SNP GENOTYPING OF RECK GENE IN LUNG CANCER

A

Thesis submitted

In partial fulfillment of the required of the degree

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Master of Science

In

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By

Kamaldeep Kaur

Roll no. 301105006

Under the supervision of

Dr. Vikas Handa

Assistant Professor/ Supervisor

DBTES, Thapar University

Dr. Siddharth Sharma

Assistant Professor/ Supervisor

DBTES, Thapar University

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Department of Biotechnology and Environmental Sciences

Thapar University, Patiala, Punjab

Certificate

This is to certify that the thesis entitled "**SNP genotyping of RECK gene in Lung cancer**" being submitted by Ms Kamaldeep Kaur (Roll No-301105006) in partial fulfillment of requirements for the award of degree of Master of Science in Microbiology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Vikas Handa and Dr. Siddharth Sharma and that no part of this thesis has been submitted for the award of any other degree.

15.07.2013

Dr. Vikas Handa
Assistant Professor/ Supervisor
DBTES, ThaparUniversity

Dr. M.S Reddy
Professor and Head
DBTES, Thapar University

Dr. Siddharth Sharma
Assistant Professor/ Supervisor
DBTES, Thapar University

Dr. S.K. Mohapatra
Dean, Academic Affairs
Thapar University

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I hereby declare that the work being presented in the thesis entitled "**SNP genotyping of RECK gene in Lung cancer**" in partial fulfillment of the requirements for the award of degree of Masters in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to July 2013, under the supervision and conception of Dr. Vikas Handa and Dr. Siddharth Sharma, Assistant Professors, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

Date: July 15th, 2013

Patiala

Kamaldeep Kaur

Roll No. 301105006

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

15.07.2013

Dr. Vikas Handa

Assistant Professor/ Supervisor

DBTES, Thapar University

Dr. M.S Reddy

Professor and Head

DBTES, Thapar University

Dr. Siddharth Sharma

Assistant Professor/ Supervisor

DBTES, Thapar University

Department of Biotechnology and Environmental Sciences

Thapar University, Patiala 147004

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ABBREVIATIONS

S.no	Abbreviation	Full form
1.	RECK gene	Reversion inducing cystiene rich protein with Kazal motifs
2.	MMPs	Matrix metalloproteinases
3.	US	United States
4.	NCRP and ICMR	National Cancer Registry Program of Indian Council of Medical Research
5.	SCLC	Small cell lung carcinoma
6.	NSCLC	Non small cell lung carcinoma
7.	SCC	Squamous cell carcinoma
8.	LCC	Large cell carcinoma
9.	AC	Adenocarcinoma
10.	GPI	Glycosylphosphatidyllinositol
11.	SPI	Serine protease inhibitor
12.	EGF	Epidermal growth factor
13.	Asn	Asparginine
14.	ECM	Extracellular matrix
15.	TIMPs	Tissue inhibitors of matrix metalloproteinases
16.	BM	Basement membrane
17.	EC	Endothelial cells
18.	PCR	Polymerase chain reaction
19.	P-stage	Pathological stage
20.	HIS	Imunohistological staining
21.	VEGF	Vascular endothelial growth factor
22.	IMVD	Intratumoural vascular density
23.	DNMTs	DNA methyl transferases
24.	HCC	Hepatocellular carcinoma
25.	TBP	TATA binding protein
26.	ERK	Extracellular kinase pathway

27.	TSA	Trichostatin A
28.	HDAC	Histone deacetylase
29.	EBV	Epstein barr virus
30.	NSAIDs	Non steroidal anti-inflammatory drugs
31.	SNPs	Single nucleotide polymorphism
32.	CI	Confidence interval
33.	HBV	Hepatitis B virus
34.	HCV	Hepatitis C virus
35.	IGF	Insulin growth factor
36.	PAI	Plasminogen activator inhibitor
37.	COX	Cyclooxygenase
38.	PGI	Post Graduate Institute of Medical Education and Research
39.	EDTA	Ethylenediaminetetraacetic acid
40.	NaCl	Sodium chloride
41.	OD	Optical density
42.	TE	Tris EDTA
43.	$MgCl_2$	Magnesium chloride
44.	dNTP's	Deoxyribonucleotide triphosphates
45.	TBE	Tris borate EDTA
46.	RFLP	Restriction fragment length polymorphism
47.	LC	Lung cancer samples
48.	LCC	Lung cancer controls
49.	RE	Restriction digestion
50.	M	Mutant genotype
51.	H	Heterozygous genotype
52.	WT	Wild type genotype

ABSTRACT

RECK is reversion inducing cysteine rich protein with Kazal motifs that inhibits matrix-metalloproteinases such as MMP-2, MMP-9 and MT-1 which in turn cause breakdown of extracellular matrix and also inhibits angiogenesis. For SNP genotyping of RECK gene, a total of 100 lung cancer samples and controls were analyzed for RECK SNPs (rs11788747) using PCR-RFLP genotyping analysis. After adjusting the co-variants, the individuals carrying RECK promoter (rs11788747) inheriting at least one G allele had higher risk of developing lung cancer as compared to wild type carriers. Individuals carrying heterozygous genotype (A/G) had significant correlation with developing of lung cancer (OR 0.398, 95% CI; 0.24-0.775, p = 0.006). Individuals with heterozygous (A/G) genotype had significant correlation with smoking in smokers (OR = 0.338, 95% CI; 0.141-0.814, p = 0.014) and But non smokers those who having mutant genotype (G/G) had 3 times more risk of lung cancer (OR = 3.000, 95% CI; 0.157-57.365, p = 0.45). RECK gene polymorphism might be a risk factor in increasing lung cancer susceptibility

Chapter 1

Introduction

Cancer is a disease in which uncontrolled growth and replication of cells or group of cells occur and invade adjacent cells or tissues and then ultimately spread to other body parts. There are main three types of carcinogenic agents physical, chemical and biological.

- Physical agents such as ultraviolet and ionizing radiations
- Chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant) and arsenic (a drinking water contaminant)
- Biological agents include infections from viruses, bacteria or parasites

There are some commonly found cancers like breast cancer, kidney cancer, skin cancer, prostate cancer, bladder cancer, lung cancer and thyroid cancer that cause millions of deaths (http://www.sheknows.com). Lung cancer is a type of cancer which originates in lungs. Lung cancer is very common type of cancer in United States in both men and women. Main cause of lung cancer is smoking which accounts 85% of lung cancer deaths of United States. There are some other lung cancer causing carcinogens found in workplaces. These include bischloromethyl ether and chloromethyl ether in chemical workers, arsenic in copper smelting and asbestos in shipbuilders and other asbestos workers (Khurana. 2010). The relative risk of developing lung cancer is 2.64 folds for bidi smokers and 2.23 for cigarette smokers with 2.25 overall relative risks. In cytogenetic studies chromosomal changes with numerical abnormalities and structural aberrations including deletions and translocations have been found in lung cancer. These mutations include the activation of proto-oncogenes of *ras* and *myc* family and inactivation of tumor suppressor genes (Behera *et al*., 2004)

RECK gene

RECK is membrane bound protein which has been found to be important in suppressing two key components in metastatic cascade, matrix-metalloproteinase (MMPs) and angiogenesis. From earlier studies it has been confirmed that *RECK* levels are significantly down-regulated in common human malignancies, compared with normal surrounding tissues. *RECK* protein is a reversion inducing cysteine rich protein with Kazal motifs. *RECK* was initially discovered by screening a human fibroblast cDNA library for genes giving rise to reversion inducing clones when transfected into v-Ki-ras transformed NIH 3T3 cells. It has been identified that *RECK* gene is present on chromosome region 9p13–p12 and length of 87 kb. It has 21 exons and 20 introns.

The protein coded by *RECK* gene is of 971 amino acid residues and sharing 93% identity (Clark *et al*., 2007).

13 single nucleotide polymorphisms (SNPs) have been detected so far in *RECK* gene, four of them present in coding region of exons 1, 9, 13 and 15 and other nine present in introns of 5, 8, 10, 12, 15 and 17 (Meng *et al*., 2008). The polymorphism in RECK gene (rs11788747) was occurred from A (wild type) to G (mutant) at exon at position 15 and no protein change was found (proline to proline) (Chung *et al*., 2012). The reversion inducing cystiene rich protein with Kazal motifs (*RECK*) is known as tumor suppressor gene. It inhibits the process of invasion and metastasis by regulating expression of many MMPs and therefore also known as key regulator of process angiogenesis in tumor development (Meng *et al*., 2008). It has also been found in many studies that *RECK* is down-regulated in many cancers except in pancreatic and hepatocellular cancer in the process of regulation of MMPs and angiogenesis (Clark *et al*., 2007). One of the elements responsible for oncogene-mediated down-regulation of *RECK* gene is the Sp1 site, where Sp1 and Sp3 factors bind. Sp1 transcription factor family is involved in basal level of promoter activity of many genes, as well as dynamic regulation of gene expression (Sasahara *et al*., 1999).

In many studies it has been also seen that *RECK* expression is known as good prognostic factor in many cancers, for example in breast cancer it has been found that poor prognosis means there was low *RECK* expression and if good prognosis was there it means *RECK* expression was high, so it suggest that *RECK* can be used as good prognosis marker (Zhang *et al*., 2012). Because *RECK* expression known as improved prognostic factor in many cancers so it suggests that this protein as therapeutic potential (Clark *et al*., 2007).

Chapter 2
Review of literature

Background of lung cancer

Lung cancer is the most commonly diagnosed cancer since 1985. Worldwide, there are 1.61 million new cases of lung cancer per year, with 1.38 million deaths, making lung cancer the leading cause of cancer-related mortality. In India, approximately 63,000 new lung cancer cases are reported each year. The major risk factor for developing lung cancer is tobacco use and this disease is often viewed solely as a smoker's disease (Noronha *et al*., 2012). It is estimated that smoking is the principle cause of about 90% of lung cancer in men and almost 80% in women. Smoking also can contribute to cancer of the larynx, mouth and throat, esophagus, bladder, kidney, pancreas, cervix, and stomach, and acute myeloid leukemia (http://www.cdc.gov).

Table no 2.1:- Incidence of lung and bronchus cancer in United States

YEAR	MEN CASES	WOMEN CASES	DEATH (MEN)	DEATH (WOMEN)
2007	109,643	93,893	88,329	70,354(http://www.cdc.gov)
2009	110190	95784	87694	70387 (http://www.cdc.gov)
2010	116750	105770	86220	71080 (Cancer facts and figures, 2010)
2011	115060	106070	85600	71340 (Cancer facts and figures, 2011)
2012	116470	109690	87750	72590 (Siegel Rebecca *et al*., 2012)
2013	118080	110110	87260	72220 (http://www.cancer.org)

Lung cancer in India

It was estimated that lung cancer was rare in the beginning of the last century but latter on it was diagnosed in various patients. But now days it has become almost epidemic resulting in greater number of deaths than those caused by colorectal, breast and prostate cancers. The data collected by National Cancer Registry Program of Indian Council of Medical Research, from six different parts of country including both rural and urban areas showed varying degree of incidence. In the figure given below shown that Delhi has highest number of total cancer cases studied. Total

number of cancer patients reported in Delhi was 13920 having 6815 and 7105 males and females respectively. Mumbai showed the second number of cancer patients including 4170 and 4335 males and females respectively. Bangalore occupied third position with 2262 and 2998 males and females respectively.

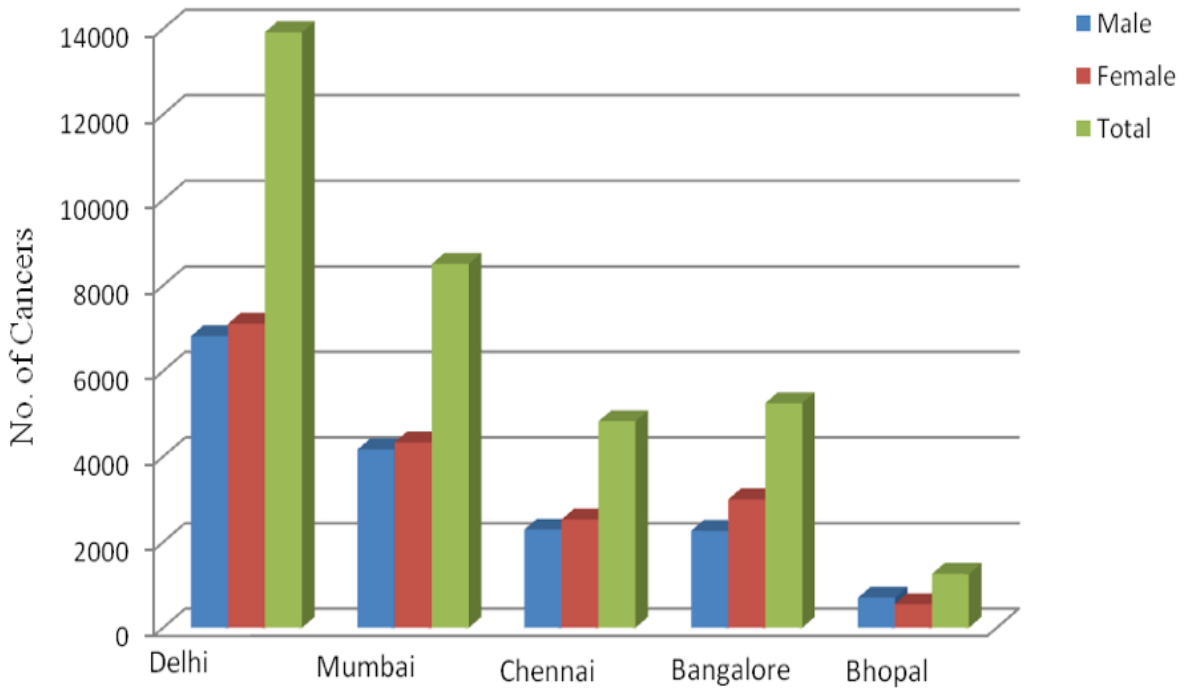

Figure 2.1: Cancer prevalence in five metropolitan cities of India (Ali *et al*., 2011).

Chennai stood at forth position (4824 total patients) with 2296 and 2528 males and females respectively. Next is Bhopal with total number of 1255 with 701 and 554 males and females respectively. This difference was may be because of different environment, food and life style (Ali *et al*., 2011).

Lung cancer can spread to other parts of respiratory tract. Some main parts in which lung cancer spreads are trachea and bronchi. But in most cases lung cancer starts in lining of bronchi. The American Cancer Society classifies lung cancer into two main subtypes. These are given as:-

Small cell lung cancer (SCLC):- This cancer basically starts in bronchi near the centre of chest and spread to lymph node. This cancer is also known as oat cell carcinoma because of its shape and also Small Cell Undifferentiated Carcinoma. This carcinoma includes small cells of lung which divide very fast and form large tumor. SCLC accounts 10 - 15% of all lung cancers.

Non small cell lung cancer (NSCLC):- It is any type of epithelial lung cancer. It has further three types as

- Squamous cell carcinoma (SCC): this is a type of NSCLC and this cancer begins in squamous cells. It is more common in males as compared to females. It accounts 25 - 30% of all non small cell lung cancers
- Large cell carcinoma (LCC): it is also a type of non small cell lung cancer and it starts in several kinds of large cells of lung. It accounts 10 - 15% of non small cell lung cancer. This type of cancer grows and spread quickly which makes it harder to treat
- Adenocarcinoma (AC): this type of cancer starts in alveoli. It accounts 40% non small cell lung cancer. (Khurana. 2010)

Symptoms of lung cancer: Primary symptoms of lung cancer are cough, shortness of breath, blood in sputum and pain. In some types, the cancer cells themselves produces some hormones or other substances that can create an imbalance and result of various symptoms.

Diagnosis and treatment: Diagnosis of lung cancer is mainly done by physical examination like chest X-ray, CT scan, lung biopsy and bronchoscopy. Treatment basically consists of surgical excision of tumor alone or in combination with either external beam radiation therapy or chemotherapy using one or more anticancer drugs (Winter. 2013).

Gene structure

It has been identified that *RECK* gene is present on chromosome region 9p13–p12 and length of 87 kb. It has 21 exons and 20 introns. The coding region of the gene starts at the 110th base of the exon 1 and ends at the 221st base of the exon 21. The upstream 52-base region contains a promoter activity. This region contains two Sp1-binding motifs, one cEBPb-binding motif, and one CAAT box (Meng *et al*., 2008). The protein coded by *RECK* gene is of 971 amino acid residues (Clark *et al.*, 2007) and sharing 93% identity (Takashashi *et al*., 1998).

There are hydrophobic regions at both ends (NH_2 and COOH ends) of the protein. It also has 5 cystiene rich repeats at NH_2 end and also has two regions with epidermal growth (EGF)-like activity while three regions with serine protease inhibitor activity are present in the middle of the

gene. The COOH end allows glycosylphosphatidylinositol (GPI) anchoring to the cell membrane.

The first third sequence of protein (5 cystiene repeats) appears to have particular importance in that it also contains a number of glycosylation sites at asparagines (Asn) residues. These sites are required for proper interaction with MMPs like MMP-2 and MMP-9. Serine protease inhibitors (SPIs) like domains are proteins which inhibit the action of proteases by trapping reactions and reversible tight binding interactions. The first three SPIs are identical to Kazal motif, which is a particular family of peptidase inhibitors containing disulphide- rich proteins with small alpha and beta folds (Clark *et al.*, 2007). Peptide sequencing of the mature human protein expressed in mammalian cells indicated that NH_2 terminal hydrophobic region (26 residues) serves as a signal peptide. The COOH terminal hydrophobic region (29 residues) appears to serve as signal for glycosylphosphotidylinositol anchoring. Based on its activity and structural features this gene is known as RECK for reversion inducing cystiene rich protein with Kazal motifs (Takashashi *et al*., 1998).

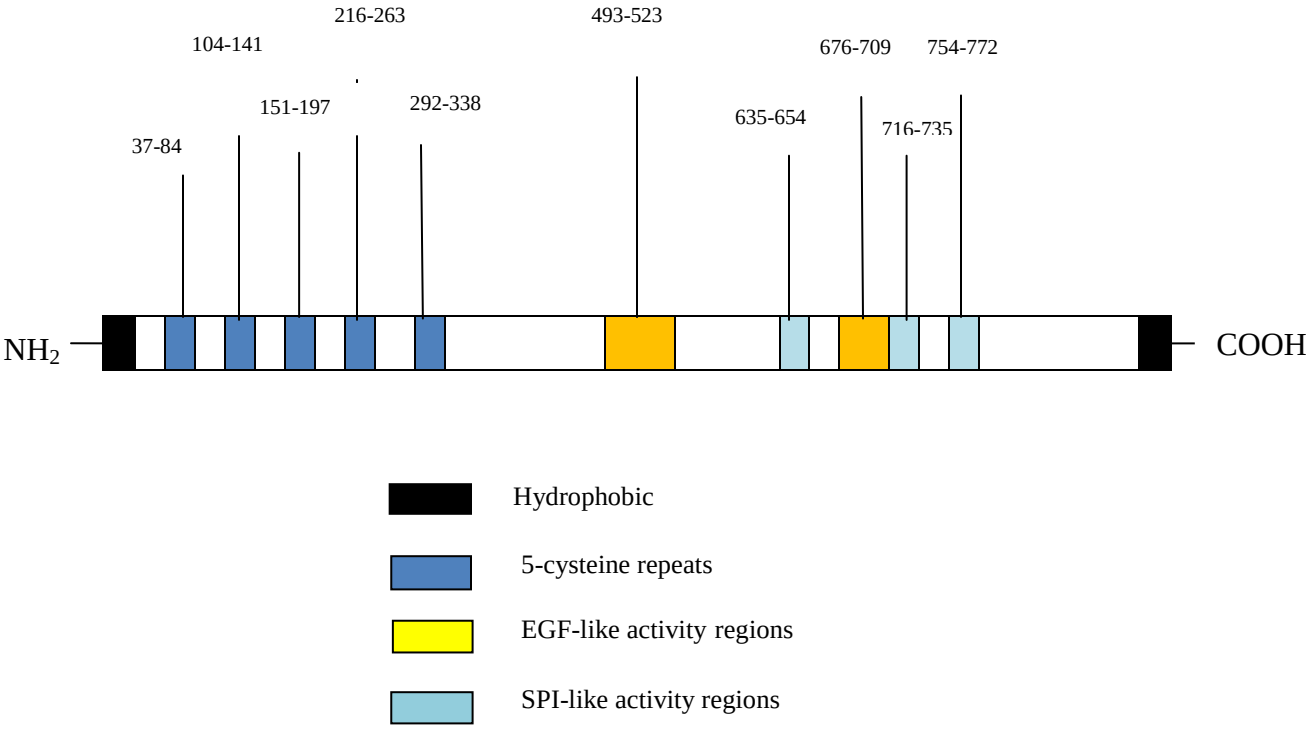

Figure 2.2: Structure of *RECK* gene (Clark *et al.*, 2007)

RECK inhibits MMPs activity and angiogenesis

Matrix metalloproteinases (MMP) family comprises 24 members (Rhee *et al.*, 2002). Matrix metalloprotiense is family of proteinases that were first described in 1962 when Gross and Lapiere identified an enzyme from a vertebrate source (tadpole tail) with proteolytic action capable of attacking collagen. Initially MMPs were known as interstitial collagenases because they cleaved collagen type I, II and III at a single site in the triple helix of the molecule (Foda *et al*., 2001). MMPs cleave the protein component of extracellular matrix and thereby, play important role in the tissue remodeling. It has been also studied that MMPs function as primarily regulators of extracellular matrix (ECM) composition and also facilitate cell migration by removal of barriers such as collagen (Stamenkovic *et al*., 2003). These enzymes require zinc ion at their active site and are inhibited by zinc and calcium chelating agents. MMPs are secreted in latent form and thus require activation for proteolytic activity. They are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (Foda *et al*., 2001).

It has been reported (Meng *et al*., 2008) that *RECK* basically inhibits activity of three types of MMPs (Zn dependent endopeptidases), MMP-2, MMP-9 and MT1-MMP (membrane type MMP). *RECK* inhibits MMPs activity by several mechanisms such as Direct inhibition of protease activity, regulation of their release from the cell and possibly through sequestration of MMPs at the cell surface and direct inhibition of two enzymes MT1-MMP and MMP-2. One particular group of MMPs, the gelatinase A and B, also known as MMP-2 and MMP-9, have the capacity to degrade native collagen type IV, a major component of basement membrane (BM). MT1-MMP plays a dual role in pathophysiological digestion of several ECM components (including collagen I) through direct cleavage of the substrates *in vivo* and activation of MMP-2. During the process of activation of MMP-2, the effect of TIMP-2 (tissue inhibitor of MMPs) is very important. TIMP-2 makes bridge between MT1-MMP and pro-MMP-2 then another MT1-MMP come and cleaves the prodomain portion of pro-MMP-2 and due to this an intermediate is formed MMP-2 protein and after that with the mechanism of autoproteolytic mechanism it gets changed into its fully activated enzyme form. Reduction of active MMP-2 is probably due to direct inhibition of its two processing enzymes, MT1-MMP and MMP-2, by *RECK*. *RECK* negatively regulates MMP-9 in two ways by suppression of pro-MMP-9 secretion from the cells and direct inhibition of its enzymatic activity, which inhibit tumor cells invasion and metastasis.

RECK directly regulates MMP activity and can indirectly modulate membrane localized growth factor availability (Meng. *et al*., 2008).

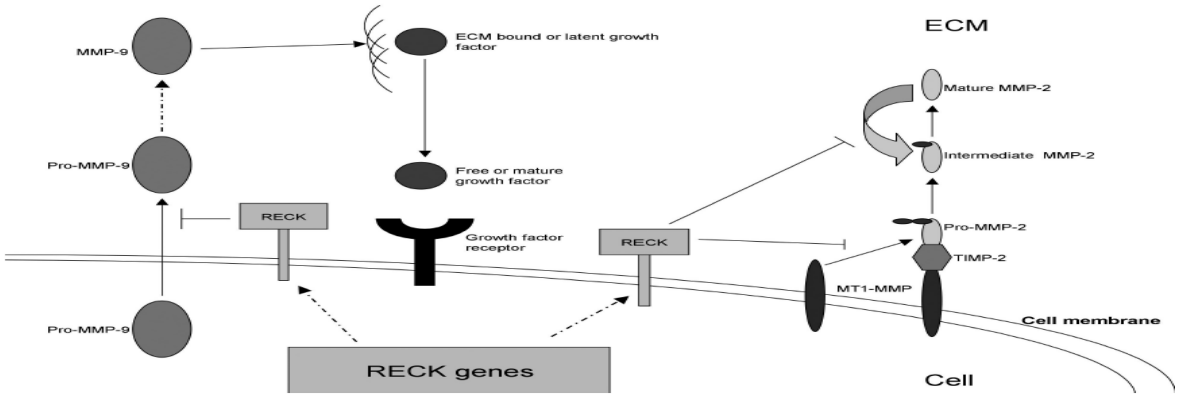

Figure 2.3: Reversion inducing cystiene-rich protein with Kazal motif (*RECK)* regulation three matrix metalloproteinases (MMPs), ECM (extracellular matrix) TIMPs (tissue inhibitors of matrix metalloproteinases) (Meng. *et al*., 2008).

It has been proved (Oh *et al*., 2001) that RECK inhibit MMP-2, MMP-9 and MT-1 MMP. He revealed that a membrane anchored ternary complex consisting of MT-1 MMP, TIMP-2 and pro-MMP-2 is formed on the cell surface and pro-MMP-2 is cleaved at specific site by a second MT1-MMP molecule to yield intermediate form of MMP-2. The removal of the residual prodomain is achieved by intermolecular autoproteolytic cleavage and is accompanied by release of active MMP-2 from the membrane (Oh *et al*., 2001)

New growth in the vascular network is very important since the proliferation, as well as metastatic spread, of cancer cells depends on the adequate supply of oxygen and nutrients and the removal of waste products. Formation of new blood vessels from pre-existing blood vessels is known as angiogenesis. Angiogenesis is very important step in cancer progression. Angiogenesis involves following four steps: the basement membrane is injured locally. There is immediate destruction and hypoxia, endothelial cells activated by angiogenic factors migrate, endothelial cells proliferate and stabilize and angiogenic factors continue to influence the angiogenic process (Nishida *et al*., 2006). This process is required so that the endothelial cells migrate to surrounding tissues (important for the metastasis) and also for nutrient and oxygen supply. ECM is very important for regulation of cellular proliferation, migration, adhesion,

invasion which affects embryonic development, tissue morphogenesis, angiogenesis, tumor transformation and metastasis. The balance between ECM breakdown and deposition is critical for EC homeostasis and contributes to vasculogenesis and angiogenesis. In the presence of high *RECK* expression, MMP activity is reduced to the point where ECM remodelling is compromised and the ability of preexisting blood vessels to send out capillary sprouts and produce new vessels is inhibited. Thus, RECK can also be regarded as a regulator of ECM remodelling by suppressing related MMPs activity, and seems to be playing essential role in angiogenesis (Meng *et al*., 2008).

RECK expression in different cancers and as prognosis factor

In has been studied that many types of tumors have been linked to *RECK* down- regulation and particularly down- regulation was associated with reduced survival. It has been also shown that at least 19 tumor cell lines have already demonstrated absent RECK mRNA. But exceptions to this rule are being identified as *RECK* is analyzed in a greater spectrum of tumors, for example in hepatocellular carcinoma, where *RECK* level was actually higher than normal liver in 40% of specimens.

Takeuchi *et al* showed in their study that colorectal carcinoma tissue samples obtained from 53 patients were analyzed for *RECK* and MMP-9 using immunohistochemistry, with MMP-9 being found to be predominant galatinase on gelatin zymography. In colon carcinoma specimen *RECK* was found down- regulated in majority of colon cancer specimens compared with surrounding normal tissue and in tumor samples with high levels of expressed *RECK* there was no poorly differentiated tissue. A higher frequency of lymph node metastasis in low *RECK* group and an inverse relationship between *RECK* levels and Duke's staging has been demonstrated. There was no relationship between *RECK* expression and MMP levels, however there was a correlation between prognosis and the *RECK*/ MMP-9 ratio. On the basis of this hypothesis that tumors with the highest *RECK* and lowest MMP-9 should have least angiogenesis, invasion and metastasis.

A more recent study on *RECK* in colorectal carcinoma found an inverse relationship between *RECK* and MMP-2 but not with MMP-9. This may be because of conserved Asn^{297} glycosylation but absent glycosylation of Asn^{352}. Span et al found in his study significantly reduced *RECK*

mRNA levels in invasive breast carcinoma specimens as compared to normal surrounding tissues.

Riddick *et al* in their study found expression of MMPs and their inhibitors, either up or down-regulated in prostate cancer. *RECK* and MMPs were both significantly down- regulated in malignant tissue when compared with levels in benign tumor tissue. It was also demonstrated that there are differences between levels of observed MMP-2 mRNA and MMP-2 protein. This may relate to fact that *RECK* is known to inhibit MMPs at post- transcriptional level (Clark *et al.*, 2007). It has been (Sun *et* al., 2006) proved that a significant negative correlation between the *RECK* expression level and invasive capacity *in vitro* and RECK expression showed an inverse proportion to that of MMP-2 and MMP-9 genes. Transwell method was used to determine invasive capacity of breast cancer cell lines including HBL-100, MCF-7 and MDA-MB 435S and revealed expression of *RECK*. MMP-2 and MMP-9 genes were measured by immunocytochemical methods. It was found that order of invasive capacity of breast cell lines was MDA-MB-435S being highest and HBL-100 being lowest. The protein expression level of *RECK* gene in HBL-100 cell lines was highest and no expression was detected in MDA-MB-435S cells and expression of *RECK* gene was negatively correlated with the expression of MMPs genes. It was also proved that function of the *RECK* gene to inhibit the cancer cell line invasive capacity is probably related to its inhibitory effect on secretion and activity of MMPs and therefore suppress the angiogenesis (Sun *et al*., 2006).

In further studies Takenaka *et al*. (2004) have shown that *RECK*- status is a significant prognostic factor correlated with tumor angiogenesis in non small cell lung carcinoma patients. To assess the clinical significance of *RECK*- expression in non small cell lung cancer, a total of 171 patients with completely resected pathological stage (p-stage) I - IIIA were retrospectively examined. Immunohistological staining (HIS) was used to assess expression of *RECK* and vascular endothelial growth factor (VEGF) in tumor tissues. Intratumoural microvessal density (IMVD), a measurement of angiogenesis, was determined by HIS using an anti- CD34 antibody. A significant inverse correlation between *RECK*- expression and tumor angiogenesis was documented. The IMVD in tumors with strong expression of *RECK* (157.1) was significantly lower than that observed in tumors with lower *RECK* expression (194.5, p = 0.008). This inverse correlation was seen only when VEGF was strongly expressed, which suggests that *RECK* gene could suppress the angiogenesis induced by VEGF. The 5 years

survival rate for patients with tumors with strong *RECK*-expression (75.8%) was significantly higher than that for the patients with weakly expressing tumors (54.3%, $p = 0.016$). further analysis showed that *RECK*- status was evident in patients with either adenocarcinoma, poorly differentiated tumors or p-stage IIIA disease. A multivariate analysis showed that reduced *RECK*-expression was an independent and significant factor in predicting poor prognosis (Takenaka *et al*., 2004).

RECK-expression is a significant prognostic factor correlated with long term survival for patients with invasive breast cancer. *RECK*-expression is therefore a potentially useful prognostic marker for breast cancers. To assess the clinical significance of *RECK*-expression in invasive breast cancer, a total of 119 patients with invasive breast cancer were examined. In this study immunohistochemical staining was used to assess the *RECK* expression in tumor tissues and after examination a significant correlation between *RECK* expression and 5 year survival rate was documented. The 5 year survival rate for patients with strong *RECK* expression was significantly higher than that for the patients with weakly expressing tumors. Univariate and multivariate analysis confirmed that reduced *RECK* expression was independent and significant factor in predicting poor prognosis (Zheng *et al*., 2012).

Epigenetic effects on *RECK* gene in different cancers

Du *et a*l. (2010) have revealed that epigenetic alteration like DNA methylation in the promoter CpG islands of tumor suppressor genes where the DNA is transcribed to RNA causes its silence and lead to cancer. This alteration is induced by DNA mehyltransferases (DNMT) catalyzing methylation at 5' position of cytosine ring using S-adenosylmethionine as donor molecule for the methyl group. It play important role in tumorigenesis and progression. It has been shown that aberrant methylation of *RECK* gene may provide useful information for early diagnosis and treatment of peritoneal metastasis of gastric cancer. Methylation specific PCR (MSP) was used to detect methylation of *RECK* gene in 40 paired samples of gastric cancer and its corresponding adjacent normal mucosa, lymph nodes and peritoneal irrigation fluid. In this study aberrant methylation of *RECK* gene was detected in 27.5% (11/40) of adjacent normal mucosa samples, in 47.5% (19/40) of gastric cancer samples, in 57.1% (12/21) of lymph node samples and in 35% (14/40) of peritoneal irrigation fluid samples respectively with a significant difference between adjacent normal mucosa and lymph node samples ($p = 0.023$). Presence of *RECK* methylation in

primary tumor samples was significantly correlated with tumor invasion (p = 0.023). The accuracy of *RECK* methylation in peritoneal lavage fluid samples for the diagnosis of peritoneal metastasis of gastric cancer was 72.5% (26/40), with a sensitivity of 66.7% (6/9) and specificity of 74.2% (23/31) (Du *et al*., 2010).

Cho *et al* (2007) have studied that down regulation of metastasis suppressor RECK in colon cancer is associated with promoter methylation and that DNA methyltransferase inhibitors may restore *RECK* expression to inhibit cell invasion. It has been also seen that down-regulation of *RECK* has been found in human colon cancer. In this study it was investigated whether down-regulation of *RECK* is caused by epigenetic inactivation via promoter methylation and tested the effect of DNA methyltransferase inhibitor on *RECK* expression and cell invasion. The mRNA and protein levels of *RECK* in colon tumor tissues and their normal counterparts were compared and found that down-regulation of *RECK* was found in 48% of twenty five tumors analyzed. MSP analysis demonstrated that methylation of *RECK* promoter was detected in 44% of tumor tissues and strong correlation between down-regulation and promoter methylation was also found (Cho *et al*., 2007).

Zhang *et al* (2012) have also revealed that hypermethylation may lead to promoter silencing of *RECK* mRNA and associated with poor survival in HCC. In this study *RECK* methylation by MSP and *RECK* mRNA by real time PCR was analyzed in 74 HCC and it was seen that *RECK* mRNA was lower in HCC tissues with the hypermethylation than that samples with hypomethylation and also found that *RECK* hypermethylation was higher in HCC patients as compared to normal tissues and cause gene silencing (Zhang *et al*., 2012).

Regulation of mouse *RECK* gene expression by Sp1 transcription factor family

Based on several studies on analysis of mouse *RECK* gene promoter has indicated the oncogene induced down-regulation of *RECK* gene is at least mediated by Sp1/Sp3 binding sites immediately downstream of transcription initiation site (Sarashara RM, Takashahi C and Noda M). Sp1 transcription factor activates transcription by associating with one of TATA binding protein (TBP) co-activators in TFIID complex. Interaction between glutamine-rich activation domains of Sp1 and TBP associated factor dTAF is an important component of Sp1 transactivation activity. In majority of promoters containing Sp1 binding elements, Sp1 appears to provide basal level of transcription, but only when acting in conjugation with other

transcription activators or regulatory proteins. Sp1 activates transcription by cooperatively interacting either with itself or with other transcriptional factors such as E2 protein bound to the bovine papilloma virus enhancer. GATA-1, the major erythyroid transcription factor activates transcription in a synergistic manner. Sp1 has also been shown to be involved in Ras/Raf pathway. So Sp1 acts as positive regulatory element in contrast to oncogene responsive Sp1 site in mouse RECK promoter.

Sp3, a member of this family was initially found to be suppressing Sp1 mediated transcription activation by competitively binding to Sp1 consensus elements or functioning as repressor by protein-protein interaction. In case of down-regulation of mouse *RECK* gene expression through Sp1 site would be that Sp3 is induced or somehow activated in oncogene-transformed cells, thus occupying Sp1 site. In the oncogene mediated down-regulation of mouse RECK gene, a slight reduction in binding of Sp1 to critical Sp1 site was also observed. Thus multiple mechanisms may exist in oncogene mediated transcriptional suppression through Sp1 sites. Sp1 can be regulated via post-transcriptional modifications of its trans-activation domain such as O-linked glycosylation and phosphorylation. One possibility is that oncogene products suppress mouse RECK expression by affecting such post-transcriptional modifications and other possibility is that oncogene signaling affects the interaction between Sp1/Sp3 and their regulatory proteins (Sasahara *et al*., 1999).

Mechanisms by which *RECK* is down regulated in cancer

It has been reported that *RECK* and its ability to counteract MMPs has major implication for treating cancer. *RECK* can inhibits both metastasis and angiogenesis. And it has been also reported that *RECK* is down- regulated in many tumors. The mechanism of how *RECK* is down-regulated in tumors is multi-factorial and also tumor specific and have a common target of Sp1 site on *RECK* promoter sequence.

In early studies Sasahara *et al*, reported that Oncogenic RAS facilitates phosphorylation or other modification of Sp1/ Sp3 factors by activation of extracellular kinase (ERK) pathway by which affinity of Sp1/ Sp3 factors increases for Sp1 site on *RECK* promoter thus this *RECK* expression get reduced. Sasahara *et al* also hypothesized that transcriptional repression of *RECK* occurs due to interaction of histone deacetylation with Sp1 site. So they used HDAC inhibitors which is Trichostatin A (TSA), in NIH3T3 cells but found no specific correlation with *RECK*

levels. It has been recently shown that transcription factor Sp1 is over-expressed in number of cancers and its over-expression contributes to malignant transformation. Sp1 regulates the expression of a number of genes that participating in multiple aspects of tumorigenesis such as angiogenesis, cell growth and resistance to apoptosis.

From the earlier studies Liu *et al*, tested a similar hypothesis in CL-1 human lung cancer cells and found that after addition of TSA, *RECK* cell surface expression increases. This is thought to occur because TSA inhibits the interaction between HDAC and Sp1 and which in turn reduces binding to Sp1 promoter site. So, once *RECK* was up- regulated, a corresponding inhibition of MMP-2 was noted.

A further study done by Chang *et al*, demonstrated evidence to support *RECK* expression inhibition via *ras* and histone deacetylation. They found that *RECK* promoter activity increases actually by Sp1 and Sp3 rather than inhibited. Oncogenic *ras* activity via ERK phosphorylation pathway, resulted in increased Sp1 protein associated with HDAC and this is believed to increase in binding of HDAC to Sp1 site on *RECK* promoter.

RECK is also inhibited by LMP-1 which is a product of Epstein Barr Virus (EBV). It acts in the similar manner by binding to Sp1 site in promoter region of *RECK* gene to inhibit promoter function. It is found that ERK pathway is also stimulated by LMP-1, but when this pathway was inhibited by PD98059 which is an ERK pathway inhibitor, there was a reduced inhibition of *RECK*. This suggests that when ERK pathway is overactive then it will be responsible for *RECK* down- regulation. HER- 2/ neu protein reduces *RECK* expression by increasing the binding of Sp1 proteins to Sp1 site in the same mechanism used by LMP-1. It does this by inducing ERK pathway to phosphorylate the Sp1 proteins because of this their affinity increases for Sp1 promoter site of RECK gene and thus inhibiting the *RECK* expression. *RECK* expression may not directly down-regulated by action of Sp1 protein, HER- 2/ neu also recruits the HDAC to Sp1 proteins, so this combination represses expression of *RECK* gene by binding the promoter site (Clark *et al*., 2007).

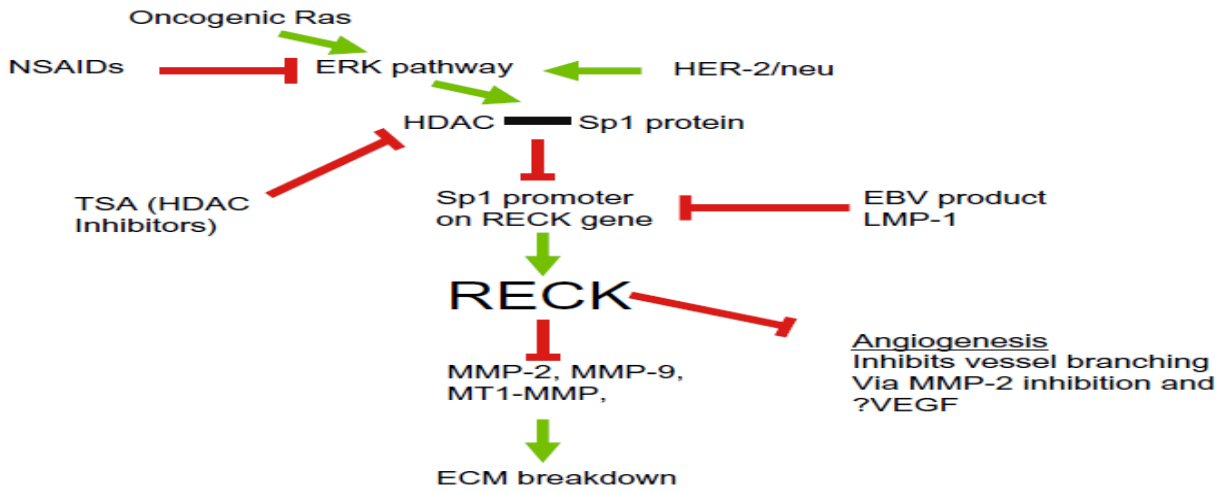

Figure 2.4: Summary of *RECK* control. *RECK* expression is inhibited by a number of mechanisms on Sp1 site. Oncogenic Ras activates the ERK pathway which leads the combined action of HDAC and Sp1 protein on Sp1 site in promoter region of *RECK* gene. And NSAIDs and HDAC inhibitors act against this common pathway and leads to reduction in *RECK* down- regulation and used as cancer therapy (Clark *et al*., 2007).

Relationship of *RECK* gene with polymorphism

Chung *et al* (2012) proved that *RECK* polymorphisms might be a risk factor increasing HCC (Hepatocellular carcinoma) susceptibility and distant metastasis. They took a total of 135 HCC cancer patients and 501 cancer controls which were analyzed for four *RECK* single nucleotide polymorphisms (SNPs) (rs10814325, rs16932912, rs11788747 and rs10972727) using Real- time PCR and PCR-RFLP genotyping analysis. After adjusting for other co-variants, the individuals carrying *RECK* promoter rs10814325 inheriting at least one C allele had a 1.85 fold (95% confidence intervals (CI), 1.03-3.36) risk of developing HCC as compared to TT wild type carriers. The HCC patients, who carried rs11788747 with at least one G allele, had a higher distant metastasis risk than wild type probands. It was also found that SNPs found in the rs16932912, rs11788747 and rs10972727 were found within coding sequence of exons 9, 13 and 15 respectively. These polymorphisms are non- synonymous and change amino acid sequence as well as *RECK* protein structure.

They revealed that *RECK* down-regulation has been confirmed in many types of human cancers and is clinically associated with metastasis. This study investigates the potential associations of *RECK* SNPs with the hepatocellular carcinoma (HCC) susceptibility and its clinicopathologic

characteristics. They also revealed that HCC is the sixth most common cancer worldwide, with 750000 newly diagnosed cases in 2008. HCC is the third most common cause of cancer mortality. Taiwan has the third highest incidence area in world, with a $35.7/10^5$ age-standardized rate in 2008. The development of HCC is a multistep and complex process. Multiple environmental risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections cirrhosis, carcinogen exposure and a variety of genetics contributes to hepatocellular carcinoma. For example, insulin-like growth factor (IGF)-2, IGF-2R, plasminogen activator inhibitor (PAI)-1 and matrix- metalloproteinases (MMP)-14 are HCC risk factors.

RECK is a novel transformation suppressor gene against activated *ras* oncogenes, and induces flat reversion in v-K-ras-transformed NIH/3T3 cells. The RECK gene encodes a membrane anchored glycoprotein that can negatively regulates matrix-metalloprotienases (MMPs) and inhibits tumor invasion, angiogenesis and metastasis. RECK down-regulation have been seen in many types of cancers pancreatic cancer, breast cancer, lung cancer, colorectal cancer, gastric cancer, prostate cancer, oral cancer, esophageal cancer and osteosarcoma and correlates with metastasis and poor prognosis (Chung *et al.*, 2012)

Chung *et al*. (2011) proved that gene-environment interactions between the *RECK* polymorphisms, smoking and betel quid may alter oral cancer susceptibility and metastasis. He took hospital-based case control study, the demographic RECK genotype and clinicopathologic data from 341 male oral cancer patients and 415 cancer free controls that were investigated. He found that *RECK* rs10814325, rs16932912, rs11788747 and rs10972727 polymorphisms were not associated with oral cancer susceptibility. Among the 488 smokers, *RECK* polymorphism carriers with betel quid chewing have a 7.62-fold (95% confidence intervals (CI), 2.96-19.64) to 25.33 fold (95% confidence intervals 9.57-67.02) risk to have oral cancer compared with RECK wild-type carriers without betel quid chewing. Among 352 betel quid chewers, RECK polymorphism carriers with smoking have a 6.68-fold (95% confidence intervals, 1.21-36,93) to 18.57-fold (95% confidence intervals 3.80-90.80) risk to have oral cancer compared with those who carried wild-type without smoking. In 263 betel quid chewing oral cancer patients, *RECK* rs10814325 polymorphism have a 2.26-fold (95% confidence intervals, 1.19-4.29) risk to have neck lymph node metastasis compared with *RECK* wild-type carriers.

They also proved that oral cancer is the fourth most common male cancer since 2003 and usually associated with carcinogens. *RECK* has a significant effect on tumorigenesis by limiting

angiogenesis and invasion of tumors through extracellular matrix. RECK down-regulation has been confirmed in many types of cancers and associated with lymph node metastasis clinically. Crude mortality rate of oral cancer was 10.1 per 100000 in 2007 and ranked as sixth cause of cancer death. Betel quid chewing, tobacco smoking and alcohol consumption have documented as risk factors for oral cancer development. Single-nucleotide polymorphisms (SNPs) in genes encoding for oral cancer susceptibility factors have been documented to influence gene expression, protein function and disease susceptibility in certain individuals. It also reflects the individual differences of response to drug and environmental factors. Several gene polymorphisms combined with betel quid or tobacco environmental carcinogens to increase oral cancer susceptibility had been documented. They were performed for four SNPs which located in promoter or exon region of *RECK* gene to analyze the associations between *RECK* gene SNPs, environmental risk factors and oral cancer clinicopathologic characteristics (Chung *et al*., 2011)

RECK as anticancer therapeutic agent

While it is still early days, the consistent association of *RECK* with improved prognosis in multiple cancers suggests that this protein may have therapeutic potential. This approach initially in cell culture and after that preclinical (animal) models and finally in clinical trials, will be necessary to demonstrate it. There are number of therapeutic possibilities that are now discussed here.

Firstly, recombinant *RECK* could be used for therapy, similar to insulin and erythroprotein administration. There could be two ways for this soluble *RECK* administration that are systemic or by local infusion. There are several methods available for protein delivery, some even currently used clinically. There is another possibility for gene transfer would be delivery of viral or non- viral vectors *in- vivo* towards gene therapy. While this technique seems less likely given the limited control over the actual amount of *RECK* produced. Studies with other proteins like PEDF demonstrate its latent promise. There is a potential for further neoplastic change to occur with viral vectors, When transfecting an already unstable genome. Thus, there are some non-viral vectors such as liposomes, microplexes, cyclodextrins and nanoparticles may be appropriate for gene transfer.

In preclinical studies the effects of *RECK* over-expression and treatment with r*RECK* on normal tissues should be first elucidated. Furthermore, its effects on developing versus established vasculature needs to be examined.

As previously studied, the new evidence that HDAC inhibitors which is TSA are able to increase *RECK* levels by minimizing promoter inhibition. This fact increases the possibility for HDAC inhibitors to be used therapeutically. For increase of *RECK* expression NSAIDs (non- steroidal anti inflammatory) also used. Liu et al hypothesize that this observation may be due to NSAID inhibition of ras/ERK/Sp1 pathway. They also noted that mechanism behind RECK up-regulation is independent of NSAID action on cyclooxygenase (COX) since (PGE_2) or over-expression of COX-2 by transfection in the lung cancer cells, did not change the levels of *RECK* (Clark *et al*., 2007).

Chapter 3

Aim of study

OBJECTIVES OF THE STUDY

- To study the genotypic frequencies of the RECK gene SNP in lung cancer cases and controls
- To evaluate the effect of RECK gene polymorphism towards lung cancer susceptibility
- To study the effect of smoking on RECK gene and risk for lung cancer
- To analyze the RECK gene polymorphism in relation to the clinico-pathological features of Lung cancer

Chapter 4
Materials and methods

Sample collection

The cases with histologically confirmed primary lung cancer were recruited from September 2012 to May 2013 from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, Punjab. The current study was ethically approved by the Institute ethics committee of PGIMER, Chandigarh. Written informed consent was obtained from all participants or from patients' representatives if direct consent could not be obtained. Each participant was personally interviewed to obtain detailed information on demographic characteristics and lifetime history of tobacco use. 5ml of blood sample was withdrawn from each individual. Intravenously blood samples were taken from patients of lung cancer and also from the controls. Patients that have chosen for lung cancer patients were mostly smokers and age group for controls and also for patients was 30-80 years.

100 lung cancer blood samples were collected from the PGI, Chandigarh.

100 controls were also collected.

DNA isolation

After the collection of blood from lung cancer samples and lung cancer controls then next step was the isolation of DNA from blood of both lung cancer samples and lung cancer controls. For the isolation of DNA from blood Phenol: choloroform method was used. Reagents used in DNA isolation are given below

Reagents:

1. Washing buffer
2. Lysis buffer
3. Phenol: choloroform: isoamyl alcohol solution
4. Choloroform: isoamyl alcohol solution
5. Isopropanol
6. 70% Ethanol
7. TE buffer

Composition:

Washing buffer:

Table 4.1: Different reagents used for washing buffer preparation	
Working concentration	**Stock concentration**
320 mM Sucrose	1M Sucrose
1% TritonX-100	100% TritonX-100
5 mM $MgCl_2$	100 mM $MgCl_2$
10 mM Tris Cl (pH=8)	100 mM Tris Cl (pH=8)

Lysis buffer:

Table 4.2: Different reagents used for lysis buffer preparation	
Working concentration	**Stock concentration**
400 mM Tris Cl (pH=8)	1M Tris Cl (pH=8)
1% SDS	10% SDS
60 mM EDTA	0.5M EDTA
150 mM NaCl	5M NaCl
100 µg/ml Proteinase K	10 mg/ml Proteinase K

DNA isolation procedure

First blood was taken in tube and then equal volume of washing buffer was added and then mixed this solution properly for 5 minutes. After mixing, solution was centrifuged at 3500 rpm for 5 minutes. After centrifugation supernatant was discarded and washing buffer was added to the pellet and centrifuged. This step was repeated three times. Then after proper washing of cells equal volume of lysis buffer was added and incubated overnight at 42 °C. Next day after incubation phenol: chloroform: isoamyl alcohol (25:24:1) solution was added in equal amount and proper mixing was done and then left it for 5 minutes in ice and after that centrifuged the solution at 8000 rpm for 10 minutes and repeated this step two times. After centrifugation two layers were formed and aqueous layer was extracted in the new tube and then solution of choloroform: isoamyl (24:1) alcohol was added in equal amount of layer which was separated and mixed properly. Then solution was centrifuged at 6500 rpm for 5 minutes. Again two layers

were formed upper layer was extracted in the new tube and isopropanol was added to it in equal volume and mixed properly and then again centrifuged the solution at 12000 rpm for 10 minutes. Then pellet was formed and supernatant was discarded and washing of pellet was done with 70% ethanol with 2ml of it and centrifuged at 12000 rpm for 5 minutes, then again supernatant was discarded. This step was repeated two times and when pellet was air dried and dissolved in TE buffer and then solution was transferred in microfuge tube for DNA quantification (Chung *et a*l., 2012)

Agarose gel electrophoresis

After the DNA isolation gel electrophoresis was done for the qualitative estimation of the isolated DNA. 0.7% agarose gel having ethidium bromide at the concentration of 0.5 µg/ml was made in 6X Tris Borate EDTA (pH = 8.0). The DNA samples were mixed with the 5X loading dye (bromophenol blue) and with water. The samples were loaded into wells and allowed to run at 8 V/cm. The gel was observed under UV trans-illuminator for the presence of DNA. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

DNA quantification

Quantitative estimation of DNA was done by Spectrometric analysis of the sample. The absorbance of samples was taken at 260 nm, to determine the concentration of sample. 1 OD is equivalent to 50 µg/ml DNA sample accordingly the concentration of sample is calculated.

$$\text{Concentration (µg/ml)} = \text{O.D}_{260\text{nm}} \times 50\ \text{µg/ml} \times \text{dilution factor}$$

The purity of the sample was checked by taking the ratio of its absorbance at 260 nm and 280 nm. The ratio if is less than 1.8, then it means there is protein contamination, if the ratio is more than 1.8 it means there is RNA contamination and if the ratio is equal to 1.8 that mean DNA is pure.

$$\text{Ratio} = A_{260/}A_{280}$$

And for amount = concentration/1000 × volume of TE in which pellet was dissolved. And dilution factor taken was 300 times. For dilution of DNA 1.5 µl of DNA sample was taken and 498.5 µl of TE buffer was taken.

PCR amplification

Polymerase chain reaction is one of the mainstays of molecular biology. The main reason for the wide adoption of PCR is the simplicity of reaction and relative ease of practical manipulation steps. The PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually known as template DNA.

Primer used were, forward primer 5'- GTAGAAGAAGTGACTCATCC- 3' and reverse primer used was 5'- ATCTCACTCCGAAGATAACC- 3' (Chung *et a*l., 2012)

Table 4.3: Different concentrations of Forward and Reverse primers

Primer	Stock concentration	Working concentration
Forward primer	10 µM	0.5 µM
Reverse primer	10 µM	0.5 µM

PCR buffer ($MgCl_2$) used was 1.5 mM. Reaction volume used was 25 µl. concentrations and solutions were used for optimization of PCR are given below:-

Table 4.4: Different reagents used for PCR optimization with their different concentrations

Name of reagents	Stock concentration	Working concentration
BSA	100X	1X
Buffer	15 mM	1.5 mM
dNTP's	10 mM	0.2 mM
Forward primer	10 µM	0.5 µM
Reverse primer	10 µM	0.5 µM
Taq. Polymerase	3 U/µl	0.8 U

The PCR cycling conditions consisted of initial denaturation at 95 °C for 5 minutes followed by 29 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 45 seconds followed by final extention at 72 °C for 5 minutes. PCR temperature profile is given below:

Table 4.5: Showing temperature profile of PCR reaction

S.no of step	Step name	Temperature	Time
Step 1	Initial Denaturation	95 °C	5 min
Step 2	Denaturation	94 °C	30 s
Step 3	Annealing	55 °C	30 s
Step 4	Extension	72 °C	45 s
Step 5	Step 2 to step 4 repeated 29 times		
Step 6	Final Extension	72 °C	5 min
Step 7	Store	4 °C	∞

The PCR products were examined using gel electrophoresis in 1.7% agarose gel dissolved in 0.5X TBE at 60 volts for 1 hour. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software. An approximately 242 bp PCR product was obtained at the temperature 55 °C which gave sharp band as compared to other temperatures so PCR was optimized at temperature 55 °C.

After the optimization of PCR, then PCR amplification of both lung cancer samples and lung cancer controls was done of 85 samples and 88 controls. But for samples and controls reaction volume used was 20 µl, 16 µl was master mix and 4 µl was template DNA. And for gel electrophoresis 5 µl of sample and 2 µl of loading dye and 3 µl of ladder (100 bp) was taken (Chung *et a*l., 2012)

Restriction fragment length polymorphism (RFLP)

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different length on the gel electrophoresis after digestion of DNA samples with a specific restriction endonuclease. Enzyme will have its specific restriction site.

First restriction digestion was done for both lung cancer samples and also for lung cancer controls. First restriction digestion was done for both lung cancer samples and also for lung cancer controls. The reaction mixture consists of PCR product (10 µl), 1X NEB 4 buffer having a stock concentration 10X, 3U *Rsa*I having a stock concentration of 10 U/µl, the total reaction volume was made up to 20 µl using sterile water. The reaction mixture was incubated at 37 °C overnight.

Enzyme used was *Rsa*I and its restriction site is GTAC
CATG

First mixture of buffer and water was added and then enzyme was added into each PCR tubes and then incubated these for overnight at 37 °C. Then next day 2.0% gel was run, by 8 µl DNA of digestion and 2 µl loading dye (6X) and kept it running at 60 volts seen under UV trans-illuminator and imaging was done under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software. In the heterozygous three bands of 242 bp and for mutant two bands of 242 bp and 142 bp and for wild type one band of 242 bp (which is actual size of PCR product) were seen.

Statistical analysis

For the SNP analysis Hardy-Weinberg equilibrium was tested among patients and controls separately with χ^2-test. We computed odds ratio (OR) (to check risk factor of developing lung cancer in patients and controls, range of OR was taken above 2) with 95% confidence interval (95% CI). And *p* value was also calculated to check the significance association of variables like smoking and small cell carcinoma and also of mutant, heterozygous and wild type genotypes with our study (lung cancer) (Chung *et al*., 2012)

Chapter 5

Results and Discussion

PCR AMPLIFICATION

PCR condition of annealing temperature was at 55 °C and then lung cancer samples and lung cancer controls genomic DNA were amplified at this temperature with the use of forward primer 5'-GTAGAAGAAGTGACTCATCC-3' and reverse primer 5'- ATCTCACTCCGAAGATAA CC- 3'. PCR amplification was performed for total 82 lung cancer samples and out of which 71 samples were amplified and 11 samples failed to get amplified. For lung cancer controls, total 88 controls were amplified out of which 85 controls were amplified while 3 controls failed to get amplified. After amplification 242 bp PCR product was obtained.

Table 5.1: Showing results of PCR amplification of lung cancer samples and controls

Samples	Total run	Amplified	Unamplified
Lung cancer samples	82	71	11
Lung cancer controls	88	85	3

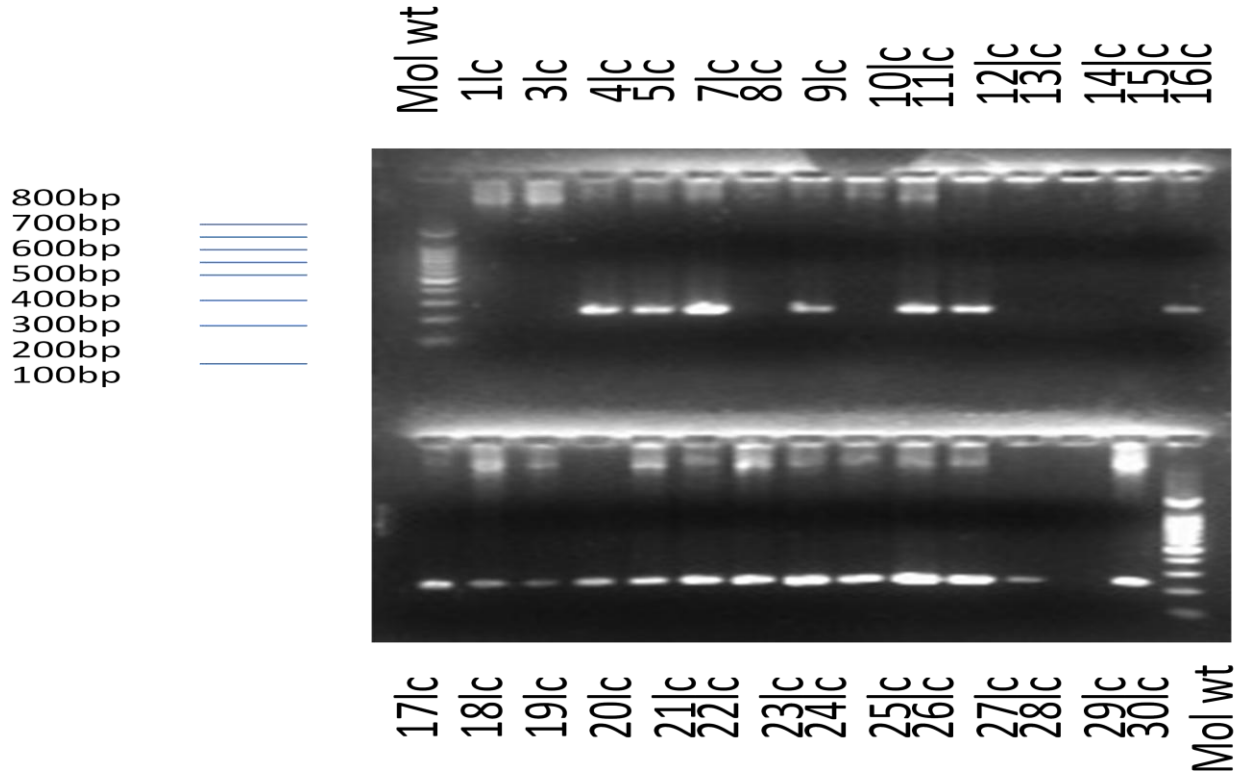

Figure 5.1: PCR amplification gel picture of lung cancer samples

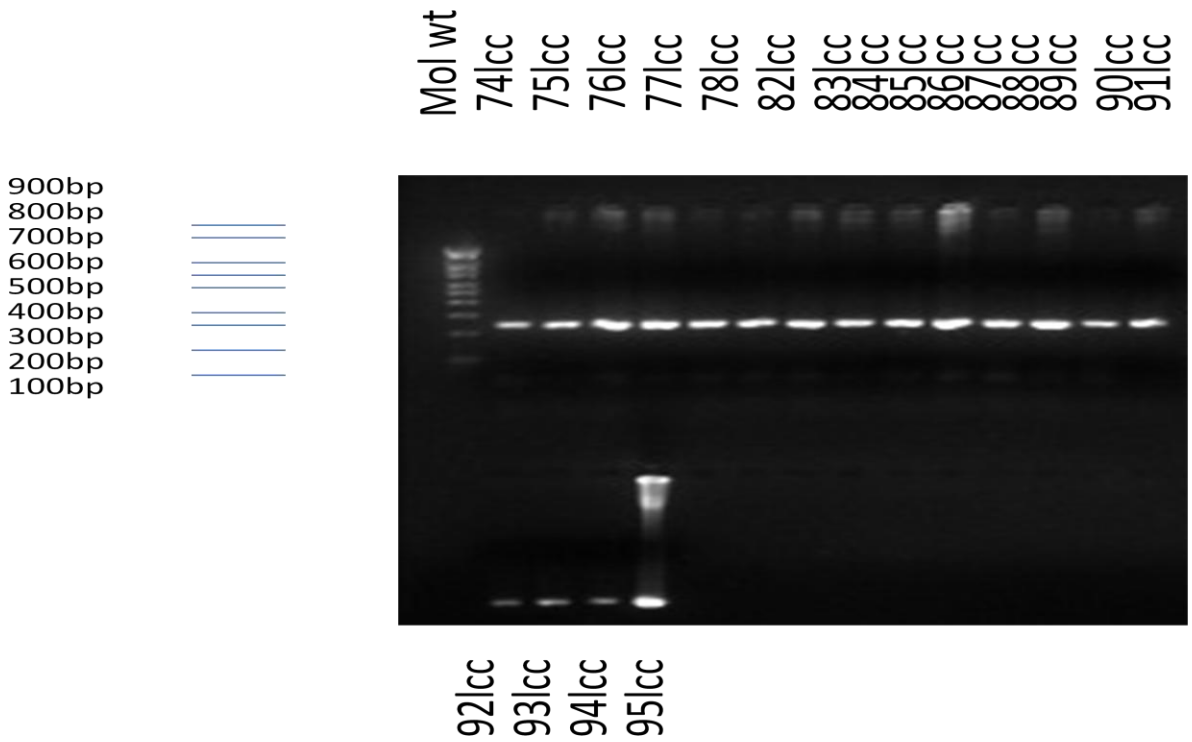

Figure 5.2: PCR amplification gel picture of lung cancer controls

RFLP (restriction fragment length polymorphism)

After PCR amplification restriction fragment length polymorphism (RFLP) was performed by using *Rsa*I restriction enzyme. The restriction enzyme digested PCR products were electrophoretically analyzed and visualized in 2.0% agarose gel. The electrophoresis was carried out at 8 V/cm. In this restriction gel three types of results were observed. In heterozygous genotypes three bands of 242 bp (uncut), 140 bp and 102 bp were seen. A to G polymorphism results in *Rsa1* site generation and as a result 140 bp and 102 bp restriction fragments were observed. In wild type (A/A) only one band was seen of 242 bp and in mutant type in which change was (G/G) and two bands were seen of 140 bp and 102 bp.

Table 5.2: Showing the results of restriction digestion of lung cancer samples and controls			
Samples	**Heterozygous**	**Mutant**	**Wild type**
Lung cancer samples	30	4	37
Lung cancer controls	55	2	27

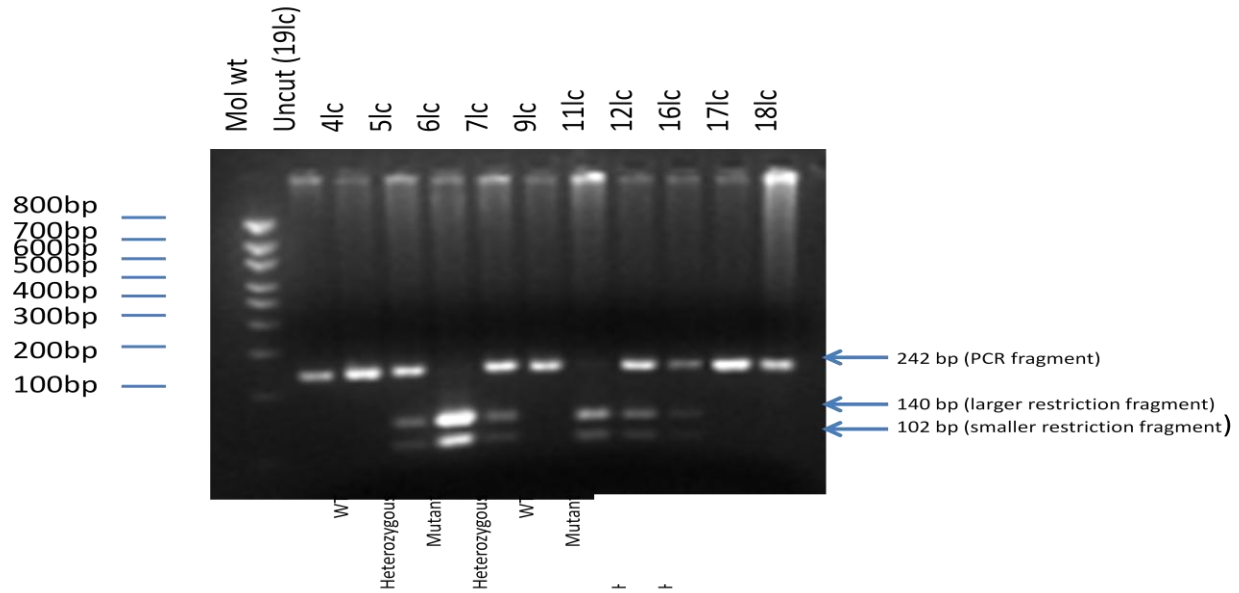

Figure 5.3:- Restriction digestion gel (2%) picture of lung cancer samples

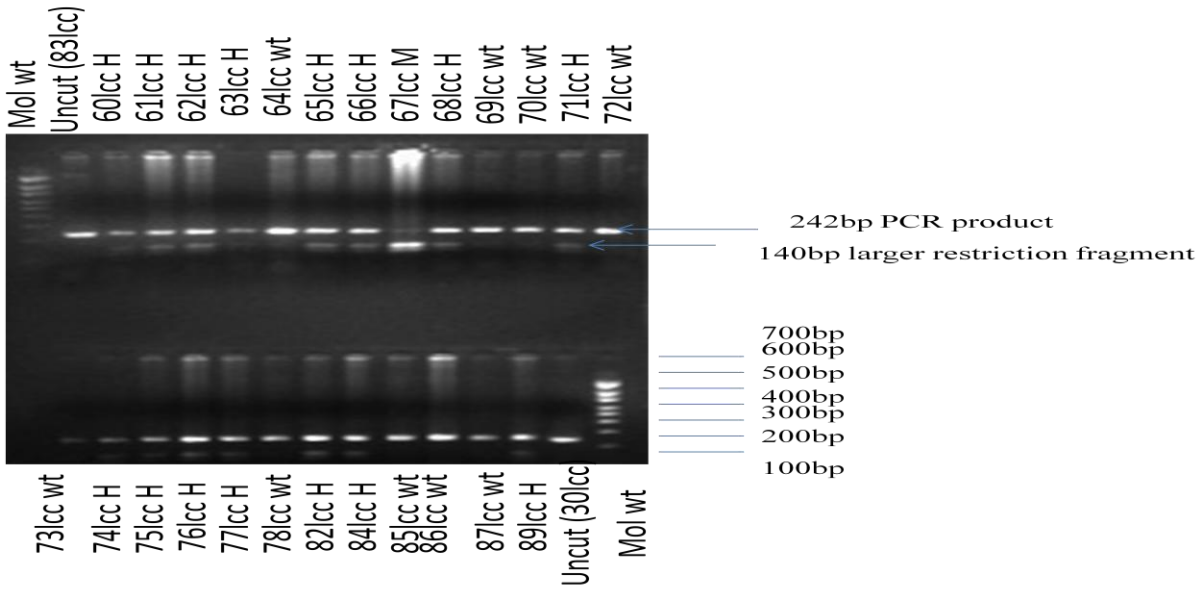

Figure 5.4: Restriction digestion gel (2%) picture of lung cancer controls

STATISTICAL ANALYSIS

EPIDEMOLOGY

The case control study pertains to 71 lung cancer patients and 84 lung cancer controls. Average age was (54 ± 26.87) among the cases and among the controls age average was (53 ± 25.45). 80.28% of lung cancer cases were more than 50 years or 50 years of age and only 19.72% of cases were of below 50 years and in controls 26.19% of population was above 50 years or 50 years and 71.43% was below 50 years. The relevant characteristics of subjects studied are shown below in Table no 5.3

Table 5.3: Distribution of Demographic variables for patients and controls		
CHARACTERSTICS	**CASES (N%)**	**CONTROLS (N%)**
GENDER		
MALE	60 (84.51%)	81 (96.429%)
FEMALE	11 (15.49%)	1 (1.19%)
UNKNOWN		2 (2.38%)
AGE		
<50	14 (19.718%)	60 (71.429%)
≥50	57 (80.28%)	22 (26.19%)
UNKNOWN		2 (2.38%)
RANGE		
SMOKING		
NO	13 (18.31%)	43 (51.19%)
YES/EX-SMOKERS	55 (77.46%)	39 (46.429%)
UNKNOWN	3 (4.23%)	2 (2.4%)
SMOKING INDEX		
≤400	28 (39.437%)	
>400	19 (26.76%)	
UNKNOWN	24 (33.80%)	
HISTOLOGY		

AC	17 (23.94%)	
SCLC	7 (9.86%)	
SCC	33 (46.48%)	
OTHERS	4 (5.634%)	
UNKNOWN	10 (14.085%)	

N number of cases and controls, SCC squamous cell carcinoma, AC adenocarcinoma, SCLC small cell carcinoma.

There are various risk factors contributing to susceptibility towards lung cancer development, out of which smoking is the major one. In present study, 71% of cases were smokers which were divided into two groups *i.e.* heavy smokers and light smokers on the basis of their smoking index. Those subjects which had smoking index greater than or equal to 400 were considered as heavy smokers and those having smoking index less than 400 were considered as light smokers.

The frequency of male subjects in lung cancer population was 84.5% and female subject was 15.4% while in controls frequency of male subjects was 96.43% and females subject was 1.19% thus indicating more or less similar stratification of subjects in terms of sex in both cases and controls population. On the basis of histology 23.94% of cases were found to be Adenocarcinoma, 9.85% were Small cell carcinoma, 46.48% were Squamous cell carcinoma and 5.634% had other carcinomas.

On the basis of TNM staging, 25.35% (18) cases were found to have stage 4, 1.41% (1) had stage 1A, 1.41% (1) had stage 2A, 2.82% (2) had stage 2B, 8.45% (6) had stage 3A, 5.63% (4) had stage 3B, 2.82% (2) had stage 4B, 1.14% (1) had stage IIB, 4.22% (3) had stage IIIA and 2.82% (2) had stage IIIB. The genotype distribution between stages is given in table:

Table 5.4: Frequency distribution of genotypes among patients on the basis of TNM staging			
Stage	**Wild type (A/A)**	**Heterozygous (A/G)**	**Mutant (G/G)**
Stage 4 (18)	8 (44.44%)	6 (33.33%)	4 (22.22%)
1A (1)	1 (100%)	0	0
2A (1)	1 (100%)	0	0
2B (2)	1 (50%)	1 (50%)	0
3A (6)	3 (50%)	3 (50%)	0
3B (4)	1 (25%)	3 (75%)	0
4B (2)	1 (50%)	1 (50%)	0
IIB (1)	0	1 (100%)	0
IIIA (3)	1 (33.33%)	2 (66.66%)	0
IIIB (2)	1 (50%)	1 (50%)	0

RELATIONSHIP OF LUNG CANCER RISK WITH GENOTYPES OF *RECK* GENE

The genotypes of patients and controls for *RECK* gene were recorded by PCR-RFLP. Out of 71 cases 52.11% of individuals were found to have wild type genotype (A/A), 42.25% had heterozygous genotype (A/G) and only 5.63% individuals had mutant genotype (G/G), while in case of controls 32.14% of individuals had homozygous wild type genotypes. 65.48% of individuals had heterozygous genotype and only 2.38% of individuals had mutant genotype. The genotypic and allelic frequencies of *RECK* gene (A>G, rs11788747) and their association with risk of lung cancer development is given below in Table 5.5

Table 5.5: Frequency distribution of *RECK* genotypes and their association with risk of lung cancer development				
GENOTYPES	**NUMBER (%)**		***p***	**O.R (95% C.I)**
***RECK* GENE**	**CASES**	**CONTROLS**		
Total	71	84		
A/A	37 (52.11)	27 (32.14)		
A/G	30 (42.25)	55 (65.48)	0.006	0.398 (0.204-0.775)
G/G	4 (5.63)	2 (2.38)		
GG/AG	34 (47.49)	57 (67.86)	0.011	0.435 (0.227-0.836)
G allele	0.479		0.65	
A allele	0.943		0.97	

The genotype distributions of SNPs both in controls and patients was not in agreement with that of the Hardy-Weinberg equilibrium (p = 0.01 χ^2=8.5). Our results are showing that A/G+G/G genotype (crude OR = 0.43, 95% CI; 0.227-0.84, p = 0.012) is significantly associated with lung cancer and heterozygous genotype (A/G) also have significant association (OR = 0.398, 95% CI; 0.204-0.775, p = 0.00623) with lung cancer development while mutant genotype has no significant correlation (OR = 1.45, 95% CI; 0.249-8.5, p = 0.67) with lung cancer development.

The previous case-control studies have investigated the association between *RECK* gene rs11788747 polymorphism and risk of various cancers including hepatocellular carcinoma (Chung *et al*., 2012) and oral cancer with environmental effects on oral cancer (Chung *et al*. 2011). In the studies done by Chung *et al.* (2012) they found the alleles with highest distribution frequency for RECK gene with rs11788747 were homozygous A/A in both HCC patients and controls. They found RECK gene polymorphism showed no higher risk of HCC as compared to wild type individuals. But with this they also found that RECK gene with rs11788747 SNPs had higher risk of distant metastasis (p = 0.003). In the studies done by Chung *et al* (2011) they found that the frequency of genotypes RECK gene was in Hardy-Weinberg equilibrium (p > 0.05). They also found that allele distribution for RECK gene with rs11788747 were A/A in both controls and oral cancer patients. Our results are showing that there is significant association between heterozygous A/G genotype (*RECK* rs11788747 polymorphism) and risk for lung

cancer and also with A/G+G/G combined genotype. The differences in result of studies can be related to the differences in the population, sample size and environmental factors.

DISTRIBUTION OF GENOTYPES AMONG PATIENTS WITH DIFFERENT HISTOLOGIES OF LUNG CANCERS

Among the cases studied 46.48% (33) had suffered from Squamous cell carcinoma, 23.94% (17) had suffered from Adenocarcinoma, 9.85% (7) had suffered from small cell carcinoma and 5.63% (4) had suffered from other types of carcinomas. There was less evidences of small cell carcinoma and also other kind of carcinomas. On further stratification, genotypic analysis was carried out for each type of lung cancer, where it was seen that out of total subjects having Adenocarcinoma 41.18% were homozygous, 52.94% heterozygous and 5.88% mutant genotype, subjects having Small cell carcinoma 57.14% were homozygous, 28.57% heterozygous and 14.28% mutant genotype and those subjects having Squamous cell carcinoma 57.58% were homozygous, 39.39% heterozygous and only 3.03% had mutant genotype. On the other hand there was also significant association for combined A/G+G/G was also found with Squamous cell carcinoma (OR = 0.35, 95% CI; 0.152-0.799, p = 0.011) while there was no significant association for mutant G/G genotype with Squamous cell carcinoma (OR = 0.71, 95% CI; 0.060-8.410, p = 0.78). On the other hand, significant association was very less for Small cell carcinoma and Adenocarcinoma because sample size was very small.

Table 5.6: Frequency distribution of *RECK* gene genotypes among different histological types of lung cancer patients

	A/A	**A/G**	**G/G**	***p* value**	**O.R (95% CI)**
CASES	37	30	4		
SCC (33)	19 (57.76%)	13 (39.39%)	1 (3.03%)	0.0097	0.336 (0.145-0.780)
				0.0113	0.349 (0.152-0.799)
SCLC (7)	4 (57.14%)	2 (28.57%)	1 (14.28%)		
AC (17)	7 (41.18%)	9 (52.94%)	1 (5.88%)		

DISTRIBUTION OF GENOTYPES *RECK* GENE AMONG PATIENTS AND CONTROLS ON THE BASIS OF SMOKING LUNG CANCERS

On the basis of smoking, patients and controls were divided into two groups those who had smoked as smokers/Ex-smokers and those who had not smoked as non smokers. On the basis of this factor in the cases smokers were 77.46% (71) and in controls 46.43% (84) on the other hand non-smokers in cases were 18.31% (71) and in controls non-smokers were 51.19% (84).

In our study based on genotypes of *RECK* gene for smokers and non smokers in cases and controls it was found that in cases smokers who had homozygous (A/A) genotype were 42.25% (30), 30.99% (22) had heterozygous genotype (A/G) and 4.23% (3) had mutant genotype while in controls smokers who had homozygous genotype were 14.29% (12), 30.95% (26) had heterozygous genotype and 1.19% (1) had mutant genotype. On the other had non smokers in cases who had homozygous genotype were 7.04% (5), 9.86% (7) were heterozygous and 1.41% (1) had mutant genotype while in case of controls who had homozygous genotype were 17.86% (15), 32.14% (27) had heterozygous and 1.19% (1) had mutant genotype.

In Statistical analysis based on smoking it was found that individuals with heterozygous genotype (A/G) had significant association with smoking (OR = 0.338, 95% CI; 0.141-0.814, p = 0.014) and those individuals having combined genotype (A/G+G/G) also shown significant association with smoking (OR= 0.370, 95% CI; 0.156-0.878, p = 0.02) but mutant genotype had a weak association, which was however statistically non-significant (OR = 1., 95% CI; 0.113-12.711, p = 0.87). But in non smokers those individuals having mutant genotype (G/G) had 3 times more risk towards lung cancer (OR = 3.0, 95% CI; 0.157-57.3, p = 0.44).

Table 5.7: Frequency distribution of *RECK* gene genotypes among controls and patients on the basis of smoking

	CASES				CONTROLS			
	N	A/A	A/G	G/G	N	A/A	A/G	G/G
TOTAL	71	37	30	4	84	27	55	2
NON-SMOKERS	13	5 (38.46%)	7 (53.85%)	1 (7.69 %)	43	15 (34.88%)	27 (62.79%)	1 (2.56%)
SMOKERS	55	30 (54.55%)	22 (40%)	3 (5.45%)	39	12 (30.71%)	26 (66.67%)	1 (2.56%)
UNKNOWN	3	2	1	0	2	0	2	0

In the other studies done by Chung *et al* (2011) significant difference in distribution of betel quid chewing and to tobacco consumption ($p < 0.001$) between oral cancer patients and controls was observed. It was also found that allele distribution for RECK gene with rs11788747 was A/A in both controls and oral cancer patients and it was also found that the smokers with polymorphic gene and betel quid chewing habit had risk of 9.37 folds (95% CI; 3.92-22.39) to have oral cancer compared with smoker with wild type gene without betel quid chewing habit and risk of 6.78 folds (95% CI; 1.57-30.05) to have oral cancer as compared with betel quid chewer with wild type without smoking habit. No significant correlation was found in gene polymorphism and clinicopathologic status and in studies done by Chung *et al.* (2012), also there was no significant association between tobacco consumption with HCC.

Chapter 6

Conclusion

In the present study, significant associations has been reported for heterozygous genotype (A/G) (OR = 0.398, 95% CI; 0.204-0.775, *p* = 0.006) and for combined genotype (A/G+G/G) (OR = 0.435, 95% CI; 0.227-0.836, *p* = 0.012) with lung cancer development and also with squamous cell carcinoma in heterozygous genotype (OR = 0.336, 95% CI; 0.145-0.780, *p* = 0.009) and in combined genotype (OR = 0.349, 95% CI; 0.152-0.779, *p* = 0.11). Significant association was also reported in smokers with smoking for heterozygous genotype (OR = 0.338, 95% CI; 0.141-0.814, *p* = 0.014) and also for combined genotype (OR = 0.370, 95% CI; 0.156-0.878, *p* = 0.022), while in non smokers 3 times more risk was reported for mutant genotype (G/G) (OR = 3.000, 95% CI; 0.157-57.365, *p* = 0.45).

Chapter 7

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27. (http://www.cancer.org)
28. (http://www.cdc.gov)
29. (http://www.sheknows.com)

APPENDIX- I

1. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaved.
2. Magnesium chloride ($MgCl_2$) (100mM): Dissolved 0.41gms of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaved.

3. Triton X- 100 (10%): Taken 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.

4. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionsed water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilize the solution by autoclaving.

5. 10% SDS: Dissolved 1g of SDS in 10ml of deionsed water.

6. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilize the solution by autoclaving.

7. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionsed water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilize the solution by autoclaving.

8. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionsed water. Sterilize the solution by autoclaving.

9. TE buffer (pH 8.0): added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deionsed water. Sterilize the solution by autoclaving.

10. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionsed sterile water and kept at 4℃ overnight.

11. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.

12. DMSO: Mixed 50ml of 100% DMSO in 50ml of deionsed sterile water. Sterilize the solution by autoclaving and stored at -20˚C.

13. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilize the solution by autoclaving.