughout cell division. Successive mutations then take place in the KRAS oncogene, which affects cell growth, differentiation, motility, and survival. Over time, the p53 gene may lose its function as a master regulator of transcription and apoptosis by these mutations' impacts affecting an extensive range of cellular functions that ultimately lead to carcinogenesis (Morin et al., 1997; Huang et al., 2004; and Simon, 2016).

On the contrary, the SSPs' development has a tendency to commence with the BRAF gene mutations, which leads to modified growth signaling and loss of apoptosis. KRAS mutations may occur in SSPs too, but their relationship with SSPs is much fewer than with adenomas (Kinzler and Vogelstein, 1996; Simon, 2016).

One more common epigenetic adjustment witnessed in serrated lesion-based CRC is deviant hypermethylation of gene promoter region. Methylation of the promoter region restrains transcription, functionally deactivating influenced genes. Disabling gene affects several genes including regulators of other growth genes.

Amongst others, N-Myc downstream-regulated gene 4 (NDRG4) and bone morphogenic protein 3 (BMP3) are abnormally methylated genes, which related with the CpG island methylator phenotype.

A different mechanism is microsatellite instability (MSI). MSI is triggered by DNA repair genes' disorder resulting in a genetical variety of CRC. MSI may lead to irregular replication of repetitive DNA sequences in small, noncoding areas (microsatellites) and an increase in the susceptibility to extra genetic mutations. MSI could happen in each of adenomas and SSPs and is linked to germline genetic alterations in genes of DNA mismatch repair, for example in hereditary nonpolyposis CRC, as well as sporadic mutations owing to abnormally methylated promoter regions of MLH1 'associated with CpG island methylator phenotype' (Simon, 2016).

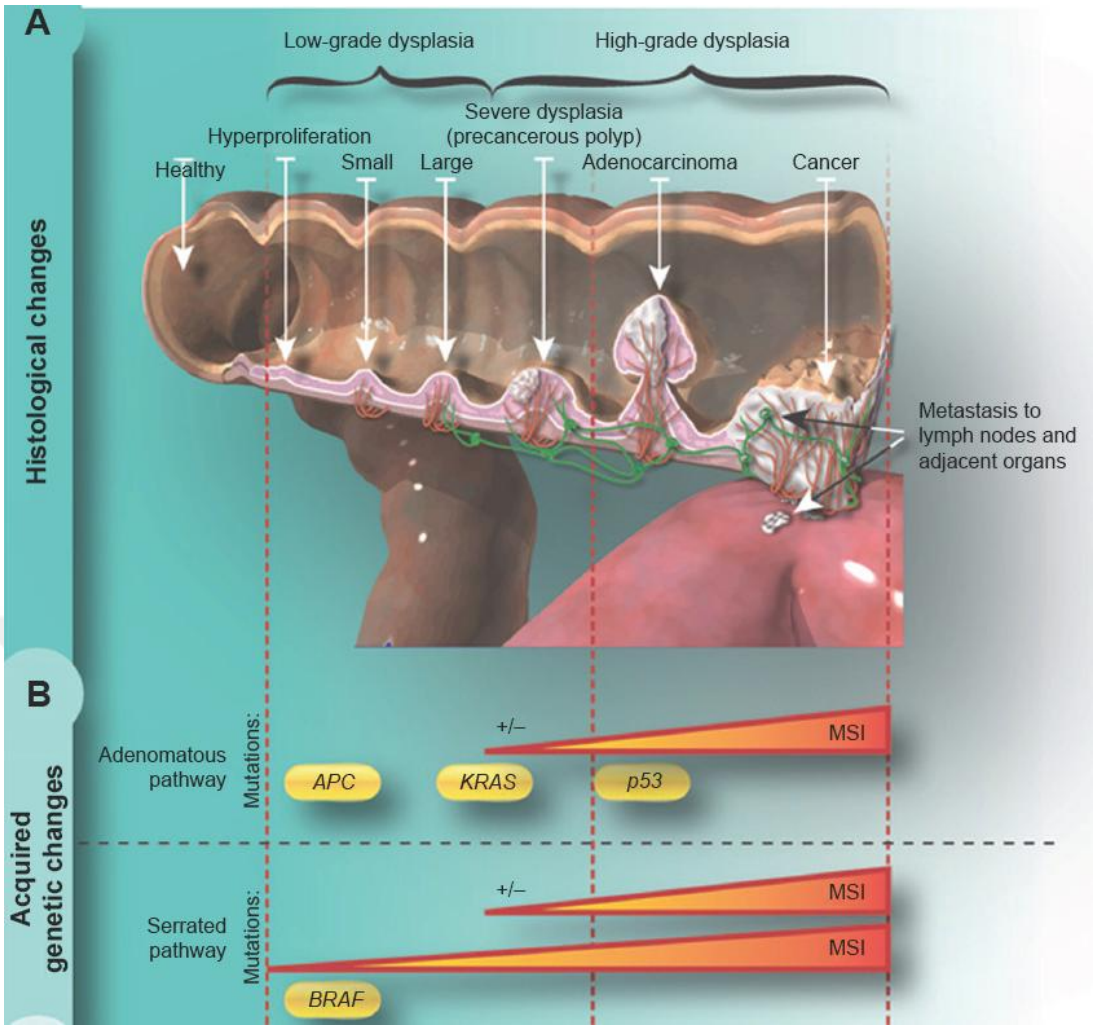

Figure 1.4 Steps of CRC development (Simon, 2016).

1.8 Transcription Factors

The interacting proteins with cis-acting elements and with other proteins at the promoter region of genes are known as transcription factors. The association between transcription factors and cis-acting elements assists in recruiting and assembling the pol II transcription start complex along with promoter permission for transcription commencement. During the absence of enhancers or upstream regulatory elements obtainable basal transcription activity requires transcription factors. Different cis elements and factors' distinctive combinations, for example, coactivators, deliver specificity and harmonize regulation of DNA polymerase II (pol II) transcription. Transcription factors are able to function as activators/repressors (enhancers or silencers) performing from both distal and proximal sites. Numerous transcription factors don't bind to DNA instantly. Instead, they act together with DNA bound

proteins via protein-protein connections and facilitate the regulation of gene transcription.

The current model proposes that reciprocal action of factors restricted to remote locations with proteins bound at proximate sites and/or basal transcription factors comprising the TATA-box-binding protein-associated factors (TAFs) can be simplified by DNA looping leading to influence transcription (Su et al., 1991). A number of forms of conserved domains or motifs identified exist in transcription factors of a eukaryotic gene. Activation domain, a DNA binding domain and sometimes a ligand binding domain together are classically composing transcription factors.

Activation domains overwhelmingly hold negatively charged rich regions in their structures also their organization still not fully understood. DNA of an activation domain usually cooperates with other trans-acting factors or immediately with pol II to influence several features of transcription. Ligand binding domains can bind hormone, heavy metals or other ligands, for example, estrogen receptor binds estrogen. Generally, the binding of a ligand alters protein amendment and either modifies the binding activity of the DNA or possible to interact with other proteins (Shang, 2002; and Riggs and Hartmann, 2003). There are numerous varieties of DNA binding domains. The zinc finger motif that is coded by DNA small stretches folds into loops or fingers in a Znt-r ion-dependent manner.

1.9 Structure of Post-Translational Sp Family Proteins

Sp1 is a member of an increasing family of nuclear proteins that control transcription of genes, and the Sp/Krüppel-like factors (KLFs) are categorized by the similarity in their modular structures. Sp1–Sp4 create a subgroup (Fig. 1.5) which encompass some discrete intersecting features or regions which comprise activation domains (AD), the C-terminal zinc finger DNA-binding region, and an inhibitory domain (ID) in Sp3 that is involved in suppressing the activity of Sp3. Sp5–Sp8 are similar in structure and seem to be trimmed shapes of Sp1–Sp4 in which fractions of the N-terminal regions are deleted. Chromosomally the locations of Sp1–Sp8 are neighboring to a homeotic (HOX) gene cluster. As a minimum 15 KLFs have been described, and these proteins also comprise the three zinc finger motifs but demonstrate a significant structural unevenness. KLF subfamilies encompass the basal transcription element binding (BTEB) proteins and the transforming growth factor β (TGFβ)-inducible early gene (TIEG) proteins (Safe and Abdelrahim, 2005).

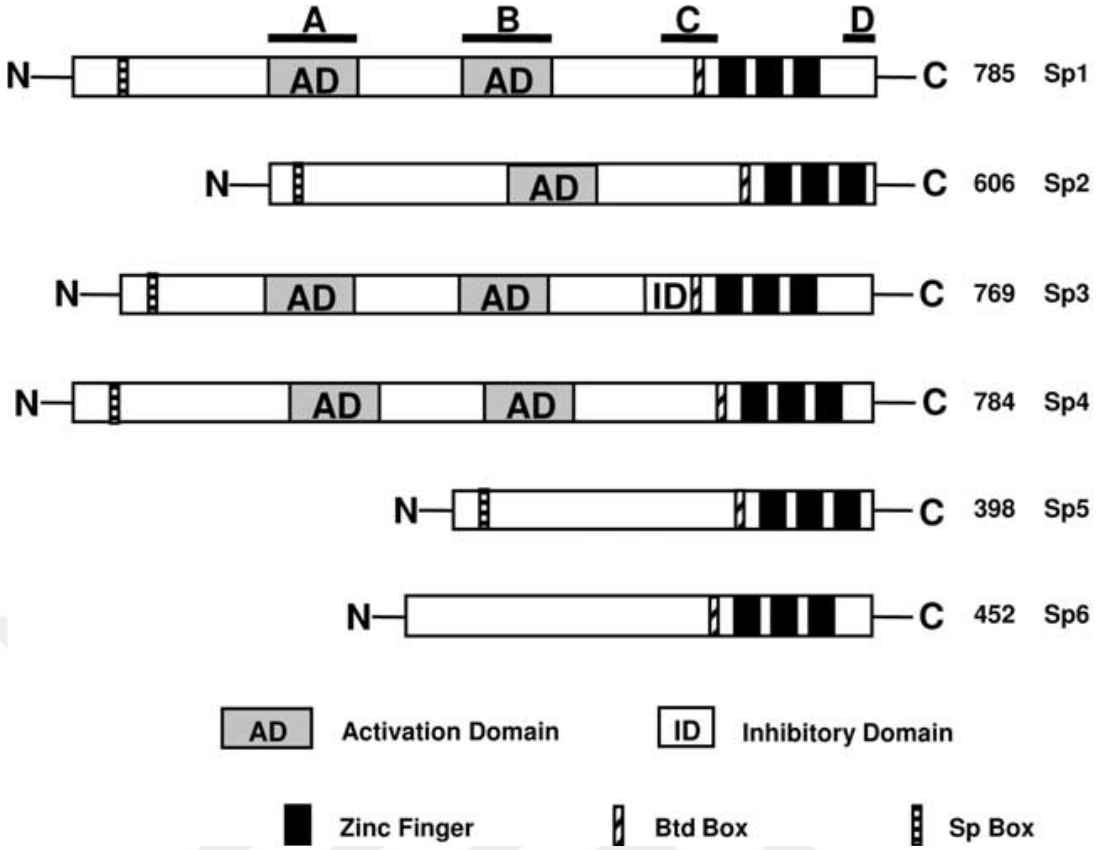

Figure 1.5 Structural features of Sp proteins. Sp1–Sp6 proteins comprise a number of mutual domains in the C-terminal region, while Sp5 and Sp6 display a truncated N-terminal structure. in all Sp proteins, buttonhead (Btd) and Sp boxes are conserved regions (Safe and Abdelrahim, 2005).

1.10 Action of Sp1 and Sp3

The consensus Spl binding DNA element sequence (CCCCGCCCC, GC box) was first recognized at the polyomavirus simian virus 40 (SV40) promoter (Dynan and Tjian, 1983a, 1983b). The general transcription factor Spl regulate gene expression through binding GC boxes and/or GT boxes (Kadonaga et al., 1987; Kadonaga et al., 1988; and Courey and Tjian, 1988). For a long time, Spl was documented as a constitutionally transcription factor activating housekeeping genes and other TATA-less genes which are commonly not extremely regulated. Though growing proof proposes that these GC/GT boxes are not only necessary for regulating the transcription of many housekeeping, but also tissue-specific, viral, and inducible genes (Philipsen and Suske, 1999; Suske, 1999; Bouwman and Philipsen, 2002; Wierstra, 2008). One chip-chip study investigating the distribution of Sp1 binding within human chromosomes 21 and 22 revealed a big digit of Sp1/Sp3 binding sites (Cawley et al., 2004). No less than 12,000 Spl binding sites were assessed by this study in the entire genome which are

situated on genes implicated in nearly all the cellular processes (Cawley et al., 2004). Remarkably, merely approximately 20% of the Spl/Sp3 sites were those at the 5' end of protein-coding genes, with almost 40% of the sites positioned at the 3' end of noncoding RNA genes.

Both Sp1 and Sp3 are auto-regulated genes. Sp1/Sp3 binding sites are existent in the proximal promoters of both Sp1 and Sp3 genes. Knocking down one of these factors will cause a decrease in the expression levels of both factors. Knocking out Sp1 and Sp3 is fatal, and the complex heterozygous Sp1/Sp3 in mice is also not viable, which advises that the correct quantity of both Sp1 and Sp3 transcription factors is crucial to maintain proper gene expression series. Sp1 and Sp3 levels change in a diverse form in mouse germ cells in the course of spermatogenesis. Sp1 and Sp3 have an overlapping but distinctive functions in the regulation of gene expression (Li and Davie, 2010).

Sp1 and Sp3 are transcription factors that either improve or limit the promoters' activity of genes engaged in differentiation, the progression of the cell cycle, and oncogenesis. The protein levels of Sp1 and Sp3 are frequently higher in cancerous than in normal cells. Comparing to normal cells, Sp1 levels are greater in breast carcinomas, thyroid cancer, hepatocellular carcinomas, pancreatic cancer, gastric cancer and lung cancer (Li and Davie, 2010).

1.11 Regulation of Sp1 and Sp3

Various mechanisms have been described or recommended illustrating the functionality of Sp1 and Sp3 (Li et al., 2004; Davie et al., 2008).

a) Adjustments in relative levels of Sp1 and Sp3

Sp1/Sp3 can regulate the genes' expression by modifying the profusion of the Sp1/Sp3 and their relative levels (Li et al., 2004). Rising the Sp1 level relative to that of Sp3 induced the E2F-associated phosphoprotein gene promoter activity, while the opposite inhibited it (Schwarzmayr et al., 2008). Higher levels of the long isoforms of Sp3 have been witnessed in differentiated Caco-2 cells compared with its short isoforms (Gartel et al., 2000).

b) The affinity of Sp1 for Sp-binding sites

GGGGCGGGG is the consensus binding sequence of Sp1/Sp3. In crystal structure and nuclear magnetic resonance (NMR) studies, it was mentioned that each one of the three zinc fingers of the Sp protein DNA binding domain can recognize the three bases in one strand and a single base in the complementary strand of the Sp1-binding DNA

sequence (Pavletich and Pabo, 1991; Narayan et al., 1997). The last C-terminal finger has the ability to bind only two bases and has lower specificity, which explains the variety of Sp1/Sp3 loading on promoter sequences (Oka et al., 2004).

According to other studies, during certain cellular processes the DNA-binding activities of Sp1/Sp3 change to regulate gene expression (Chadjichristos et al., 2003; Wu et al., 2003). The alterations in the interactions 'protein-protein' and post-translational modifications (PTMs) can regulate the DNA-binding activity of Sp1/Sp3.

c) Proteins that interact with Sp1 and Sp3

Through its D domain, Sp1 can synergize transcriptional activation by forming a tetramer and further collecting multiple stacked tetramers (Li et al., 2004). The Sp1 multimer may offer many docking sites for a number of Sp1-associated proteins, which will determine the Sp1 role as an activator or a repressor (Porter et al., 1997; Doetzlhofer et al., 1999; Xiao et al., 2000; Li et al., 2004). On the other hand, Sp3 itself does not form homo-multimer even with a similar D domain (Yu et al., 2003). Thus, while Sp3 doesn't have the capacity to be a synergistic activator, Sp1 has. This capacity 'the Sp1 forming multimers' enables only Sp1 to regulate the looping of chromatin between enhancer and upstream promoter regions of genes but not Sp3 (Deshane et al., 2010). Transcription factors, transcriptional regulators, and chromatin remodeling factors are interacting with Sp1 and Sp3 directly or indirectly (Li et al., 2004). In order to initiate transcription through remodeling the chromatin to be accessible to transcription machinery and other cofactors to, Sp1 possess the capability of recruiting chromatin remodeling complex family proteins (Chen et al., 1994; Lu et al., 2003). But to repress gene transcription, both Sp1 and Sp3 can recruit Sin3A HDAC1/HDAC2 repressor complexes (Zhang and Dufau, 2003; Clem and Clark, 2006). Alternately, coactivators such as p300 or CBP can be recruited by Sp1 and Sp3 so as to activate transcription (Ammanamanchi et al., 2003; Nunes et al., 2010). Sp1 and Sp3 associate with the HDAC1, HDAC2, and the E2- responsive ZR-75 cells estrogen receptor alpha (ERα) in MCF-7 breast cancer cells (He et al., 2005). The affinity of Sp1 DNA-binding for the SK3 gene promoter in L6 cells was increased by the ERα and Sp1 interaction (Jacobson et al., 2003). Likewise, the binding of Sp1 to the upstream promoter region of fatty acid synthase gene was improved by estradiol-ERα (Lu and Archer, 2010).

The number of Sp1 and Sp3 interactive proteins has been growing (Li et al., 2004). Sp1 interacts with DNA cytosine methyltransferase (DNMT1) directly via the seven

consensus amino acids conforming with the N-terminal region of the transcription factors of the Sp group (Estève et al., 2007).

d) Modifying the Sp1 and Sp3: sumoylation, acetylation, and methylation

Phosphorylation, glycosylation, sumoylation, and acetylation form the Sp1/Sp3 modifications (Li et al., 2004) (Figure 1.6). Proteins related to ubiquitin in structure and also similar in size are known as small ubiquitin-related modifiers (SUMOs). Yet, SUMOs typically do not function as an indication for protein degradation. Sumoylation is more possible to be involved in various ruling functions, for instance, subcellular categorization, protein steadiness, chromatin structure arrangement, and transcription factor activity (Gill, 2003; Verger et al., 2003). Three SUMOs have been described in mammals; SUMO-1/Smt3C, SUMO-2/Smt3A, and SUMO- 3/Smt3B. These SUMOs functionally strive discrete consequences (Müller et al., 2001; Saitoh and Hinchey, 2000). Although the precise mechanisms are not clear yet, sumoylation classically effects negatively on transcription factors' activity (Verger et al., 2003). SUMO-1 can sumoylate both of the endogenous Sp1 and Sp3 (Ross et al., 2002; Sapetschnig et al., 2002; Spengler and Brattain, 2006; Wang et al., 2008). The location of Sp1 modification by SUMO-1 was found at lysine 16 in the Sp1 N-terminal negative regulatory domain (Spengler and Brattain, 2006). The same residue controls the Sp1 N-terminal cleavage in vivo, which can relieve the constantly repressed Sp1 activity produced by sumoylated lysine-16 residue. Spengler and Brattain (2006) proposed that negative regulatory domain integrity is preserved by sumoylation in order to inhibit Sp1-dependent transcription, which recommends that post-translational competition at lysine 16 controls Sp-dependent transcription (Spengler and Brattain, 2006). Even so, one more study revealed that sumoylation of Sp1 could also facilitate Sp1 degradation via modifying its subcellular position and further cooperating with proteasome subunit rpt6, which leads to the final degradation of Sp1 (Wang et al., 2008). Excitingly, one study reconnoitering the mechanism of Sp1 processing explained that cyclin A/cdk2 phosphorylation of serine-59 could stimulate Sp1 proteolytic processing, which further leads to a desumoylated, derepressed and unstable Sp1 product (Spengler et al., 2008). The protein inhibitor of activated STAT1 (PIAS1), an E3 ligase, sumoylates Sp3 by a single lysine (K551) in its inhibitory domain (Sapetschnig et al., 2002). Alternative small sumoylation site is at lysine 120 that is only found in the N-terminal of the two long forms of Sp3. Sumoylation of all Sp3 forms can happen at K551 (Ross et al., 2002). This alteration acts as a silencer or significantly decreases Sp3 activity (Ross et

al., 2002; Sapetschnig et al., 2002; Spengler et al., 2008). In concurrence, Ellis et al. (2006) found that the short Sp3 isoforms can be converted into vigorous transactivators of the SRC 1A gene promoter by mutatively blocking sumoylation. Unexpectedly, sumoylation had slight influence on the Sp3 long forms' transcriptional features (Ellis et al., 2006). This result is more supporting the idea that transcription mediated by Sp3 is extremely reliant on the isoform bound, sumoylation status and the promoter situation. There is an inconsistency about the probability of correlation exists between Sp3 sumoylation and its nuclear localization (Ross et al., 2002; and Sapetschnig et al., 2002). However, one study indicated that the Sp3 sumoylated has repressed transcription through stimulating the establishment of compacted repressive chromatin (Stielow et al., 2008b, a). With the help of ChIP-qPCR, Li and Davie (2010) succeeded to show that both of the sumoylated Gal4 transgene and mouse Dhfr gene (mDhfr) promoters have recruited one series of chromatin remodelers (for instance, Mi-2, MBT-domain proteins), and these recruitments lead to form a repressive chromatin with heterochromatic features (Li and Davie, 2010). This occasion was also escorted with the creation of histone alterations related with repressed genes. The DNA accessibility to the transcription machinery is blocked by these chromatin adjustments (Stielow et al., 2008a, b).

Stimulatingly, Sp3 is acetylated by the KAT p300 at K551 'the same sumoylated lysine' (Ammanamanchi et al., 2003; Braun et al., 2001). In Drosophila Schneider SL2 cells, the mutation of K551 transformed the Sp3 activity from a weak to a stronger activator. One study mentioned that acetylating the Sp3 makes it a transcriptional activator (Ammanamanchi et al., 2003). It is probable that acetylating Sp3 prevents the inhibition by sumoylation moreover stops the sumoylation silencing function. p300 also can acetylate Sp1 (Suzuki et al., 2000; Ryu et al., 2003; Song et al., 2003). Providing mammalian cells with HDAC inhibitors or treating them with the stimulation of the oxidative stress lead to the acetylation of Sp1 in situ (Dempsey et al., 2003; Ferrante et al., 2003; Ryu et al., 2003). In vitro studies propose that acetylation improves Sp1 DNA-binding activity and transcription activity of Sp1. Though, the accurate acetylational role of Sp1 stays vague.

e) Methylation of CpG regions

Methylation of DNA plays a title role in regulation of gene expression in mammalian healthy and malignant cells, frequently causing transcriptional silencing. The demethylated GC/GT boxes, Sp1/Sp3 sites, are essential to keep the adenine

phosphoribosyltransferase (APRT) gene active (Brandeis et al., 1994; Macleod et al., 1994). Methylation of Sp1/Sp3 sites or near them is one way to control gene expression (Mudduluru and Allgayer, 2008; Wang et al., 2010). In MCF-7 cells, Sp3 binding was increased at the demethylated intron 2 region of Rad9 gene and reduced its mRNA expression (Chang et al., 2004). Some studies presented that the binding of Sp1/Sp3 witnessed no influence by the methylation within the consensus Sp1/Sp3 site (Harrington et al., 1988; Zhu et al., 2003). But the methylation that happened outside the GC boxes reduced Sp1/Sp3 binding clearly and also generated repression of gene expression (Douet et al., 2007; and Zhu et al., 2003).

Captivatingly, one study highlighted that forming a high level of chromatin methylation on the distal promoter region resulted in upregulating the expression of cell-type specific podoplanin in human osteoblast-like MG63 cells (Hantusch et al., 2007).

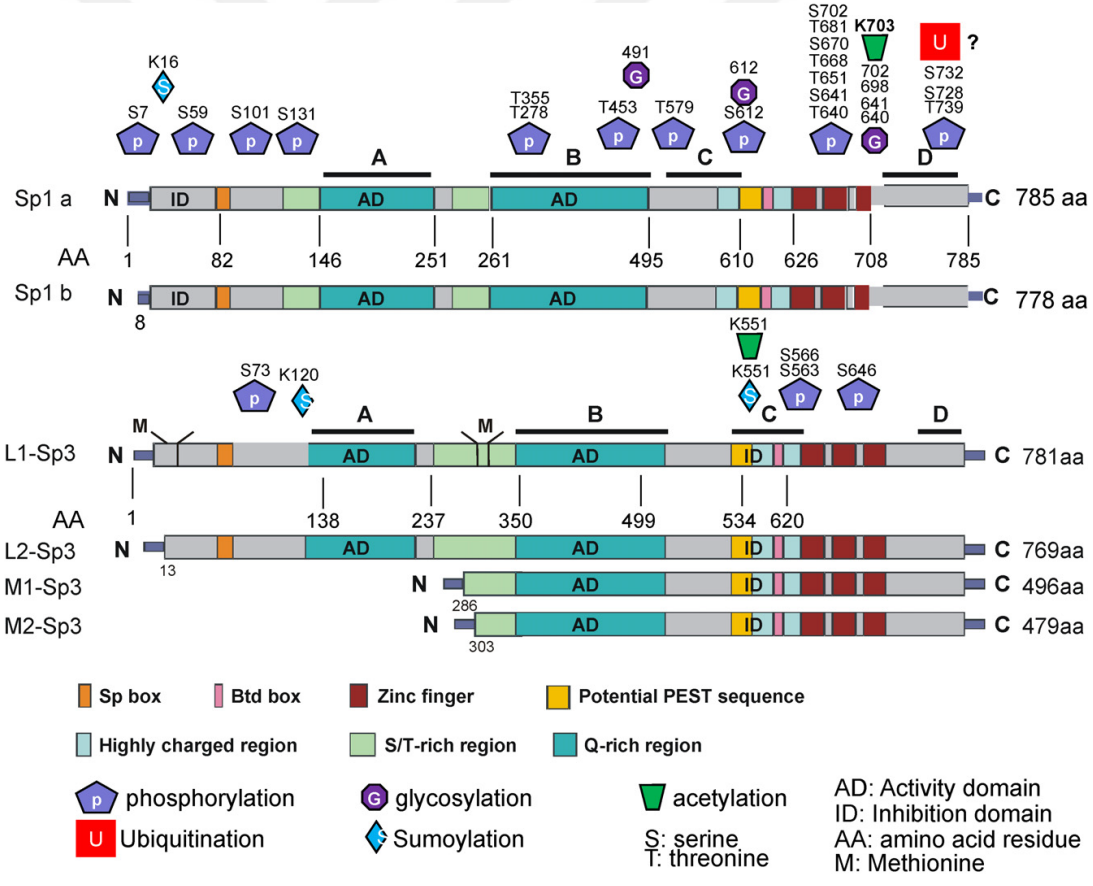

Figure 1.6 Human Sp1 and Sp3 post-translational modifications PTMs (Li and Davie, 2010).

1.12 The Study Objectives

The objectives of this study are given as below;

- Determination of both Sp1 and Sp3 gene expression levels in surgical specimens of CRC and their matched non-cancerous normal tissues.
- Investigation of the presence of possible association between Sp1 and Sp3 in normal and tumorous tissues of patients with CRC.
- Evaluation of clinicopathological characteristics of patients with respect to Sp1 and Sp3 activity.
- Investigation of the correlation between the expressions of Sp1 and Sp3 genes in CRC tissues.

CHAPTER 2

LITERATURE REVIEW

2.1 Sp1 and Sp3 in Cancers

For many years, the Sp1 was considered as a basal transcription factor and referred to a regulating role so-called the housekeeping genes. And while the recent estimates indicate that the majority genes of mammalian lack TATA boxes in its structures, the recognition of the role of Sp1 as a recruiter of the general transcription machinery rose its value in gene regulation (Beishline and Azizkhan-Clifford, 2015).

Sp1 has been identified for the first time by Dynan and Tjian in 1983 as an essential (a promoter-specific binding) factor for transcribing the SV40 major Immediate Early (IE) gene (Dynan and Tjian, 1983b, 1983a). The largest number of C2H2 motifs containing proteins behave as regulatory factors for transcription for instant, human Sp1 gene's product. According to functional analysis, these proteins were demonstrated to serve as positive or negative transcription regulators depending on the concentration (Dovat et al., 1998). Sp1 did not expressed differently in both the SW613-S human colon carcinoma cell line and the cells of non-tumorigenic clones. The same case was witnessed in the genes of the factors of the preinitiation complex interacting with Sp1 protein (Prochasson et al., 1999). Years later, while Chiefari *et al.* were studying the expression levels of both AP2 and Sp1 in thyroid cancer, they've found that Sp1 expression levels were higher in tumor cells than what in normal cells of thyroid. Chiefari *et al.* concluded that the reason of both Sodium/iodide symporter (NIS) expression and the subsequent iodide transport are reduced in thyroid tumors might be due to modifications in the binding activity of AP2 and Sp1 transcription factors to NIS promoter (Chiefari et al., 2002). In 2003, Wang et al. investigated the expression levels of Sp1 in 196 samples of gastric tissues, including human gastric cancer, lymph node metastasis specimens, and normal gastric tissue specimens. Briefly, they found that Sp1 is infrequently expressed in normal human gastric cells versus high expression levels in human gastric cancer cells. Also, they suggested that there is a probability of the abnormally activated Sp1 being a potential molecular

marker for poor prognosis and a direct contributor to development and progression of gastric cancer, as they found that the elevated expression levels of Sp1 in human gastric cancer were correlated reversely with survival of patients. As a result, the determination of Sp1 expression levels prior to surgeries may be useful for decision making in the modality and extent of postoperative therapy (Wang et al., 2003). Similarly, during the evaluation of the relationship between the expression of Sp1 and vascular endothelial growth factor (VEGF), along with their influence on survival in human gastric cancer patients, the expression of Sp1 was found high in late stages of cancer but not in early stages. Also, the Sp1 high expression was detected in higher ranks of lymph node metastasis compared to the lower ranks with negative or low expression, and its high expression was found associated with N2/N3 ranks of lymph node metastasis and with inferior survival. Regarding to their results, Yao and his colleagues suggested that dysregulated Sp1 expression and activation play important roles in VEGF overexpression and, thus, gastric cancer development and progression (Yao et al., 2004). In 72 samples of breast cancer, a study was implemented by Wang *et al.* in 2007, they showed the elevated level of expression for Sp1 in both tumorous and normal tissues of breast with different ratios. Likewise, they showed the positive correlation of Sp1 high expression to TNM stage, tumor invasion, and lymph node metastasis. thereafter, statistically overall survival rate was found significantly lower in samples with positive expression of Sp1 than in those with negative expression of Sp1. Finally, Wang and his colleagues have reached to the opinion that Sp1 could contribute in the breast cancer's invasion and metastasis, and is accepted as one of the valued markers pointing the poor prognosis of breast cancer. The exploration of Sp1, considering the tumor invasion and clinical stage, might rise the reliability of predicting prognosis in breast cancer patients (Wang et al., 2007).

Thereafter, Jiang and his colleagues had worked on Sp1 expression in primary pancreatic adenocarcinoma. According to their findings, Sp1 over-expression indicated in a subset of primary pancreatic adenocarcinoma in addition to its association to several clinicopathological factors, paid Jiang *et al* to conclude that Sp1 is a new biomarker that can be used to identify clinically aggressive adenocarcinoma and patients with a bigger likelihood of cancer metastasis and short overall survival (N. Y. Jiang et al., 2008).

Almost the same findings of Wang *et al.* but in glioma cell lines and tissues were revealed by Guan *et al*. as the paraffin-embedded archival glioma specimens

demonstrated an Sp1 positive expression and 58.6% of the specimens showed high expression level of Sp1. Additionally, Guan *et al.* explained in their study statistically the strong correlation of Sp1 high expression with the cancer grades, and with the survival status of patients with glioma. Again statistically, the high Sp1 expression was observed in patients with better overall survival while low Sp1 expression was detected in worse overall survival patients. Collectively, Guan et al. suggested that Sp1 may characterize a valued prognostic marker for glioma and that Sp1 may be involved in the modulation of tumor invasion (Guan et al., 2012).

At the same year, Hsu *et al.* performed their study on lung adenocarcinoma patients. They found an elevated Sp1 expression in 58.5% and low levels of Sp1 expression in 41.5% of 118 patients. Oppositely, in the 118 normal matched tissues taken from the same patients, high rates of Sp1 expression were observed in 11%, while 89% of the normal tissues seemed to have a negative and low expression. According to statistical analysis applied, the I, II and IV stages of lung adenocarcinomas witnessed a significant correlation between the Sp1 low expression and the low survival. The final conclusion of Hsu and his group was that meanwhile, the Sp1 was necessary for the growth of lung tumor, Sp1 inhibited metastasis by inducing the expression of E-cadherin (Hsu et al., 2012).

The next year, Lee and his colleagues worked on HSC-2 human oral cancer cells. Sp1 expression was reduced after samples were treated with methanol extracts *C. officinale* Makino (MECO) or methanol extracts of *C. bursa*‑*pastoris* (MECB). Using siRNA, the Sp1 was downregulated resulting in growth inhibition and reducing the total expression of poly (ADP-ribose) polymerase (PARP). The Sp1 downregulation was enough to prevent the cell growth and to induce the apoptosis. As a final point, depending on their results, Lee *et al.* mentioned that the treatment HSC-2 human oral cancer cells by MECO and MECB repressed the cell growth and induced apoptosis through the downregulation of Sp1 (Lee et al., 2013).

Other than that, in the latest study of Hedrick and his colleagues, using the RNA interference (RNAi) results, each of the Sp1, Sp3, and Sp4 was shown separately as an independent role player in the growth, survival and immigration/invasion of the (breast, kidney, pancreatic, lung and colon) cancer cell lines. The high Sp expression in tumor compared to low expression in normal tissues together with the genomic and functional results and the decrement in expression of Sp1 with age indicate that none

of the Sp1, Sp3 or Sp4 are oncogene addiction (OA) genes and that they are attracting drug targets for singular and complicated cancer chemotherapies (Hedrick et al., 2016). From results of their study, Bakovic et al. mentioned that, through sequence-specific binding within three promoter domains, CTα gene transcription is activated by Sp1 and Sp3. And in Sp1-mediated transcription, Sp3 performs as an activator in a dose-dependent manner and vice versa. Also, the Sp1-/Sp3-driven transcription in Drosophila SL2 cells is repressed by Sp2, but the transcription in C3H10T1/2 mammalian cells is stimulated by Sp2. Overall, Bakovic et al. concluded that CTα gene expression levels depend on cell type, Sp proteins availability, and structure/arrangement of three cis-acting elements (Bakovic et al., 2000).

Essafi-Benkhadir and her colleagues have evaluated the expression of Sp3 in generated tumor and normal cell lines. Also, they have analyzed the cell growth in vitro and after the inoculation in nude mice. They found that both the expression of genes implicated in the regulation of cell cycle and pro/anti-apoptotic genes and the apoptosis were respectively modified and induced by the Sp3 conditional over-expression. And the tumors development in nude mice was strongly reduced by the over-expression of Sp3 which confirms the pro-apoptotic potential of Sp3 *in vivo*. However, through the selective Sp3 cleavage by caspase, cells were able to survive to apoptosis. In created tumors, the induction of Sp3 led to transient regression then progression. The re-accumulation of the full-length form of Sp3 corresponds with progression. The presence of high levels of the full-length form of Sp3 indicates a poor prognosis for overall survival of patients with head and neck tumors. According to Essafi-Benkhadir et al., the conclusion of their study was that the accumulation of full-length Sp3 underlines the tumor cell ability to bypass apoptosis and is an indicator of aggressiveness in tumors of head and neck (Essafi-Benkhadir et al., 2009).

Summarily, abnormal levels of Sp1 protein are detected in numerous cancers and are highly correlated with the stage and poor prognosis of the cancers (Wang et al., 2003; Yao et al., 2004; and Safe and Abdelrahim, 2005). Suppression or knocking down Sp1 to regular cellular level ordinarily reduces the formation growth and metastasis of tumor (Jiang et al., 2004; Lou et al., 2005; Yuan et al., 2007). Reducing the expression of Sp1/Sp3 transcription factors results in lowering the expression of Sp1/Sp3 target genes comprising receptor of epidermal growth factor, cyclin D1, factor of vascular endothelial growth, sterol regulatory element binding protein 2 and the CD151 gene ‘the role player genes in metastasis of liver cancer’ (Kang and Chen, 2009;

Chadalapaka et al., 2010; Wang et al., 2010). Also, Sp1 is involved in the pathology of several human diseases containing Huntington's disease (HD) and Human Burkitts' lymphoma (Freiman and Tjian, 2002; Hu et al., 2002). The full-length Sp3 accumulation indicates the head and neck tumors aggressiveness (Essafi-Benkhadir et al., 2009). CTα gene expression levels rely on cell type, Sp proteins availability, and structure/arrangement of three cis-acting elements (Bakovic et al., 2000).

Another study demonstrated that the reduction of or losing the expression of human reduced folate carrier gene in multiple tumor cell lines is caused by the increase in levels of the Sp3 short isoforms (Rothem et al., 2004).

Studies have concentrated mostly on protein and mRNA expression of Sp1 but not on Sp3. Yet, Sp1 and Sp3 dysregulation has been highlighted in different cancers and diseases.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Patients

A total of 41 patients' approvals were signed for this study prior to tissue sampling. Tumor and normal matching tissues were obtained from each patient after they were undertaken surgeries at Gaziantep University Hospital. In addition, the available clinicopathological data of patients were obtained from Gaziantep University Hospital. All tissue specimens were fresh-frozen by liquid nitrogen and were kept under -80°C. This study was approved by the Local Ethics Committee of Gaziantep University (Decree no: 2017/191).

3.1.2 Chemicals and Solutions

3.1.2.1 RNA Isolation Kit

For the contents of the PureLink RNA Mini Kit used for this study, as seen in Table 3.1.

Table 3.1 Contents of the PureLink RNA Mini Kit.

PureLink® RNA Mini Kit Contents	**Supplier**
Lysis Buffer	**Cat. No. 12183018A**
Wash Buffer I	**Invitrogen, USA**
Wash Buffer II	
RNase-Free Water	
Spin Cartridges (with collection tubes)	
Collection Tubes	
Recovery Tubes	

3.1.2.2 cDNA Synthesis Kit

The High Capacity cDNA Reverse Transcription Kit was used for the synthesis of the cDNA as shown in Table 3.2

Table 3.2 Contents of High Capacity cDNA Reverse Transcription Kit.

Component	**Supplier**
10X RT Buffer, 1.0 *mL*	**All components were supplied by Applied Biosystems, (Ref 4368814)**
10X RT Random Primers, 1.0 *mL*	
25X dNTP Mix (100 mM)	
MultiScribe™ Reverse Transcriptase, 50 U/*μL*	
RNase Inhibitor 20 U/*μL*	**N8080119, Applied Biosystems**

3.1.3 Real-Time PCR

We used all components of kits shown in Table 3.3 for implementing the gene expression assays.

Table 3.3 The Real-Time PCR kits used for determining the gene expression levels.

Component	**Supplier**
TaqMan Fast Advanced Master Mix (2X)	Applied Biosystems
TaqMan Sp1 Assay (20X) 5′-GCGAGAGGCCATTTATGTGT-3′ 5′-GGCCTCCCTTCTTATTCTGG-3′	Thermofisher
TaqMan Sp3 Assay (20X) 5′-ATTCTGGAGAACGCCCTTTT-3′ 5′-TATGTTTGGCAAGGTGGTCA-3′	Thermofisher
TaqMan GAPDH Assay (20X)	Applied Biosystems
Nuclease-Free Water	Applied Biosystems

3.1.4 Additional Solutions and Materials

Table 3.4 The additional solutions used.

Solution	Reference	Supplier
2–Mercaptoethanol	**31350-010**	**Life Technologies, USA**
Ethanol (70%)		
Ethanol (96–100%)		
Nase/RNase free H_2O	**10977-035**	**Invitrogen, USA**
MicroAmp Fast 96-Well Reaction Plate	**4346907**	**Applied Biosystems**

3.1.5 Equipment

1. Vortexer
2. Rotor-stator homogenizer (TissueLyser LT/Qiagen).
3. Spectrophotometer NanoDrop (Nano, maestrogen)
4. Centrifuge (Universal 320 r, Hettich, Germany)
5. Thermal cycler (ProFlex PCR System, Applied Biosystems, USA)
6. Real-Time PCR System (StepOnePlus, Applied Biosystems, USA)

3.2 Methods

3.2.1 Buffers Preparation

- Before we used the Wash Buffer II for the first time, we added 60 *mL* of 96–100% ethanol as required in (Cat. no. 12183018A).
- We prepared a Lysis mix by adding 10 µL of 2-mercaptoethanol to 1000 µL of Lysis Buffer for each purification procedure.

3.2.2 Tissue Lysis and Homogenization

1. The tissue harvest was done by obtaining about 25 mg of sliced tissue from each specimen (all materials used during the tissue harvest were sterile scalpels, Petri-dishes...etc.)
2. We transferred the tissue slices harvested to 2 *mL* tubes.
3. We added a mix of 1000 µL Lysis Buffer with 10 µL 2-mercaptoethanol to every single tissue harvested.
4. We vortexed the lysate mix for few seconds.
5. Mix was kept in -80 °C for few minutes till it was frozen.

6. All mix tubes were transferred to rotor-stator homogenizer (TissueLyser LT/Qiagen).
7. Homogenization was achieved through high-speed shaking of samples for 10-15 minutes occasionally the mix-freezing and homogenization procedures were repeated according to type of tissue provided.
8. The homogenate was centrifuged at 26,000 × g for 5 minutes; then we transferred the supernatant to a clean RNase-free tube.

3.2.3 RNA Purification and Storage

1. We added one volume of 70% ethanol to each volume of cell homogenate.
2. In order to disperse any visible precipitate that may form after adding ethanol and to mix thoroughly the homogenate-ethanol mix, we vortexed it.
3. Repeatedly and till the whole mix is processed, we transferred about 700 µL of the sample (including any remaining precipitate) to the spin cartridge (with the collection tube), and we centrifuged it at 12,000 × g for 15 seconds at room temperature, then we discarded the flow-through, and reinserted the spin cartridge into the same collection tube.
4. We added 700 µL Wash Buffer I to the spin cartridge, and centrifuged at 12,000 × g for 15 seconds at room temperature, the we discarded the flow-through and the collection tube.
5. Before we added 500 µL Wash Buffer II with ethanol to the spin cartridge, we placed the spin cartridge into a new collection tube.
6. We centrifuged at 12,000 × g for 15 seconds at room temperature and discarded the flow-through (steps 5 and 6 were repeated for one time).
7. The spin cartridge was centrifuged at 12,000 × g for 1–2 minutes in order to dry the membrane with the bounded RNA, then we discarded the collection tube and inserted the spin cartridge into a recovery tube.
8. We added 50 µL RNase-free water to the center of the spin cartridge and incubated at room temperature for one minute; then the spin cartridge was centrifuged for two minutes at ≥12,000 × g at room temperature so as to elute the RNA from the membrane into the recovery tube.
9. All concentrations and qualities of the extracted RNAs were determined by using the (ND1000) Nano-Drop spectrophotometer at 260 nm.
10. Dilutions were done to most of the extracted RNA samples in accordance with the minimum concentration obtained to be 30 ng/µL.

11. All purified RNA samples were stored at -80 °C deep freezer.

3.2.4 cDNA Synthesis

First, we prepared a 20-μL reaction of the 2X Reverse Transcription (RT) master mix for each sample as below:

1. We allowed the components of the kit to be thawed on ice.
2. Referring to Table 3.3, all calculations for the volumes of components required to prepare the needed number of reactions.
3. The 2X RT master mix was gently mixed.
4. All steps above were done on ice.

All calculations were done as shown in Table 3.5

Table 3.5 Calculations of the 2X Reverse Transcription (RT) master mix volume.

Component	**Volume/Reaction *(μL)***
10X RT Buffer	**2.0**
25X dNTP Mix (100 mM)	**0.8**
10X RT Random Primers	**2.0**
MultiScribe Reverse Transcriptase	**1.0**
RNase Inhibitor	**1.0**
Nuclease-free H2O	**3.2**
Total per Reaction	**10**

Additional reaction volumes were included in the calculations to provide extra volume for the loss that might happen throughout reagent transfers (Ref 4368814).

Secondly, we performed RT procedure as below;

1. We mixed 10 µL of extracted RNA with 10 μL 2X RT master mix in a 0.25 mL PCR tube separately to produce a total reaction volume of 20 μl for each sample.
2. We programmed the thermal cycler (ProFlex PCR System) conditions according to the High Capacity cDNA Reverse Transcription Kit protocol as mentioned in Table 3.6
3. We set the reaction volume to 20 μL
4. We loaded the reactions into the thermal cycler.

5. We started the reverse transcription run.
6. Finally, we stored the cDNA RT tubes prepared for long-term storage at -20 *°C* deep freezer.

Table 3.6 Conditions of the thermal cycler (*C* = Celsius, *min* = minutes)

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

3.2.5 Quantitative Real-Time PCR (qPCR)

1. The qPCR reaction mix preparation.

- First of all, total number of reactions was determined.
- We combined the following components as shown in Table 3.7 relating to the number of reactions required, including no-template controls (NTCs) for each assay, plus 10% overage.
- Three qPCR reaction mixes were prepared separately, containing 3 different assays (Sp1, Sp3, and GAPDH) in a duplicate manner for each sample.
- We applied brief vortexing to the mix.
- In order to ensure elimination of any possible air bubbles in the mix tube, a brief centrifuging was done.

Table 3.7 The components and volumes of qPCR reaction mix.

Component	**Volume/Reaction *(µL)***
TaqMan Fast Advanced Master Mix (2X)	**10**
TaqMan Assay (20X)	**1**
Nuclease-Free Water	**7**
Total volume per reaction	**18**

2. Preparation of the qPCR reaction plate.

- We transferred 18 μL of qPCR reaction mix to each well of an optical reaction plate.
- We added 2 μL of cDNA template to each well.
- We added 2 μL of Nuclease-Free Water instead of cDNA Template to one well for each assay due to preparing the NTC well.
- We slightly applied some pressure manually after sealing the reaction plate with optical adhesive film; then a brief centrifuge was done so as to bring the qPCR reaction mixes to the bottom of the wells and to eliminate any potential air bubbles.

3. qPCR system settings.
    - We set the qPCR conditions as given in Table 3.7 using StepOnePlus Real-Time PCR System.
    - We defined the blocks depending on how we distributed samples in wells previously.
    - We selected the quantitation as the experiment type.
    - We selected TaqMan as the reagent to be used in system.
    - According to the protocol of the supplier, we selected the run mode as Fast.
    - Reaction volume was defined as 20 μL.
4. Running the reaction plate in PCR.
    - Reaction plate was loaded then we run the StepOnePlus Real-Time PCR System.
5. Data Analysis
    - Although we used auto threshold settings, all amplification plots were viewed, in order to verify the amplification validity.
    - The comparative Ct method was used to analyze data in qPCR software.
    - Calculation of expression levels of target genes normalized to the endogenous control gene (GAPDH) were done by applying the ΔΔCt method where:

$\Delta\Delta Ct_{taget\ gene}$ = ($Ct_{target\ gene}$, tumor tissue – $Ct_{endogenous\ control}$, tumor tissue) - ($Ct_{target\ gene}$, normal tissue - $Ct_{endogenous\ control}$, normal tissue).

Ct = Cycle Threshold value, Target gene = Sp1 or Sp3, Endogenous control = GAPDH

- Then we calculated the relative gene expression (fold change) according to the method as below:

Relative Gene Expression (fold change) = $2^{-\Delta\Delta Ct}$

3.2.6 Statistics

Depending on the SPSS software (v.22, IBM) to investigate any probable associations between Sp1 and Sp3 and any of the clinicopathological characteristics, also, for the presence of possible correlation between the two genes, we applied Student-*t* Test, Chi-Square (χ^2) Test, and Spearman's Test, respectively.

CHAPTER 4

RESULTS

4.1 Clinicopathological Characteristics

Depending on the patients' information gathered from Gaziantep University Hospital, all information has been arranged, classified, and evaluated as seen in Table 4.1.

Table 4.1 Groups of the clinicopathological characteristics (n = number of samples, % = percentage).

Variable	**Group 1**	***n***	**%**	**Group 2**	***n***	**%**
Age	Mean 54.4 (range 26-84)				41	-
	50 years and below	15	36.6	Above 50 years	26	63.4
Gender	Male	27	65.9	Female	14	34.1
Tissue Location	Colon	24	58.5	Rectum	17	41.5
Stage of Cancer	Early	22	53.7	Advanced	19	46.3
Lymph node Involvement	Yes	18	43.9	No	23	56.1
Distant metastasis	Yes	10	24.4	No	31	75.6
Other diseases	Yes	20	48.8	No	21	51.2
Smoking habit	Smoker	16	39	Non-Smoker	25	61

4.2 Total RNA Concentrations

All RNA quantities and qualities detected were explained in Table 4.2, but only the uni-diluted RNA concentrations were used for the cDNA synthesis.

Table 4.2 RNA quantities measured (ng/µL) and qualities (Optical Density O.D.) in tumorous (T) and normal (N) tissue samples.

Sample No		Concentration *(ng/µL)*	O.D. (260/280)
1	**T**	921.0	2.25
	N	845.6	2.26
2	**T**	160.4	2.36
	N	164.1	2.38
3	**T**	144.5	2.35
	N	48.5	2.10
4	**T**	33.5	2.28
	N	18.1	1.48
5	**T**	116.2	2.02
	N	29.1	2.13
6	**T**	478.9	2.00
	N	32.6	2.06
7	**T**	112.1	2.12
	N	139.0	1.95
8	**T**	140.9	1.99
	N	81.4	2.00
9	**T**	94.7	2.08
	N	146.5	2.07
10	**T**	530.1	2.14
	N	244.8	2.14
11	**T**	116.9	2.17
	N	436.2	0.99
12	**T**	205.3	2.09
	N	138.9	2.13
13	**T**	226.9	2.15
	N	50.8	1.96
14	**T**	380.6	2.21
	N	657.4	2.19
15	**T**	182.6	2.14
	N	378.6	2.19
16	**T**	30.3	1.94
	N	230.7	2.14
17	**T**	39.2	2.05
	N	80.5	2.42
18	**T**	456.9	2.16
	N	76.2	1.91
19	**T**	154.7	2.04
	N	60.4	1.84
20	**T**	127.4	1.89
	N	110.8	2.1

Sample No		Concentration *(ng/µL)*	O.D. (260/280)
21	**T**	271.3	2.15
	N	151.7	2.19
22	**T**	1395.5	2.18
	N	157.5	2.43
23	**T**	59.2	2.08
	N	71.8	2.20
24	**T**	1298.7	2.16
	N	1222.5	2.15
25	**T**	906.5	2.24
	N	1274.0	2.18
26	**T**	30.0	2.09
	N	28.0	2.12
27	**T**	410.2	2.35
	N	96.2	2.64
28	**T**	1001.9	2.20
	N	336.1	2.25
29	**T**	408.8	2.21
	N	148.3	2.37
30	**T**	1363.4	2.15
	N	570.2	2.24
31	**T**	329.4	2.28
	N	694.1	2.23
32	**T**	343.3	2.23
	N	159.0	2.24
33	**T**	560.8	2.21
	N	951.6	2.17
34	**T**	50.8	2.19
	N	44.3	2.15
35	**T**	127.2	2.30
	N	615.7	2.22
36	**T**	556.4	2.21
	N	914.1	2.21
37	**T**	96.2	2.36
	N	39.1	2.19
38	**T**	758.1	2.20
	N	33.0	1.91
39	**T**	668.8	2.22
	N	56.2	2.24
40	**T**	30.3	2.00
	N	118.4	2.34
41	**T**	33.1	1.18
	N	56.9	2.36

4.3 Expression Levels of Sp1 and Sp3

After qPCR was performed, we viewed all amplification plots for Sp1, Sp3, and the endogenous gene GAPDH (Figure 4.1, Figure 4.2, Figure 4.3).

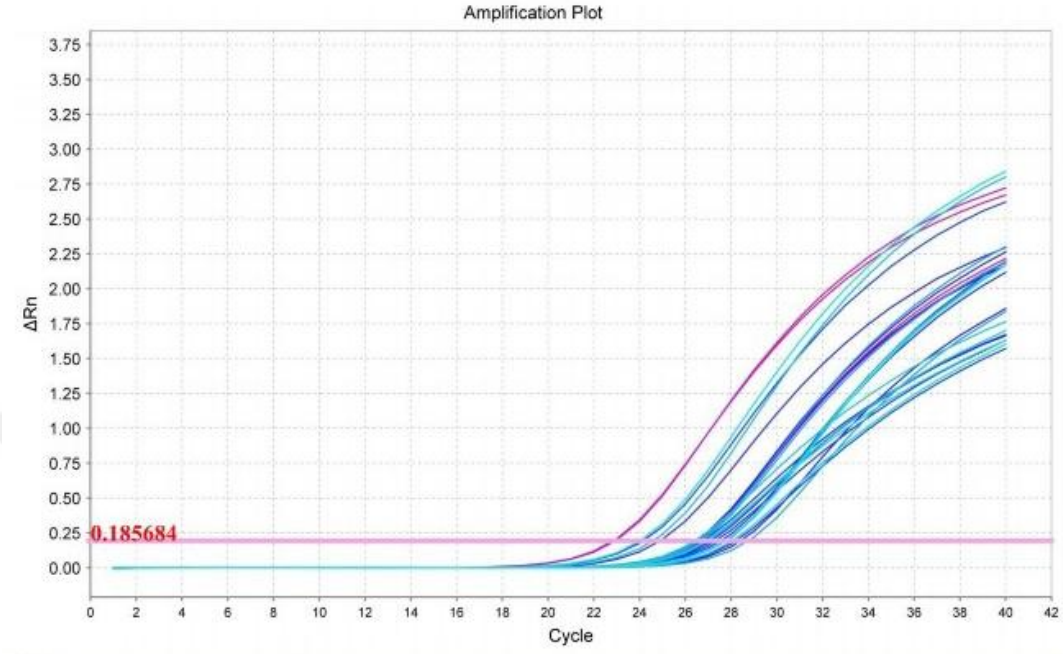

Figure 4.1 Sp1 amplification plot .

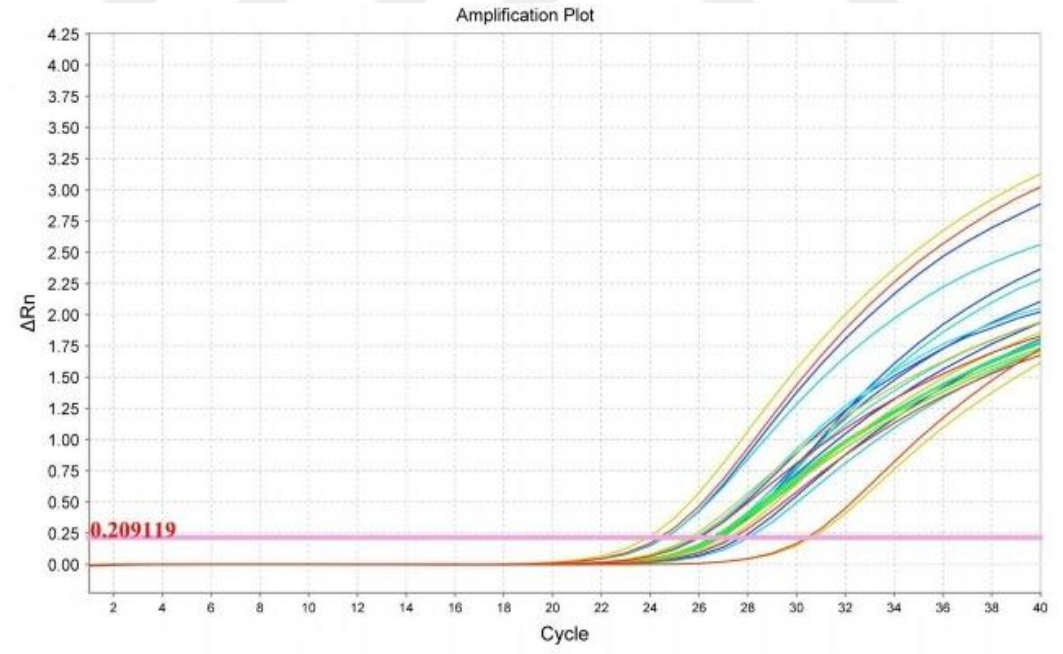

Figure 4.2 Sp3 amplification plot.

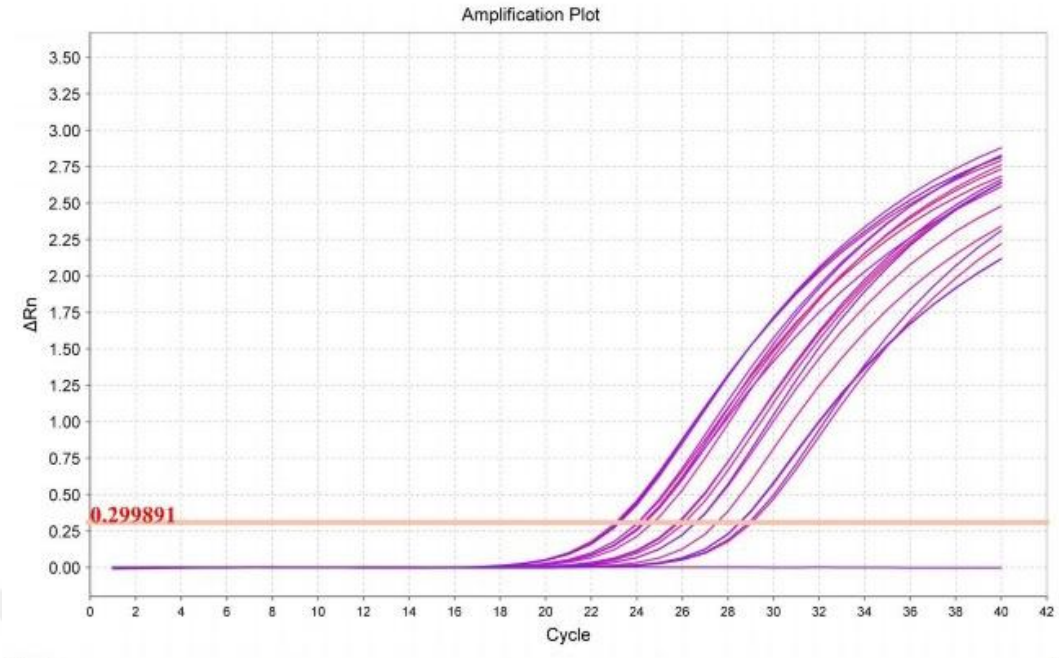

Figure 4.3 GAPDH amplification plot.

Then, the Ct mean was calculated for Ct values of all samples examined by qPCR, which were in a duplicate manner. The Ct means were used for the calculations of ΔCt for Sp1 and Sp3 in tumor and normal tissue samples. Afterward, in order to estimate the relative gene expression (fold change), the ΔΔCts of Sp1 and Sp3 were calculated by the equation:

$\Delta\Delta Ct_{\text{target gene}} = (Ct_{\text{target gene, tumor tissue}} - Ct_{\text{endogenous control, tumor tissue}}) - (Ct_{\text{target gene, normal tissue}} - Ct_{\text{endogenous control, normal tissue}})$.

and applied to the equation:

Relative Gene Expression (fold change) = $2^{-\Delta\Delta Ct}$

In summary, the range of all Sp1 ΔCts was -3.249 to 10.752 as Sp3 ΔCts ranged from -0.579 to 11.509. Also, fold change ranges were 0.013 - 32.983, 0.022 - 16.028 for Sp1 and Sp3 respectively.

4.4 Sp1 and Sp3 Expression Levels in Tumor and Normal Tissues

The first aim of our study was to determine any possible association between Sp1 or Sp3 gene expression results in both tumor and normal tissue samples statistically. Therefore, we applied Student-*t* Test including all ΔCt values of Sp1 and Sp3 compared to the group of tumor and normal tissue samples, as explained in Table 4.3. There were no statistically obvious differences between Sp1 expression in tumor

samples' group (3.173 ± 3.158) and in normal group (3.103 ± 3.608), (p = 0.39). In addition, similar result was detected for Sp3 expression in the group of cancer tissues (4.362 ± 2.947), and for normal adjacent tissues' group (4.474 ± 3.479), (p = 0.223).

Table 4.3 The absence of the relationship between Sp1 or Sp3 expression levels in tumor and normal tissue samples (n = number of samples).

Group Statistics					
Delta Ct	Tissue Type	*n*	Mean	Std. Deviation	Std. Error Mean
Sp1	Tumor	41	3.173	3.158	0.493
	Normal	41	3.103	3.608	0.563
No association (p = 0.39)					
Sp3	Tumor	41	4.362	2.947	0.460
	Normal	41	4.474	3.479	0.543
No association (p = 0.223)					

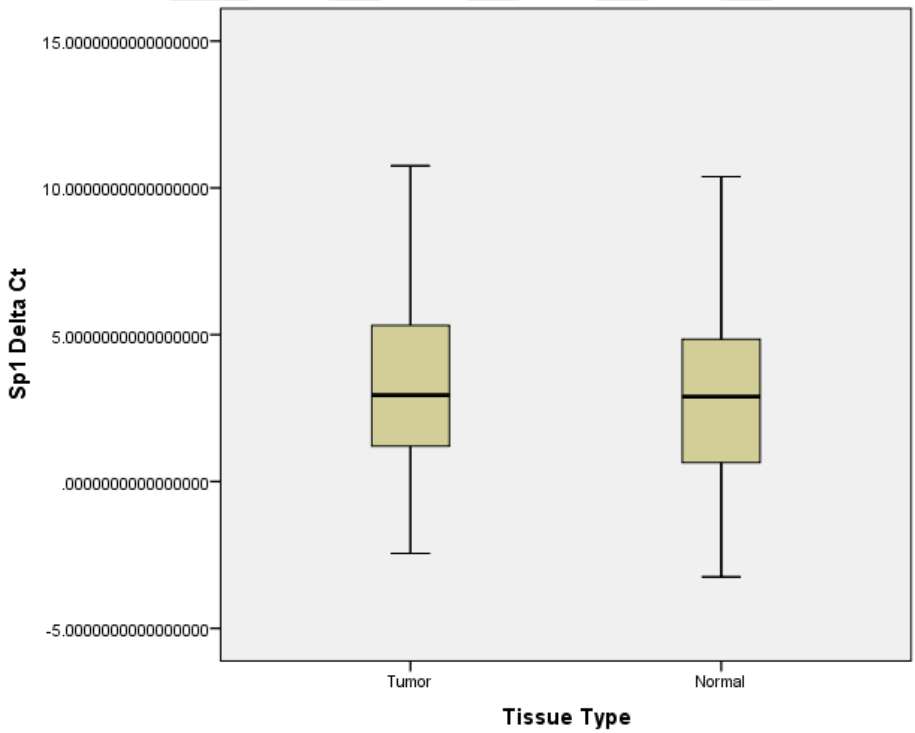

Figure 4.4 The Sp1 ΔCts plotted to the group of tissue types as tumor and normal.

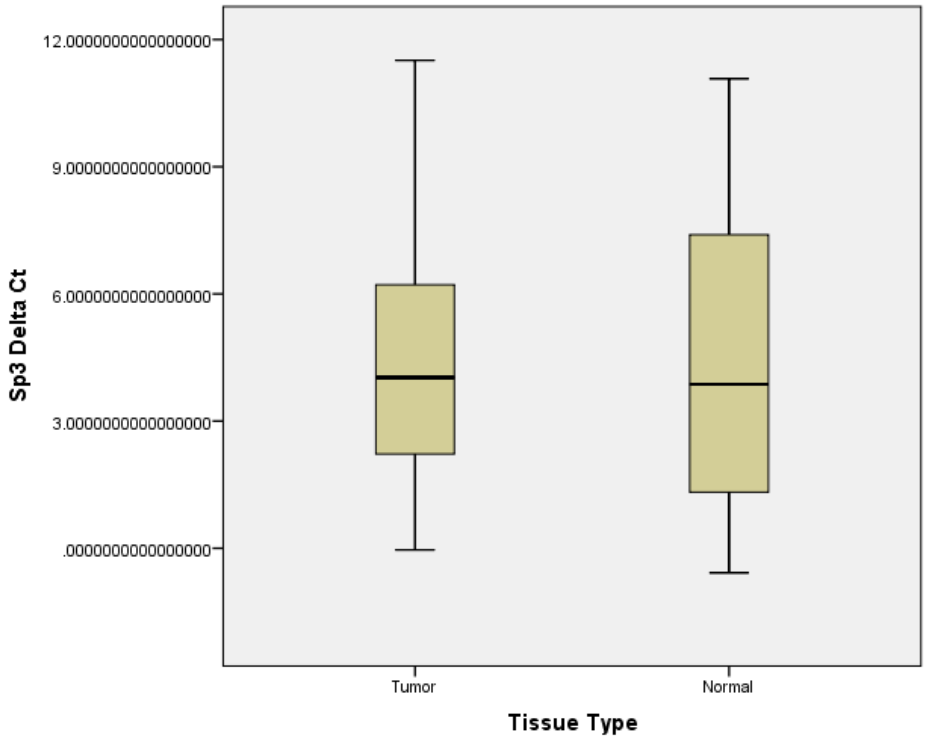

Figure 4.5 The Sp3 ΔCts were plotted to the tissue type group as tumor and normal.

4.5 The Relationship between The Clinicopathological Characteristics and The Expression of Sp1 and Sp3

In order to investigate any available association between any of the Sp1 or Sp3 expression levels and any of the clinicopathological characteristics, by using the SPSS software, we performed Chi-Square (χ^2) Test. The Sp1 and Sp3 relative gene expression levels (fold change) were labeled as High and Low according to their original values obtained earlier from real-time PCR (High > 1, Low <1) prior to χ^2 Test.

4.5.1 Clinicopathological Characteristics and The Sp1 Expression

15 patients with age of 50 years or less witnessed different levels of Sp1 expression as 5 of them were with low expression while the rest 10 patients encountered an over-expressed Sp1. Also, the other group comprising 26 patients over 50-year-old showed a variety of Sp1 expression as low-expression was noticed in 15 patients while high expression was observed in 11 patients. According to χ^2 Test applied, no significant differences were detected between Sp1 expression and the age groups ($p = 0.133$) as given in Table 4.4

Table 4.4 The relationship between age groups and Sp1 expression levels ([a] = Pearson Chi-Square).

Age Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Age Groups	50 or Less	Count	5	10	15
		% within Age Groups	33.3%	66.7%	100.0%
		% within Sp1 Expression Level	25.0%	47.6%	36.6%
		% of Total	12.2%	24.4%	36.6%
	Above 50	Count	15	11	26
		% within Age Groups	57.7%	42.3%	100.0%
		% within Sp1 Expression Level	75.0%	52.4%	63.4%
		% of Total	36.6%	26.8%	63.4%
Total		Count	20	21	41
		% within Age Groups	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.133[a]					

The second clinicopathological characteristic to investigate was gender. Totally, 14 females and 27 males were involved in this study. High expression of Sp1 was noticed in 64.3% of females and 44.4% of males, while the low expressed Sp1 occurred in the rest 35.7% females and 55.6% males. Collectively, the expression of Sp1 was high in 51.2% of the two genders together, whereas the low Sp1 expression was detected in the other 48.8%. Obviously, the p-value of 0.228 was very high, indicating no significant differences between the low and high Sp1 expression in any of the genders examined (Table 4.5).

Table 4.5 The association of gender groups with Sp1 expression levels ([a] = Pearson Chi-Square).

Gender Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Gender	Female	Count	5	9	14
		% within Gender	35.7%	64.3%	100.0%
		% within Sp1 Expression Level	25.0%	42.9%	34.1%
		% of Total	12.2%	22.0%	34.1%
	Male	Count	15	12	27
		% within Gender	55.6%	44.4%	100.0%
		% within Sp1 Expression Level	75.0%	57.1%	65.9%
		% of Total	36.6%	29.3%	65.9%
Total		Count	20	21	41
		% within Gender	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.228[a]					

For the examined tissue located in colon and rectum, the expression levels of Sp1 were found different in both locations. A total of 24 colon tissues and 17 rectal tissues were examined. Among them, 13 colon and 7 rectal tissues witnessed low levels of Sp1, though 10 rectal and 11 colonial tissues had high levels of Sp1. Statistically, there was no significant difference between Sp1 expression levels in colon and rectal tissues, p = 0.412 (Table 4.6).

Table 4.6 The relationship between tissue location groups and Sp1 expression levels ([a] = Pearson Chi-Square).

Tissue Location Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Cancer Location	Colon	Count	13	11	24
		% within Cancer Location	54.2%	45.8%	100.0%
		% within Sp1 Expression Level	65.0%	52.4%	58.5%
		% of Total	31.7%	26.8%	58.5%
	Rectum	Count	7	10	17
		% within Cancer Location	41.2%	58.8%	100.0%
		% within Sp1 Expression Level	35.0%	47.6%	41.5%
		% of Total	17.1%	24.4%	41.5%
Total		Count	20	21	41
		% within Cancer Location	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.412[a]					

All samples were classified to different stages of CRC in accordance with TNM classification system, but they were divided into two groups due to the low number of samples that was available for this study. The group labeled ‘early stages’ comprises the stages 1, 2A, and 2B, while the ‘advanced stages’ group contains the stages 3A, 3B, 3C, and 4. Low levels of Sp1 were seen in 9 of 22 early stages, whereas the rest were with highly expressed Sp1. On the other hand, advanced stages consisted of 11 low and 8 high Sp1 expression. The p-value 0.278 means no significant differences between Sp1 expressions in early and advanced stages of CRC.

Table 4.7 The stage of disease groups relationship with Sp1 expression levels ([a] = Pearson Chi-Square).

Stage of Disease Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Stage	Early	Count	9	13	22
		% within Stage	40.9%	59.1%	100.0%
		% within Sp1 Expression Level	45.0%	61.9%	53.7%
		% of Total	22.0%	31.7%	53.7%
	Advanced	Count	11	8	19
		% within Stage	57.9%	42.1%	100.0%
		% within Sp1 Expression Level	55.0%	38.1%	46.3%
		% of Total	26.8%	19.5%	46.3%
Total		Count	20	21	41
		% within Stage	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.278[a]					

According to the existence and absence of metastatic lymph nodes, one group of 18 metastatic lymph nodes samples was labeled with yes, and the other 23 were included in the no group (non-metastatic lymph nodes). Sp1 showed high levels in 8, 13 of yes and no groups respectively, while the number of samples with low Sp1 expression was equal in both groups, (10 of each). Relying on χ^2 Test performed there was no significant difference between Sp1 expressions in lymph node status groups p = 0.443 (Table 4.8).

Table 4.8 The association of lymph-node involvement groups with Sp1 expression levels ([a] = Pearson Chi-Square).

Lymph Node Involvement Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Lymph Node Involvement	Yes	Count	10	8	18
		% within Lymph Node Involvement	55.6%	44.4%	100.0%
		% within Sp1 Expression Level	50.0%	38.1%	43.9%
		% of Total	24.4%	19.5%	43.9%
	No	Count	10	13	23
		% within Lymph Node Involvement	43.5%	56.5%	100.0%
		% within Sp1 Expression Level	50.0%	61.9%	56.1%
		% of Total	24.4%	31.7%	56.1%
Total		Count	20	21	41
		% within Lymph Node Involvement	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.443[a]					

All CRC tissues were divided into two groups as metastatic (Yes) and non-metastatic (No) tumors. Sp1 high expression was found in 5 metastatic and in 16 of the non-metastatic tumors, whereas low Sp1 expression was detected in 5 samples of the metastatic group and 15 specimens of the non-metastatic tumors. The results of Fisher's Exact test applied confirmed that no significant difference between high and low Sp1 expressions in distant metastasis groups p = 1.0 (Table 4.9).

Table 4.9 The relationship of distant metastasis groups with Sp1 expression levels ([b] = Fisher's Exact).

Distant Metastasis Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Metastasis	Yes	Count	5	5	10
		% within Metastasis	50.0%	50.0%	100.0%
		% within Sp1 Expression Level	25.0%	23.8%	24.4%
		% of Total	12.2%	12.2%	24.4%
	No	Count	15	16	31
		% within Metastasis	48.4%	51.6%	100.0%
		% within Sp1 Expression Level	75.0%	76.2%	75.6%
		% of Total	36.6%	39.0%	75.6%
Total		Count	20	21	41
		% within Metastasis	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 1.0[b]					

Other diseases such as (diabetes, hypertension, heart diseases, and others) were also involved in our study. Patients were distributed to two groups as in Table 4.10, the Yes group for patients with other diseases, and the No group for patients with only CRC. Sp1 expression was high in 9 of patients with other diseases and in 12 of patients with no other diseases. Contrarily, 11 of other diseases group and 9 of without other diseases patients had low levels of Sp1. Statistically, no significant difference was observed between Sp1 levels in other diseases groups (p = 0.437) (Table 4.10).

Table 4.10 The association of the other diseases groups with Sp1 expression levels ([a] = Pearson Chi-Square).

Other Diseases Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Other Diseases	Yes	Count	11	9	20
		% within Other Diseases	55.0%	45.0%	100.0%
		% within Sp1 Expression Level	55.0%	42.9%	48.8%
		% of Total	26.8%	22.0%	48.8%
	No	Count	9	12	21
		% within Other Diseases	42.9%	57.1%	100.0%
		% within Sp1 Expression Level	45.0%	57.1%	51.2%
		% of Total	22.0%	29.3%	51.2%
Total		Count	20	21	41
		% within Other Diseases	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.437[a]					

According to the smoking habit, 16 samples constituted the smokers set while the other set consist of 25 non-smokers. Half of the smokers group over-expressed Sp1 whereas the other half showed low expression of Sp1. Sp1 expression was slightly different in non-smokers, as 12 low-expression and 13 with high expression. The p = 0.901 obtained from χ^2 Test pointed to the lack of a significant difference between Sp1 expressions in smoking habit groups.

Table 4.11 The relationship of smoking habit groups with Sp1 expression levels ([a] = Pearson Chi-Square).

Smoking Habit Groups * Sp1 Expression Level Crosstabulation					
			Sp1 Expression Level		Total
			Low	High	
Smoking Habit	Smokers	Count	8	8	16
		% within Smoking Habit	50.0%	50.0%	100.0%
		% within Sp1 Expression Level	40.0%	38.1%	39.0%
		% of Total	19.5%	19.5%	39.0%
	Non-Smokers	Count	12	13	25
		% within Smoking Habit	48.0%	52.0%	100.0%
		% within Sp1 Expression Level	60.0%	61.9%	61.0%
		% of Total	29.3%	31.7%	61.0%
Total		Count	20	21	41
		% within Smoking Habit	48.8%	51.2%	100.0%
		% within Sp1 Expression Level	100.0%	100.0%	100.0%
		% of Total	48.8%	51.2%	100.0%
No significant difference observed, p = 0.901[a]					

4.5.2 Clinicopathological Characteristics and The Sp3 Expression

15 patients with age of 50 years or less viewed different levels of Sp3 expression as 6 of them were with low Sp3 expression, while the rest 9 patients encountered an over-expressed Sp3. Also, the other group including 26 patients over 50-year-old showed a variety of Sp3 expression as low-expression was noticed in 16 patients while high expression was observed in 10 patients. According to χ^2 Test applied, no significant differences were detected between Sp3 expression levels in the age groups p = 0.183 (Table 4.12).

Table 4.12 Association of the age Groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Age Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Age Groups	50 or Less	Count	6	9	15
		% within Age Groups	40.0%	60.0%	100.0%
		% within Sp3 Expression Level	27.3%	47.4%	36.6%
		% of Total	14.6%	22.0%	36.6%
	Above 50	Count	16	10	26
		% within Age Groups	61.5%	38.5%	100.0%
		% within Sp3 Expression Level	72.7%	52.6%	63.4%
		% of Total	39.0%	24.4%	63.4%
Total		Count	22	19	41
		% within Age Groups	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.183[a]					

Investigating the gender, Sp3 high and low expressions were equally detected in 14 females, while the males group included 15 and 12 patients showing low and high Sp3 expression respectively. Collectively, the expression of Sp3 was high in 19 of the two genders together, whereas the low Sp3 expression was discovered in the other 22 patients. Obviously, the p-value of 0.735 was very high, indicating no significant differences between the low and high Sp3 expression in any of the genders inspected (Table 4.13).

Table 4.13 The relationship between gender groups and Sp3 expression levels ([a] = Pearson Chi-Square).

Gender Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Gender	Female	Count	7	7	14
		% within Gender	50.0%	50.0%	100.0%
		% within Sp3 Expression Level	31.8%	36.8%	34.1%
		% of Total	17.1%	17.1%	34.1%
	Male	Count	15	12	27
		% within Gender	55.6%	44.4%	100.0%
		% within Sp3 Expression Level	68.2%	63.2%	65.9%
		% of Total	36.6%	29.3%	65.9%
Total		Count	22	19	41
		% within Gender	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.735[a]					

Considering location of cancer studied, 58.3% of colon cancers had low expression of Sp3 while the rest 41.7% had high Sp3 expression. Whereas in rectum cancers more similar results were observed as 47.1% were low and 52.9% showed high expression. Regarding statistics, no significant differences were observed between Sp3 expressions in both colon and rectum tissues p = 0.476 (Table 4.14).

Table 4.14 The association of tissue location groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Tissue Location Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Cancer Location	Colon	Count	14	10	24
		% within Cancer Location	58.3%	41.7%	100.0%
		% within Sp3 Expression Level	63.6%	52.6%	58.5%
		% of Total	34.1%	24.4%	58.5%
	Rectum	Count	8	9	17
		% within Cancer Location	47.1%	52.9%	100.0%
		% within Sp3 Expression Level	36.4%	47.4%	41.5%
		% of Total	19.5%	22.0%	41.5%
Total		Count	22	19	41
		% within Cancer Location	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.476[a]					

Apparently, high and low Sp3 expressions were detected equally in early stages. While in advanced stages the Sp3 low expression was slightly higher than its over-expression, (57.9% low versus 42.1% high). The statistics showed no significant variances between Sp3 high and low expression in stage of disease groups p = 0.613 (Table 4.15).

Table 4.15 The relationship of stage of disease groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Stage of Disease Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Stage	Early	Count	11	11	22
		% within Stage	50.0%	50.0%	100.0%
		% within Sp3 Expression Level	50.0%	57.9%	53.7%
		% of Total	26.8%	26.8%	53.7%
	Advanced	Count	11	8	19
		% within Stage	57.9%	42.1%	100.0%
		% within Sp3 Expression Level	50.0%	42.1%	46.3%
		% of Total	26.8%	19.5%	46.3%
Total		Count	22	19	41
		% within Stage	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.613[a]					

Also, after inspecting Sp3 expression in relevance to lymph node metastasis involvement, Sp3 expression was high in 8 and low in 10 patients with metastasized lymph nodes. Likewise, in non-metastasized lymph node samples, Sp3 was over-expressed in 11 tumors and under-expressed in 12 samples. Counting on results of χ^2 Test, there was no substantial dissimilarity between Sp3 expression various levels in the groups of lymph node involvement in metastasis (p = 0.829) (Table 4.16).

Table 4.16 The relationship of lymph-node involvement groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Lymph Node Involvement Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Lymph Node Involvement	Yes	Count	10	8	18
		% within Lymph Node Involvement	55.6%	44.4%	100.0%
		% within Sp3 Expression Level	45.5%	42.1%	43.9%
		% of Total	24.4%	19.5%	43.9%
	No	Count	12	11	23
		% within Lymph Node Involvement	52.2%	47.8%	100.0%
		% within Sp3 Expression Level	54.5%	57.9%	56.1%
		% of Total	29.3%	26.8%	56.1%
Total		Count	22	19	41
		% within Lymph Node Involvement	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.829[a]					

Only 10 patients were with tumors metastasized to other organs, and 4 of them had low levels of Sp3, whereas the other 6 showed high Sp3 levels. Oppositely, the rest 31 had 13 patients with high Sp3 expression and 18 samples with low levels of Sp3. Statistically, no clear variation between Sp3 levels in distant metastasis patients' groups. Depending on Fisher's Exact test p = 469 (Table 4.17).

Table 4.17 Distant metastasis groups relationship with Sp3 expression levels ([b] = Fisher's Exact).

Distant Metastasis Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Metastasis	Yes	Count	4	6	10
		% within Metastasis	40.0%	60.0%	100.0%
		% within Sp3 Expression Level	18.2%	31.6%	24.4%
		% of Total	9.8%	14.6%	24.4%
	No	Count	18	13	31
		% within Metastasis	58.1%	41.9%	100.0%
		% within Sp3 Expression Level	81.8%	68.4%	75.6%
		% of Total	43.9%	31.7%	75.6%
Total		Count	22	19	41
		% within Metastasis	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.469[b]					

Sp3 expression was almost the same in patients whether they were with or without other diseases other than CRC. Sp3 expression was high in 45% of patients with other diseases and in 47.6% of patients with no other diseases. Contrarily, 55% of people with other diseases and 52.4% of patients without other diseases had low levels of Sp3. Statistically, no significant difference was observed between Sp1 levels in other diseases groups p = 0.867 (Table 4.18).

Table 4.18 The relationship of other disease groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Other Diseases Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Other Diseases	Yes	Count	11	9	20
		% within Other Diseases	55.0%	45.0%	100.0%
		% within Sp3 Expression Level	50.0%	47.4%	48.8%
		% of Total	26.8%	22.0%	48.8%
	No	Count	11	10	21
		% within Other Diseases	52.4%	47.6%	100.0%
		% within Sp3 Expression Level	50.0%	52.6%	51.2%
		% of Total	26.8%	24.4%	51.2%
Total		Count	22	19	41
		% within Other Diseases	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.867[a]					

Both high and low levels Sp3 was seen in the same number of smoker patients (8 samples of each). While 14 of non-smokers had low Sp3 expression, 11 smokers exhibited high Sp3 levels. the p-value of 0.707 means that there is no significant variance between Sp3 expression in smoker and non-smoker groups (Table 4.19).

Table 4.19 The relationship of smoking habit groups with Sp3 expression levels ([a] = Pearson Chi-Square).

Smoking Habit Groups * Sp3 Expression Level Crosstabulation					
			Sp3 Expression Level		Total
			Low	High	
Smoking Habit	Smokers	Count	8	8	16
		% within Smoking Habit	50.0%	50.0%	100.0%
		% within Sp3 Expression Level	36.4%	42.1%	39.0%
		% of Total	19.5%	19.5%	39.0%
	Non-Smokers	Count	14	11	25
		% within Smoking Habit	56.0%	44.0%	100.0%
		% within Sp3 Expression Level	63.6%	57.9%	61.0%
		% of Total	34.1%	26.8%	61.0%
Total		Count	22	19	41
		% within Smoking Habit	53.7%	46.3%	100.0%
		% within Sp3 Expression Level	100.0%	100.0%	100.0%
		% of Total	53.7%	46.3%	100.0%
No significant difference observed, p = 0.707[a]					

4.6 The Sp1 and Sp3 Harmonized Expression

Purposing to evaluate any likely correlation between Sp1 and Sp3 expression levels, we used the Spearman's rho Test. Statistically, in a total of 41 patients, a very significant correlation was witnessed between Sp1 and Sp3 expression levels (with an $r = 0.827$ at the $p = 0.01$) as viewed in (Table 4.20).

Table 4.20 The correlation between the Sp1 and Sp3 relative gene expression levels (fold change) (n = number of samples).

Correlations				
			Sp1 Fold	Sp3 Fold
Spearman's rho	Sp1 Fold Change	Correlation Coefficient	1.000	.827**
		Sig. (2-tailed)	.	.000
		n	41	41
	Sp3 Fold Change	Correlation Coefficient	.827**	1.000
		Sig. (2-tailed)	.000	.
		n	41	41
**. Correlation is significant at the p = 0.01 (2-tailed).				

CHAPTER 5

DISCUSSION

There is still much to be comprehended about the role of the Sp1 and Sp3 isoforms and their PTMs in gene expression regulation. So far, studies classically concern with the association status between upstream promoter regions and either Sp1 or Sp3. Too many reasons have pushed us to investigate the expressions of Sp1 and Sp3 genes in colorectal cancer. As both Sp1 and Sp3 are implicated in a great variety of the essential biological processes and have been proven important in cell growth, differentiation, apoptosis, and carcinogenesis (Vizcaíno et al., 2015). The big number of Sp1/Sp3 associated binding sites (around 12000) existed in the human genome (Cawley et al., 2004). And that Sp1 target genes of the six hallmarks of cancer (self- sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis). Also, genes involved in genomic instability and cell growth/metabolism (mobilization of resources) (Hahn and Weinberg, 2002). Likewise, Sp1:Sp3 ratio plays an important role in target genes regulation (Black et al., 2001). Another important reason was the determination of Sp1 expression levels prior to surgeries may be useful for decision making in the modality and extent of postoperative therapy (Wang et al., 2003). And Sp1 can be used as a new biomarker to identify clinically aggressive adenocarcinoma and patients with a bigger likelihood of cancer metastasis and short overall survival (Jiang et al., 2008). Also, Sp1 may characterize a valued prognostic marker for glioma and may be involved in the modulation of tumor invasion (Guan et al., 2012). The Sp1 and Sp3 are not oncogene addiction (OA) genes, and they are attracting drug targets for singular and complicated cancer chemotherapies (Hedrick et al., 2016).

And in order to accomplish this study, measuring Sp1 and Sp3 expression levels in both cancerous and normal tissues besides evaluating them statistically was the first objective in our study. The preceding discoveries were sharp in viewing the contrast in expression levels of both Sp1 and Sp3 generally between normal and cancerous

tissues, though the results of our study statistically revealed that there was no significant difference between the expression levels of Sp1 in normal and tumorous tissues examined. The Sp3 levels of expression in both cancerous and matching normal tissues also statistically did not display any significant differences. Whereas a previous study demonstrated that Sp1 levels were higher in tumor cells than in normal cells of thyroid and that the modifications in the binding activity of Sp1 transcription factors to Sodium/iodide symporter (NIS) promoter might be the reason for the decrement in NIS expression and the subsequent iodide transport in thyroid tumors (Chiefari et al., 2002). Another study also found low levels of Sp1 in normal human gastric cells compared to its high expression in human gastric cancer cells (Wang et al., 2003). Similarly, Sp1 expression was high in late stages of gastric cancer but not in early stages (Yao et al., 2004). Also, elevated level of expression for Sp1 were observed in both tumorous and normal tissues of breast with different ratios (Wang et al., 2007). Once more, Sp1 was over-expressed in a subset of primary pancreatic adenocarcinoma (Jiang et al., 2008). Briefly, Sp1 and Sp3 expressions were found high in different cancer cell lines such as (breast, kidney, pancreatic, lung and colon) however they were low in normal tissues (Hedrick et al., 2016).

On the other hand, an early study of Prochasson and colleagues in 1999 revealed that Sp1 did not show different levels between the SW613-S human colon carcinoma cell line and the cells of non-tumorigenic clones. The same case was witnessed in the genes of the factors of the preinitiation complex interacting with Sp1 protein which may reinforce our similar findings.

The second objective in our study to be achieved was the investigation of Sp1/Sp3 expression levels with respect to the clinicopathological parameters of patients involved in this study. Our results underlined that there was no association between Sp1/Sp3 expression and all the clinicopathological features of the 41 colorectal cancer patients. While various associations between Sp1/Sp3 expression and the clinicopathological factors were detected through the former studies. Moreover, the elevated expression levels of Sp1 in human gastric cancer were correlated reversely with survival of patients (Wang et al., 2003). Also, Sp1 high expression was found associated with high ranks of lymph node metastasis and with inferior survival of gastric cancer patients (Yao et al., 2004). The high Sp1 expression was positively correlated to TNM stage, tumor invasion, and lymph node metastasis (Wang et al., 2007). Again there was an association between Sp1 overexpression and higher stage,

higher grade, and lymph node metastasis in a subset of primary pancreatic adenocarcinoma (Jiang et al., 2008). Remarkably, the presence of high levels of the full-length form of Sp3 indicates a poor prognosis for overall survival of patients with head and neck tumors (Essafi-Benkhadir et al., 2009). Furthermore, high Sp1 expression was observed in patients with better overall survival while low Sp1 expression was detected in worse overall survival patients with glioma (Guan et al., 2012). There was a significant correlation between the Sp1 low expression and the low survival in the I, II and IV stages of lung adenocarcinomas (Hsu et al., 2012).

In our opinion, the absence of association between Sp1/Sp3 and the clinicopathological parameters may be referred to many explanations. Absence of data regarding the tumor grading system for our patients may be a reason for our study different results while there was an association between Sp1 overexpression and higher tumor grade (Jiang et al., 2008). Also, the huge number of Sp1/Sp3 binding sites "almost 12000" in the human genome. In addition, these sites are associated with nearly all genes of the cellular processes including cell growth, differentiation, apoptosis, and carcinogenesis (Vizcaíno et al., 2015). Furthermore, the uninvestigated roles of the other Sp family members such as Sp2, as it was early mentioned that the Sp1-/Sp3-driven transcription in Drosophila SL2 cells was repressed by Sp2 (Bakovic et al., 2000). The probability of the undocumented different lifestyles of patients involved in our study also would provide an explanation for the different expression of Sp1 and Sp3. Not forgetting the complicated interactions of the different isoforms of both Sp1 and Sp3 to their binding sites. Over 90% of DNA binding domain of Sp1 and Sp3 are homologous sequences. Hence four isoforms of Sp3 are competing with the two of the Sp1 isoforms. But because the N-terminal activation domain is absent in M1 and M2 Sp3 isoforms, the best chance for those isoforms will be poor activators (Li and Davie, 2010). Generally, cancerous cells are aneuploid, holding many copies of each chromosome (Giam and Rancati, 2015). For the Sp1/Sp3 regulation-dependent genes, the recruitment of Sp1 vs. Sp3 and which of their isoforms will determine the transcriptional activity of that allele. Thus, the rivalry among the isoforms of Sp1 and Sp3 will lead to distinctive allelic expression, for instance, the short isoforms of Sp3 were noticed over-expressed in multiple tumor cell lines but not Sp3 long isoforms (Rothem et al., 2004). Also, the formation of multimers out of Sp1 proteins may provide too many docking sites for other proteins that may determine the Sp1 function as activator or repressor (Porter et al., 1997; Doetzlhofer et al., 1999; Xiao et al., 2000; Li et al., 2004). Still, the promoter

situation and the upstream promoter transcription factor repertory may direct binding of Sp1 against Sp3 isoforms to the alleles, and consequently decrease the heterogeneity of Sp1/Sp3 relationship (He et al., 2005). Another variable is that Sp1 and Sp3 have distinctive positions in the nucleus. It is possible that the likelihood of a promoter linking with either Sp1 or Sp3 may be decided by the promoter's relative position next to an Sp1 or Sp3 foci (Li and Davie, 2010). Also, the Sp1 and Sp3 merit of being autoregulated genes as Sp1/Sp3 binding sites are already present in their proximal promoters may be an explanation of their changing behavior (Nicolás et al., 2001). Other than that, numerous epigenetic alterations involving in the regulation of Sp1/Sp3 expression such as the sumoylation alterations at specific sites in Sp1/Sp3 may also be implicated in our results as it affects negatively on both Sp1/Sp3 activity (Ross et al., 2002; Sapetschnig et al., 2002; Verger et al., 2003; Spengler and Brattain, 2006; Wang et al., 2008). Finally, the unexplored RNAi status in our patients can be another justification for our differing results as RNAi is known to cause depletion to Sp factors in other cancers (Jiang et al., 2004; and Yuan et al., 2007).

Eventually, using the Spearmen's rho test, we showed that Sp1 together with Sp3 are extremely correlated in levels of expression in both the cancerous and the wild-type tissues. The obvious correlation of Sp1 and Sp3 supports the fact that both transcription factors are genetically of the same ancestor and have the most similar structures amongst the Sp-family factors as 'Sp1 and Sp3 share over 90% DNA sequence homology' (Li and Davie, 2010). Also, it is backing up our opinion that both Sp1 and Sp3 expression levels are being affected by almost the same elements during cancer development due to the similarity in structure and affinity of their isoforms.

CHAPTER 6

CONCLUSION

Even with the results screened from up to date studies, in which Sp1 and Sp3 high expression levels were observed in cancers, the results of our study suggest that the absence of Sp1/Sp3 association with clinicopathological parameters point out that Sp1/Sp3 may not present an important role in colorectal cancer. Also, the high correlation between Sp1 and Sp3 expression levels support the idea that both of Sp1/Sp3 belong to the same family of genes and might be affected mostly by the similar elements during cancer development due to the similar structure and affinity of Sp1 and Sp3 isoforms. Further investigation need to be applied.

Suggestions

For the future studies, we suggest;

- To increase the number of samples to be investigated along with planning to detect Sp1/Sp3 protein levels in addition to mRNA levels and to determine their existed isoforms in each sample.
- More data about patients can be obtained to be included in future studies regarding CRC grading system, survival, and body mass index.
- To implicate extra genes involved in regulation of Sp1/Sp3 to be studied along with Sp1 and Sp3.
- To implicate more detailed data related to chronic/concomitant diseases in future studies as a clinicopathological characteristic.

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