Genetic Polymorphism in NQO1 gene and its Association with Lung Cancer in North Indian Population

A

Thesis submitted

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By

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CERTIFICATE

This is to certify that the dissertation entitled ***"Genetic Polymorphism in NQO1 gene and its Association with Lung Cancer in North Indian Population"*** being submitted by **Ms Divya Walia** in partial fulfilment for the requirement of degree of Masters of Sciences in Biotechnology in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala is a bonafide work carried out under the esteemed supervision and conception of **Dr. Siddharth Sharma**, Assistant Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala.

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DECLARATION

I, the undersigned, hereby declare that the research work presented in the M.Sc dissertation entitled "***Genetic Polymorphism in NQO1 gene and its Association with Lung Cancer in North Indian Population***" has been carried out by me under the supervision and guidance of **Dr. Siddharth Sharma**, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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ABBREVIATIONS

SCLC	Small Cell Lung Cancer
NSCLC	Non Small Cell Lung Cancer
NQO1	NAD(P)H dehydrogenase [quinone] 1
NQO2	NAD(P)H dehydrogenase [quinone] 2
SNP	Single Nucleotide Polymorphism
NRF2	Nuclear Respiratory Ractor 1
ARE	Antioxidant Response Elements
KEAP1	Kelch-like Erythroid cell-derived Protein with CNC homology-Associated Protein
ROS	Receptor Tyrosine Kinase
GST	Glutathione S-Transferases
HBE 1	Human Bronchial Epithelial Cells
GCLC	Glutamate—Cysteine Ligase Catalytic Subunit
GCLM	Glutamate-Cysteine Ligase Regulatory Subunit
UGT	UDP - Glucuronosyltransferases
NAT	N – Acetyltransferase 1
CYP	Catabolite Activator Protein
NOX	NAD(P)H oxidase
PARP	Poly ADP ribose polymerase

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ABSTRACT

Identification of host differences in the susceptibility to carcinogenesis, especially to cigratte smoking is essential in predicting if one is at a higher risk of acquiring the disease. NAD(P)H:quinone oxidoreductase (NQO1) is a detoxification enzyme that protects against the regeneration of reactive oxygen species chemically induced by oxidative stress, cytotoxicity, mutagenicity, and carcinogenicity. The protection conferred by NQO1 protein reduces certain environmental carcinogens, such as nitroaromatic compounds, heterocyclic amines, and possible cigarette smoke condensate.

The present study was focused to assess the vulnerability of an individual towards lung cancer having a polymorphism in the *rs 1800566* of Nqo1, which is a well documented and researched gene. A case control study was performed on the North Indian population. Odds ratio and class intervals were used to assess the potency of the association. P values were also calculated to find out the statistical significance of the analysis.

Cumulative cigarette smoking was interrogated to be a lead player along with other parametrs responsible for susceptibility towards lung Cancer. The combinatorial effect of genotype and histological forms with combined effect of polymorphism was also studied and significant associations were found to exist.

The present study provided us with wider platform to study the *rs 1800566* polymorphism which are not well studied yet.

APPENDIX 1

1. **0.5M EDTA :** Dissolved 9.306g of disodium salt of EDTA in 20ml of deionized water and then adjusted the ph to 8.0 by 1M Sodium Hydroxide. Sterilized the solution by autoclaving.
2. **10% SDS :** Dissolved 1g of SDS in 10ml of deionized water.
3. **100mM Tris-Cl (pH 8.0):** Dissolved 0.32g of Tris-Cl in 10ml of deionized water, then adjusted the pH to 8.0 by 1M Sodium Hydroxide. Sterilized the solution by autoclaving.
4. **10mg/ml Proteinase K :** Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
5. **1mg/ml BSA :** Dissolved 100mg of BSA in 100ml of deionized sterile water and kept at 4ºC overnight.
6. **5% DMSO :** Mixed 50ml of 100% DMSO in 50ml of deionized sterile water. Sterilized the solution by autoclaving and stored at -20ºC.
7. **5M Sodium Chloride (NaCl) :** Dissolved 5.85g of Sodium Chloride in 20ml of deionized water. Sterilized the solution by autoclaving.
8. **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. Sterilized the solution by autoclaving.
9. **6X loading dye :** Mixed 0.050g of bromophenol blue, 0.050g of xylene cyanol and 8g of sucrose. Mixed it in a small amount of TE buffer, then made up the volume upto 20ml by adding more TE buffer.
10. **Ethidium Bromide (10mg/ml) :** Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.
11. **Magnesium Chloride ($MgCl_2$ 100mM) :** Dissolved 0.41g of $MgCl_2$ in 20ml of deionized water and sterilized by autoclaving.
12. **Sucrose (1M) :** Dissolved 3.41g of sucrose in 10ml of deionized water and sterilized by autoclaving.
13. **TE buffer (pH 8.0) :** Added 1ml of 100mM Tris-Cl (pH-8.0) and 200µL of 0.5M EDTA solution to 8.8ml of deionized water. Sterilized the solution by autoclaving.
14. **Triton X- 100 :** 100µL of Triton X- 100 mixed with 900µL of deionized water and mixed properl

CHAPTER 1

Introduction

INTRODUCTION

The discipline of genetic and molecular epidemiology continuously evaluates the risk of known genes and identification of unknown genes contributing to the risk of various diseases. These studies focuses on various potential risk factors caused by environment and genetic factors which can be identified at a molecular level, to the etiology, spreading and prevention of various diseases amongst individuals and families. Every individual have a peculiar association of polymorphic traits that alter genetic susceptibility and react to chemicals, carcinogens and drugs. Determination of high risk genotype has become easy by the development of molecular studies that investigates various biological markers.

Cancer is a complex disease resulting in a group of other diseases in which some of the cells become abnormal, divides without any control and permeate other tissues. These abnormalities in the cells are caused due to the genetic and environmental risk factors. These risk factors are commonly termed as carcinogens in case of cancer which manipulate the activity of normal genes thus disturbing the genetic material of the cell. Forty four chemicals agents were reported by the International Agency for Research on Cancer (IARC). It was reported that out of 44 chemical agents 9 were found in cigarette smoke. These 9 agents were chromium, arsenic, benzene, nickel, 2-naphthyl-amine, vinyl chloride, beryllium, 4-aminobiphenyl, cadmium (Smith *et al.*, 1997).

Lung cancer is known as the second most dangerous type cancer all around the world. The cases of lung cancer are increasingly rapidly. It is seen that 90% of the lung cancer cases are due to the cigarette smoke but not all smokers acquire lung cancer. There are two types of lung cancer known: non-small cell lung cancer & small cell lung cancer. Around 85% of the lung cancer patients are diagnosed with non-small cell lung cancer whereas 15% of the cases accounts for small cell lung cancer. Non small lung cancer further has three histological sub forms: adenocarcinoma, squamous cell carcinoma & large cell lung cancer. The patients acquiring lung cancer due to cigarette smoke are mainly diagnosed with small cell lung cancer and squamous cell carcinoma. On the other hand the nonsmokers acquiring lung cancer are mostly diagnosed with adenocarcinoma sub form of lung cancer. A poor prognosis is seen in lung cancer patients and the overall survival time for these patients is very less. The major contribution to the lung cancer is by tobacco smoking which is a well known carcinogen. Particularly, there is irrefutable evidence that 90% of the lung cancer is due to tobacco smoking. Nearly, amongst lifetime a smoker only one in ten develops lung cancer. The growing development of biological markers may decrease/increase predisposition to smoking related carcinogens. Various epidemiologic studies indicates that family history with lung cancer directly correlates with the increase risk of lung cancer in an individual. Moreover, germ line mutations in epidermal growth factor receptors, p53, etc., contribute to high risk of lung cancer.

Every living organism is significantly exposed to various chemicals or unwanted harmful substances present in the environment or within the body which gets absorbed into the body through skin, gastrointestinal tract or lungs. These chemicals include drugs, pesticides, toxins, secondary metabolites, alkaloids, etc. The lipophilic natures of these compounds make it difficult to excrete them out of the body. Hence, it is important to convert these xenobiotic into water soluble compounds and this phenomenon is termed as biotransformation of xenobiotic, if the process of biotransformation doesn't takes place in the body the lyophilic compounds will take a very long time to excrete out of the body eventually harming the organism. These compounds if not eliminated at right time can forms toxins resulting in serious diseases inside the body which could kill the organism. To carry out these biotransformation reactions certain enzymes are required and eliminates the xenobiotics. These enzymes carrying out the biotransformation reactions plays an important role in deciding the intensity & time required for action of a drug as well as these enzymes takes care of the chemical toxicity & tumorigenesis. Biotransformation not only takes care of the elimination of xenobiotics but it also maintains the homeostasis at the time when organism is exposed to small foreign particles like drugs. The enzymes carrying out these reactions are classified as Phase I & Phase II detoxification enzymes and also Phase III transports that helps in efflux mechanisms.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a well-known phase II detoxification gene which metabolizes xenobiotics. It prevents the formation of free radicals by reduction of quinones into hydroquione and thus protecting the cell from oxidative stress. Moreover, NQO1 plays a potential role in alteration of some environmental procarinogens present in tobacco smoke like heterocyclic amines and nitro aromatic compounds. It acts as a defensive gene for the cell and is commonly called as cell-protector gene. NQO1 along with other antioxidant genes detoxify the phase I products. It not only reduces the oxidative stress but also shows scavenging activity which makes it a unique gene. It directly degrades the superoxide's thus protecting the cell. Also, it prevents significantly important protein p53 tumor suppressor from degrading which helps to protect cell from unwanted tumors.

Polymorphisms in some genes can regulate the formation of DNA carcinogen adducts and leads to the alteration in amino acids which develops lung cancer especially in smokers. The polymorphic variant of NQO1 has an important role in lung cancer. Therefore, to identify the role of polymorphic variant in NQO1 in the susceptibility of lung cancer patients in North Indian Population we evaluated the association of SNP NQO1 $Pro^{187}Ser$ rs1800566 with the risk of development of Lung cancer in the North Indian population.

CHAPTER 2

Review of Literature

REVIEW OF LITERATURE

2.1. Lung cancer

Lung cancer being the most dreadful form of cancer has a poor prognosis. It is reported that almost half of the lung cancer patients dies within the 6 months of their diagnosis and the survival rate is less than 17.8% for 5 years. It is the leading cause of deaths both in men and women (Siegel *et al*., 2014). The death rate due to lung cancer has overreached the most common breast cancer in recent years. There are two forms of lung cancer known: Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC). Of all the lung cancer cases 85% of them accounts for small cell lung cancer whereas 15% of them for non-small cell lung cancer. NSCLC have 3 more histological sub forms: Adenocarcinoma, Squamous cell carcinoma and large cell carcinoma (Sher *et al*., 2008).

1. **Adenocarcinoma:** This is most repeatedly occurring form of lung cancer both in smokers and nonsmokers despite of an individual's gender and age (Coraud *et al*., 2012). Of all the lung cancer cases 40% are diagnosed with Adenocarcinoma. Adenocarcinoma initiates from alveolar II cells (Noguchi *et al*., 1995) and develops slowly in the lungs and can be diagnosed at an early stage before proliferating outside the lungs. It develops in the periphery of the lungs (Travis *et al*., 1995) and the reason thought for this peripheral development is filters that are added in cigarettes which blocks the large particles from reaching into the lungs. These filters provide intense inhalation resulting in the peripheral tear and lesions (Stellman *et al*., 1997).

2. **Squamous-cell carcinoma:** As the name suggests this sub form emerges from the early type of squamous cells in the airway epithelial cells of the bronchial tubes right at the center of the lungs. Out of all lung cases this is the second most common type of lung cancer and 25-30% of the patients are diagnosed with squamous cell carcinoma. Cigarette smoking being the major reason for its occurrence (Keinfield *et al*., 2008).

3. **Large cell carcinoma:** It is generally known as the undifferentiated cell carcinoma with strong correlation with cigarette smoke. It comprises of 5-10% amongst all the lung cancer cases. It generally initiates from the center of the lungs moving to the closest lymph nodes and then spreading into chest wall to the other different close and distant organs (Muscat *et al*.,1995).

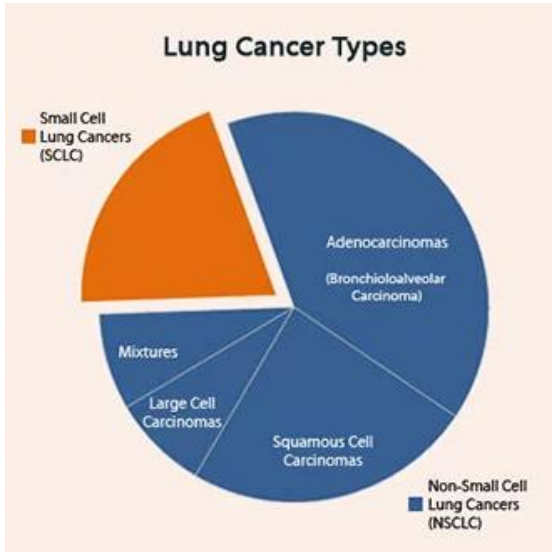


Figure 2.1: Different types of Lung Cancer

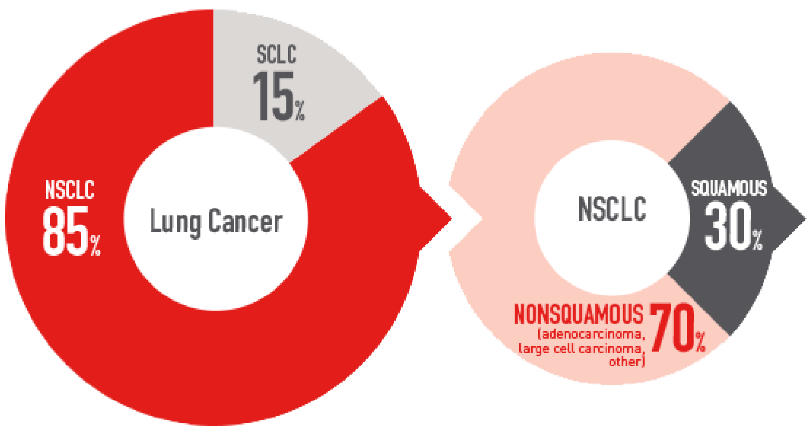


Figure 2.2: Percentage Distribution of types Lung Cancer

(Muscat et al.,1995)

2.1.1. Risk Factors associated with Lung Cancer:

1. **Cigarette Smoke:** Cigarette smoke contributes majorly towards the lung cancer leading to 90% of the cases. Since the tobacco manufacturing increased the risk for lung cancer increased simultaneously making it a common form of cancer. It constantly increased with the increased number of pack years of an individual (Hecht 1999). In a meta-analysis it was reported that the nonsmokers who lived with smokers had a risk of acquiring lung cancer from 1.14 to 5.20

(Whitrow *et al*., 2003). Moreover, it was reported by the U.S. Surgeon, there are 20-30% of chances for a nonsmoker to acquire lung cancer by living with a smoker (General surgeon report, 2014).

2. **Asbestos:** Another important risk factor for the leading cause of lung cancer is the industrial exposure of an individual. Exposure to asbestos has resulted in the increased cases of lung cancer. Mortality from lung cancer has been reported to be associated with the fiber size of asbestos (Stayner *et al*., 2008).

3. **Air pollution:** In the urban areas due to the heavy traffic, factories and industries the increased air pollution has also been reported as a risk factor for lung cancer. The continuous exposure to the harmful gases and released polycyclic aromatic compounds portrays a high risk towards lung cancer (Vineis *et al*.,2005).

4. **Genetics:** Family history with lung cancer increases the risk of lung cancer. There are various factors at molecular level that lead to increase in the risk. There are some specific genes or chromosomes, for example: a person has three fold risk of acquiring lung cancer if he is a carrier of TP53 germline sequence variations with smoking habits (Hwang *et al*.,2003).

5. **Occupational risk:** there are various carcinogenic components that an individual is exposed to while working for example: Arsenic, Uranium, Nickel (SQCC), Haematite, Chromium, etc., (Beherea *et al*., 2004).

6. **Passive smoking:** Passive smoking means the smoke encountered at work places, homes, public areas. According to a meta-analysis increased exposure to environmental tobacco shows an increased risk of 1.48 in males and 1.2 in females (Beherea *et al*., 2004).

2.1.2. Epidemiology of Lung Cancer Worldwide

According to the Globcan 2012 report there were 1.8 million new cases reported, out of which 58% cases belonged to developing countries. It was found that the disease was more prevalent in men worldwide that is about 1.2 million with the maximum incidences in central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,00) whereas in Middle and western Africa (2.0 and 1.7 per 100,00 respectively) a low incident rate was seen. The figure below shows the maximum cases in Northern

America (33.8) and in Northern Europe (23.7) and minimum cases in Western and Middle Africa (1.1 and 0.8 respectively).

Lung Cancer

Estimated Incidence, Mortality and Prevalence Worldwide in 2012

Estimated numbers (thousands)	Men			Women			Both sexes		
	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.	Cases	Deaths	5-year prev.
World	1242	1099	1267	583	491	626	1825	1590	1893
More developed regions	490	417	593	268	210	341	758	627	933
Less developed regions	751	682	674	315	281	286	1066	963	960
WHO Africa region (AFRO)	12	11	10	6	6	5	18	16	15
WHO Americas region (PAHO)	178	149	208	146	113	175	324	262	383
WHO East Mediterranean region (EMRO)	26	23	22	7	6	6	33	29	28
WHO Europe region (EURO)	323	283	343	126	105	133	449	388	476
WHO South-East Asia region (SEARO)	116	104	79	46	42	34	162	146	113
WHO Western Pacific region (WPRO)	588	528	605	251	220	273	839	748	878
IARC membership (24 countries)	514	438	582	279	219	343	794	657	925
United States of America	112	92	140	102	76	128	214	168	269
China	459	422	431	193	175	179	653	597	610
India	54	49	24	17	15	8	70	64	32
European Union (EU-28)	214	186	234	99	82	106	313	268	340

Figure 2.3: Lung cancer estimated Incidence, Mortality and prevalence Worldwide in 2012

(Source : Globocan 2012)

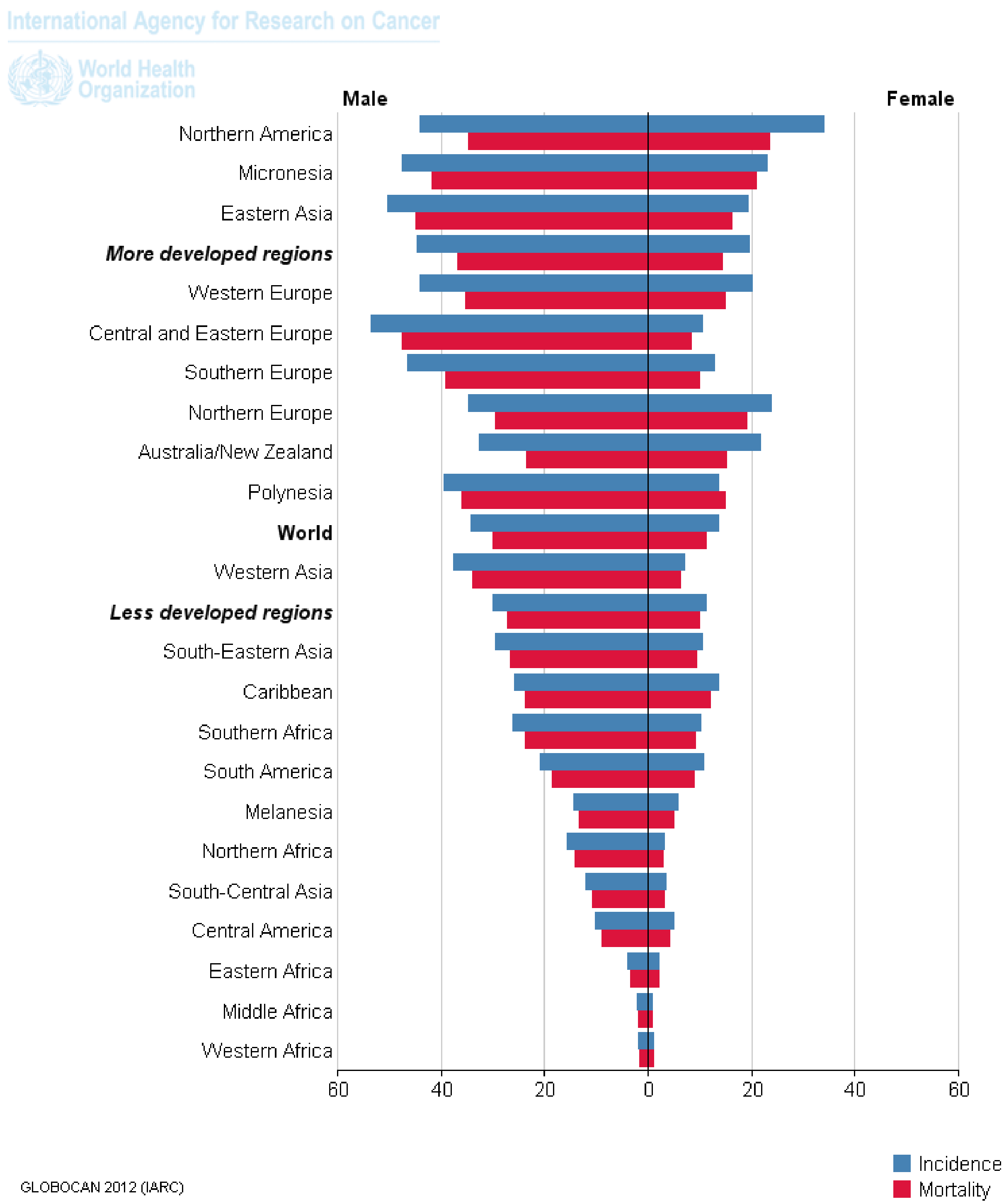


Figure 2.3: Worldwide Incidences and Mortality rate in male and females in 2012

2.1.3. Epidemiology of Lung Cancer in India

From all the new cancer cases reported 6.9% of the cases are of lung cancer with 9.3% death rate in India. Mizoram has been reported as the most prone area for lung cancer in both males (28.3 per 100,000 population) and females (28.7 per 100,000 per population) (Indian Council of Research, 2014). In recent years, a significant hike in case of both males and females has been seen in the cities Delhi, Bengaluru and Chennai. The number of cases varies from geographic region and also ethnicity plays an important role in the occurrence of the disease (Parkin *et al*., 2002).

An evidence to show the rapid hike in the lung cases was shown by cancer registry of Indian Cancer Society from Maharashtra which covers Mumbai, Pune, Nagpur and Aurangabad. In year 2011 a report was submitted covering 24,270,077 Indians. 3170 new cases were reported from these four registries. Taking these new cases and predicting to the 1.16 billion Indians, hypothetically we get 156,736 new cases in India more than double of Globocan reports (Parikh *et al*., 2016).

Lung cancer 2011	Males			Females			Total	
Maharashtra	Rank	Per 100,000 popn	Absolute No	Rank	Per 100,000 popn	Absolute No	New cases in 2011	Population in 2011
Aurangabad	3	5.9	70	7	1.9	22	92	11,79,295
Mumbai	1	10.3	1470	5	4.2	600	2070	1,42,75,780
Nagpur	2	8.8	230	6	3.9	102	332	26,14,285
Pune	3	7.8	484	5	3.1	192	676	62,00,717
Total		9.28	2254		3.78	916	3170	2,42,70,077

Figure 2.4: Incidence of lung cancer in Maharashtra in 2011 – data from Indian Cancer Society's population based cancer registries

(Source : Parikh et al., 2016)

Lung cancer comparison	Males Absolute No		Females Absolute No		Total Absolute No	
Maharashtra	2020	2011	2020	2011	2020	2011
Aurangabad	135	70	39	22	174	92
Mumbai	2176	1470	816	600	2992	2070
Nagpur	280	230	109	102	389	332
Pune	978	484	255	192	1233	676
Total	3569	2254	1219	916	4788	3170

Figure 2.5: Projected increase in incidence of lung cancer by 2020 in Maharashtra

(Source : Parikh et al., 2016)

As the data suggests a great hike in new cases by 2020, it is important that we should have potential technologies to reduce such cases in future.

2.2. BIOTRANSFORMATION OF XENOBIOTICS

Every living organism is significantly exposed to various chemicals or unwanted harmful substances present in the environment or within the body which gets absorbed into the body through skin, gastrointestinal tract or lungs. These chemicals include drugs, pesticides, toxins, secondary metabolites, alkaloids, etc. The lipophilic nature of these compounds makes it difficult to excrete them out of the body. Hence, it is important to convert these xenobiotics into water soluble compounds and this phenomenon is termed as biotransformation of xenobiotics. If the process of biotransformation doesn't takes place in the body the lipophilic compounds will take a very long time to excrete out of the body eventually harming the organism. These compounds if not eliminated at right time can form toxins resulting in serious diseases inside the body which could kill the organism. To carry out these biotransformation reactions certain enzymes are required that eliminates the xenobiotics. These enzymes carrying out the biotransformation reactions plays an important role in deciding the intensity and time required for action of a drug as well as these enzymes takes care of the chemical toxicity and tumorigenesis. Biotransformation not only takes care of the elimination of xenobiotics but it also maintains the homeostasis at the time when organism is exposed to small foreign particles like drugs. The enzymes carrying out these reactions are classified as Phase I and Phase II detoxification enzymes and also Phase III transporters that help in efflux mechanisms *(Anders, 1985)*.

Typically, phase I includes three events such as hydrolysis, reduction and oxidation. These events adds a functional group (–OH, –NH2, –SH, or –COOH) resulting in slight increase in the hydrophilicity of xenobiotics. The addition of functional groups during phase I reactions usually sites for phase II reactions. On the other hand, phase II reactions cover glucuronidation, sulfonation, acetylation, methylation, conjugation with glutathione, and conjugation with amino acids. Cofactors of phase II reactions will react with functional groups that were added in the xenobiotics in phase I reactions. This reaction between the two will result in high increase in the hydrophilicity of the xenobiotics. As a result, the foreign particles or xenobiotics are significantly removed or excreted out from the body thus maintaining the homeostasis (*Parkinson, chapter 6)*.

REACTION	ENZYME	LOCALIZATION
	Phase I	
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Reductive dehalogenation	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes
	Phase II	
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Cytosol, microsomes
	Amino acid conjugation	Mitochondria, microsomes
	Acylation	Mitochondria, cytosol
	Methylation	Cytosol, microsomes, blood

Figure 2.6: General Pathways of Xenobiotic Biotransformation and Their Major Subcellular Location

(Source : Parkinson et al, 2015)

1. **Phase I detoxification:** The various toxins or the xenobiotics present are broken down by a family of enzymes called cytochrome P450. The process takes places in the main detoxification organ called liver. Cytochrome P450 group of enzymes have Iron and Oxygen which breaks the xenobiotic into smaller metabolites thus making it polar so that it can be easily eliminated out of the body. Phase I liver detox genes includes :
    - CYP1A1 – metabolizes estrogen, polyaromatic hydrocarbons, etc.
    - CYP1A2 – metabolizes caffeine, Cymbalta, Welbutrin, etc.
    - CYP2A6 – metabolizes nicotine, coumarin, etc.
    - CYP2C9 – metabolizes warfarin, Crestor, celecoxib, etc.
    - CYP2C19 – metabolizes Plavix, some proton pump inhibitors, etc.
    - CYP2D6 – metabolizes some antidepressants, antipsychotics, etc.
    - CYP3A – metabolizes half of all prescription drugs.
    - CYP2E1 – metabolizes fatty acids, alcohol, and some aesthetics.

2. **Phase II detoxification:** This step involves the uptake of the Phase I metabolites and converting them from slight water soluble to completely water soluble metabolites resulting in an easy elimination of the xenobiotics. Phase II liver detoxification genes include :
    - Nrf2 Signaling Pathway
    - UGT genes
    - GST genes
    - NAT genes
    - NQO1

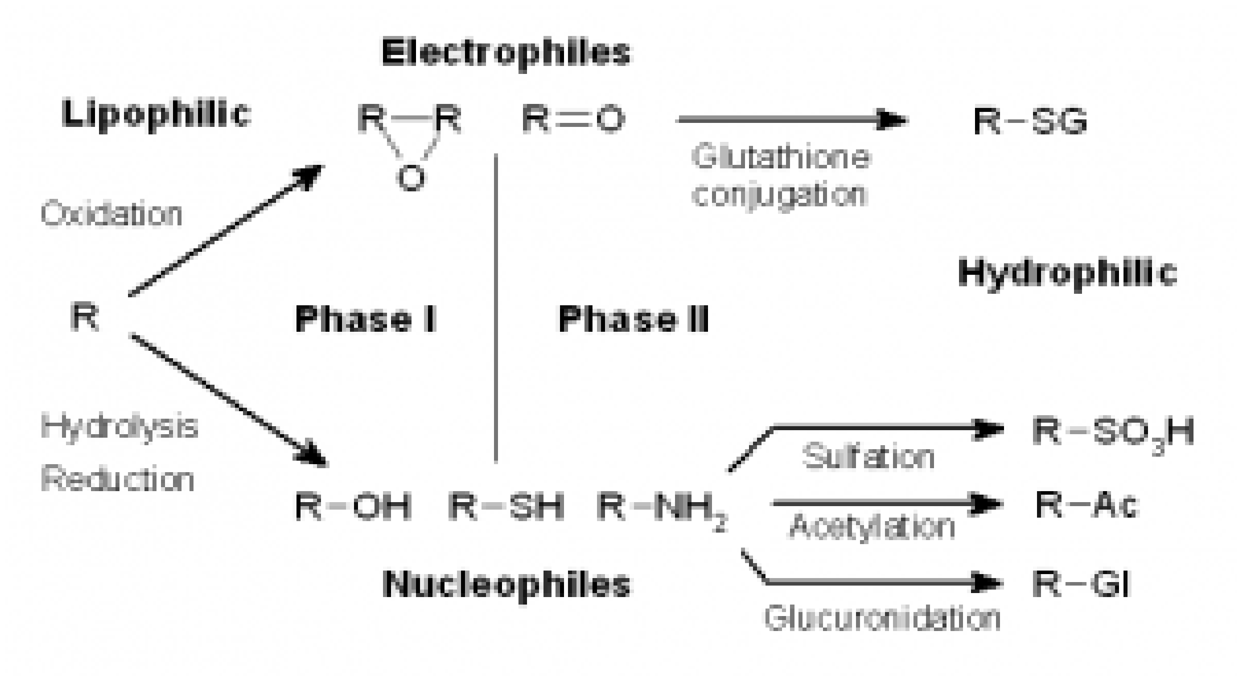


Figure 2.7: Metabolism of Phase I and Phase II detoxification

(Source : Parkinson et al., 2016)

2.3. NQO1 : INTRODUCTION & STRUCTURE

NAD(P)H:quinone oxidoreductase 1 (NQO1) formally known as DT diaphoresis is a PHASE II detoxification gene which plays a crucial role in the detoxification of quinones, protects cell from oxidative stress and inhibits the activity of carcinogens in the body. DT diaphorase was discovered by Ernster and Navazioare in late 1950s in a rat. It is now a commonly known flavoprotein which reduces quinones, azodyes, quinone imines by 2-electron reductase, an enzyme that prevents the oxidative damage of the cells and bio activates some antitumor quinones. Some endogenous quinones such as the vitamin E, α-tocopherol quinone, menadione are present within the body as electron-carrying coenzymes , flavonoids and metabolic end-products of oxidation and some exogenous like urushiol, the active chemical in poison ivy are enviornmental quinones. NQO1 catalysis 2 or 4 electron reduction and reduces these quinone into less toxic hydroquinones which generates free radicals and prevents the cell from oxidative stress (Nebert *et al*., 2002,). NQO1 is a 20kb gene where 1850 base pairs (bp) of the 5’ flanking region, and 67 bp of the 3’ flanking region have been sequenced. It is located at 16q22.1 on chromosome and encompasses 6 exons interfered by 5 introns.

Figure 2.8: Exons of NQO1

(Hongping et al., 2012)

2.3.1. *NQO1* Gene Family

Gene family of *NQO* belongs to flavoprotein clan and is present in the form of *NQO1* and *NQO2* genes in human genome. It was found that both *NQO1* and *NQO2* belong to the flavodoxin-2 family as they have a flavodoxin-like fold domain. This flavodoxin – 2 family belongs to flavoprotein clan. Two or more Pfam (similar domain structure) families arising from a single evolutionary origin is known as a clan (Mulder *et al*.,2005). Flavoprotein clan is known to have four Pfam members that are shown in the table below:

Table 2.1: Relationship between NQO gene family and flavoprotein clan

Flavodoxin-1 family	**Flavodoxin-2 family**	**Flavodoxin Ndrl family**	**FMN red family**
Nitric oxide synthases	Archaebacterial *NQO5* genes	Ribonucleotide reductases	Flavin reductases
Flavodoxins	Bacterial *NQO3* genes		
	Vertebrate *NQO1*, *NQO2* genes		
	Fungal *NQO4* genes		
	Bacterial acyl carrier protein phosphodiesterase		

(Vasilis et al .,2006)

2.3.2. Regulation of NQO1 by the Keap1/Nrf2/ARE pathway

Regulation of NQO1 is caused by a broad set of chemical inducers, $H_2O_{2,\ oxidative}$ stress, heavy metals and polycyclic aromatic hydrocarbons (cigarette smoke). Regulation of NQO1 is intervened *via* Keap1/Nrf2/ARE pathway (Krajka, *et al*., 2017; Jeddi *et al*., 2017). It is a two step cellular process:

1. When a cell is under normal conditions, a cluster of proteins keeps Nrf2 (nuclear factor erythroid 2-related factor 2) in the cytoplasm. Nrf2 is a transcription factor of the "cap-'n collar" family. Nrf2 is degraded by the proteins KEAP1 (Kelch like-ECH-associated protein 1) and Cul3 (Cullin 3) through ubiquitination. Nrf2 is ubiquitinated by Cul3 protein and the reaction is carried by the substrate Keap1. As soon as Nrf2 is ubiquitinated it moves to proteasome followed by degradation and recycling of its elements.
2. But when a cell is under stress conditions, cysteine residues in Keap1 are interrupted which further interrupts Keap1-cul 3 ubiquitination system. In this condition Nrf2 is not ubiquitinated and moves into the nucleus where it interacts with Maf proteins and forms a heterodimer. This complex of Nrf2 and Maf proteins binds to the antioxidant response element (ARE) in the promoter region of NQO1 (antioxidant gene) from where transcription and protein expression is initiated. Like, NQO1 this pathway is also responsible for the regulation of many other antioxidant genes.

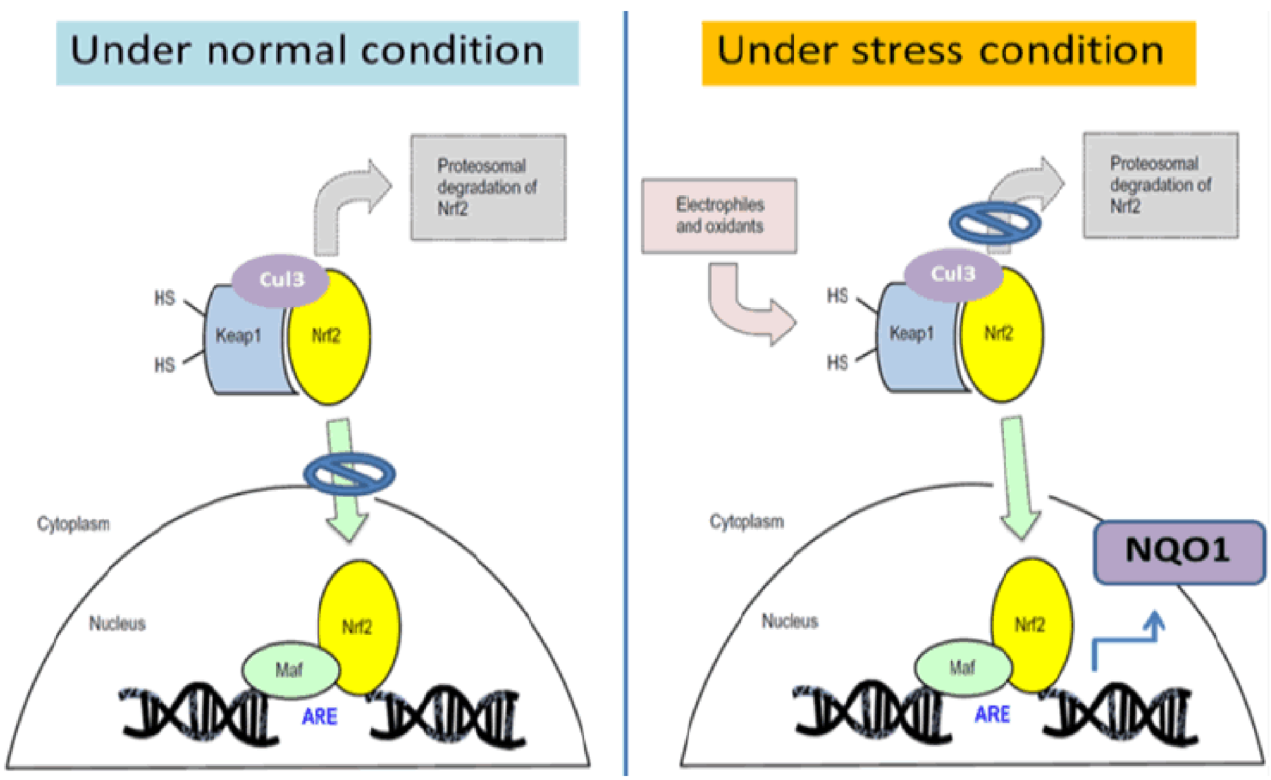


Figure 2.9: Regulation of NQO1 by Keap1/Nrf2/ARE pathway

(Gao et al., 2014)

2.3.3. Functions of NQO1:

NQO1 is known to function as a cytoprotective gene by reducing the oxidative stress caused by various unwanted quinones and its harmful derivatives.

2.3.3.1. Quinones Detoxification:

Depending upon the structure of quinones, they are electrophile species and have a potential to react with cellular nucleophiles *(*Ernester.,*1967).* This removes reactive electrophile by a two electron mediated reduction reaction from a biological system. Moreover, two-electron reduction omits the formation of semiquinones which has capability to generate vigorous oxygen and nitrogen species by interacting with molecular oxygen that leads to cellular damage. It was recently reported that hydroquinones have an ability to generate toxicity with various redox stabilities. Depending upon the stability of hydroquinones it was observed in a study of the effect of quinones on cellular protein handling systems and toxicity that NQO1 has an opposing effect. (Xiong *et al*., 2014). Figure 2.10 depicts that reduction by NQO1 will represent bioactivation step if hydroquinones shows redox activity (e.g., β-lapachone) or has an ability of rearrangement to a reactive alkylating species (e.g., mitomycin C) and not a detoxification step. This is known as bio reductive activation (Kennedy *et al*., 1980).

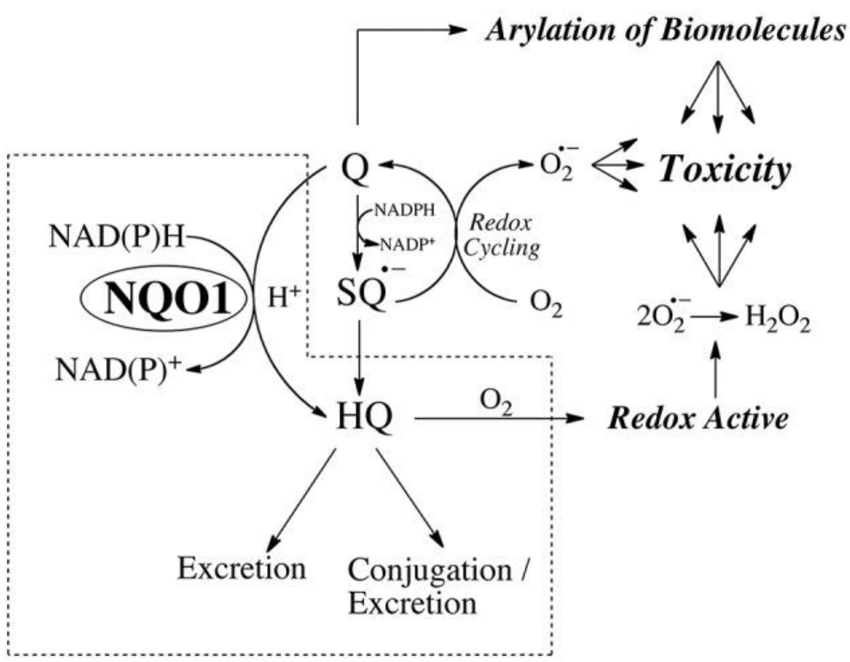


Figure 2.10: The properties of hydroquinone determines whether reduction by NQO1 leads to detoxification or toxicity.

(David et al., 2017)

2.3.3.2. NQO1 as superoxide reductase:

NQO1 being a flavoprotein have a flavin co-factor which have a direct role in scavenging of superoxide with the help of superoxide reductase enzyme (Siegel *et al.*, 2004). Experiments have shown that with direct use of EPR spectroscopy and wide range of superoxide generating systems NQO1 has a catalytic role as a superoxide reductase (Zhu *et al*., 2007). A normal cell exhibits various mechanisms to stimulate the levels of superoxide and also possess a significant enzyme system called superoxide dismutase family (SOD) that eliminates superoxide from cell *(McCord and Fridovich, 1969).* Since the rate of reaction of SOD is more than that of NQO1 there arises a question whether the direct scavenging activity of NQO1 in cellular system is relevant or not. But it has also been seen that under the basal conditions NQO1 is highly expressed in different cells. The increased levels of NQO1 (30 folds in human cells) by oxidative stress was observed by using UV radiation and X-ray (Boothman *et al*., 1993). The question of direct scavenging activity of NQO1 was solved by the experiment using an NQO1 transfected series of Chinese hamster ovary cells. An increased scavenging of superoxide was seen with increased levels of NQO1 in Chinese hamster ovary cells. This study concluded that direct scavenging activity of NQO1 has relevance in cellular system (Siegel *et al*., 2004).

2.3.3.3. Tumor suppressor p53 protein stabilizer:

p53 plays a vital role by preventing the genome from unwanted stresses. It protects the cell from tumor formation and unwanted transformation of the cell (Shu *et al.*, 2007). 20s proteasome are present inside the cell which function to degrade short lived proteins like p53. On the other hand NQO1 is physically linked with 20s proteasomes. NQO1 binds to 20s proteasome and inhibits the degradation of the short lived proteins by acting as a gatekeeper of the 20s proteasomes. By acting as a gatekeeper NQO1 stabilizes p53 protein and the conditions like apoptosis or cell cycle arrest do not take place (Gong *et al.*, 2007).

2.3.3.4. NQO1 studies with β-lapachone

There are numerous studies indicating that the regulation of $NAD(P)^+$/NAD(P)H redox balance by NQO1-mediated metabolism of β-lapachone have some therapeutic possibilities along with the capability to generate NAD^+ and stimulate PARP activities and sirtuin (figure 2.11). It has been seen that on treating with β-lapachone a number of physiological processes are regulated specifically associated with metabolic syndrome like reduction in sudden hypertension (Kim *et al*., 2011, 2013, 2015; Kim Y. H. *et al*., 2014), decrease in inflammation (Byun *et al*., 2013; Lee et al., 2015) and improvement in obesity (Hwang *et al*., 2009). Treatment with β-lapachone is shown to reduce hearing loss (Kim H. J. *et* *al*., 2014), decrease cisplatin-mediated nephrotoxicity (Gang *et al*., 2013; Oh *et al*., 2014), restenosis by suppressing vascular smooth muscle cell proliferation (Kim *et al*., 2009) and also to inhibit health drops in aged mice (Lee *et al*., 2012). The basis of all these studies was to activate AMP-activated protein kinase (AMPK) pathway by β-lapachone. In addition, these studies also shows how β-lapachone predominates the regulation of various pathways including NAD(P)H oxidase (NOX), sirtuins and poly ADP ribose polymerase (PARP). β-lapachone is significantly reduced to the hydroquinone by NQO1 which autoxidizes back to the quinone with consumption of NADH and NADPH and generation of NAD^+ and $NADP^+$. NQO1 plays an important role in the regulation of NAD^+/NADH redox balance especially by the metabolism of β-lapachone (Pink *et al*., 2000).

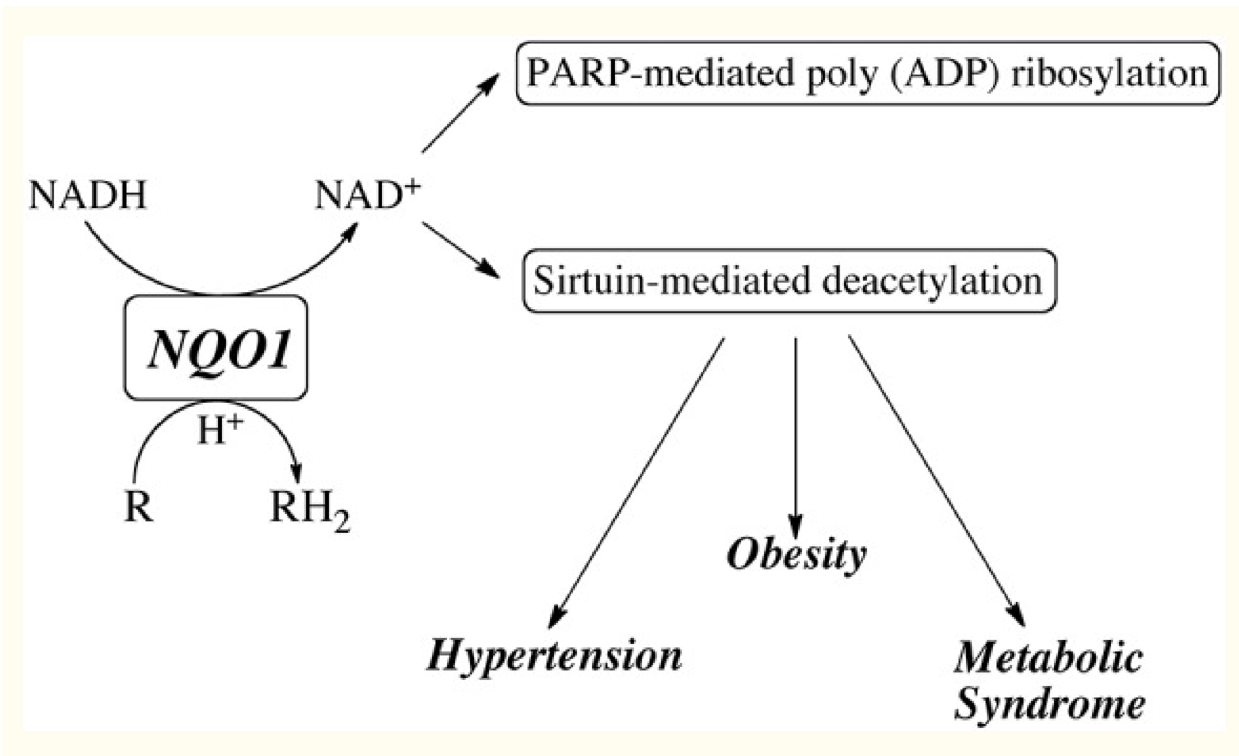


Figure 2.11: Generation of NAD^+ by NQO1

(David et al., 2017)

2.3.3.5. Protein binding as a "Non-Metabolic" function of NQO1

Various examples are seen where NQO1 is found to be able to connect with different other proteins undergoing degradation with suitable cellular functions. Protein-protein interaction between a tumor suppressor p53 protein and NQO1 stabilizes p53 by acting as a gate keeper that protects it from ubiquitin-independent 20S proteasomal degradation (Asher *et al*., 2001, 2002). Not only p53 but other proteins such as p63, p73, PGC-1 α and ornithine decarboxylase are also stabilized by NQO1 (Asher *et al*., 2005). 20S proteasome degrades many NQO1 interacting proteins having intrinsically disarranged regions (Asher and Shaul, 2006; Moscovitz *et al*., 2012). It has also been reported that NQO1 also suggest a modulatory role in the direct interation with 20S proteosome. Asher and shaul in 2006 found the relation between NQO1 with 20S proteasome in mouse liver by cofractionation techniques and co-immunoprecipitation (Asher and Shaul, 2006). Then again in 2007 Sollner reported Yap4 (transcription factor) stabilization in Yeast by the binding of Lot6 (Human ortholog of NQO1) with 20S proteasome which activated Yap4 (Sollner *et al*., 2009). In 2012, interations between purified NQO and purifies 20S proteasome in a cell free system were found by using mass spectrometry (Moscovitz *et al*., 2012).

2.3.3.6. Regulation of Humoral and Autoimmunity by NQO1

Karim *et al.*, in his report suggested the in vivo role of NQO1 in humoral and autoimmunity. They worked on NQO1 – null nice and after performing various tests and studies they found out decrease of B cells in peripheral blood, impaired antibody responses, elevated response to autoimmune disease, a decrease expression and inactivation of NK- κB cells. Hence, it was concluded that NQO1 plays a vital role in the modulation of humoral and auto immune responses. A decline in the activity of NQO1 will lead to defective immune response and more response to autoimmune diseases (Karim *et al*., 2006).

2.3.4. NQO1 Polymorphisms

In NQO1 gene over 22 Single Nucleotide Polymorphisms (SNPs) are reported. The wild type allele *NQO1*1* codes for a normal enzyme and function. Whereas, *NQO1*2* allele is responsible for P187S mutation which results in loss of NQO1 activity (Nebert *et al*., 2002). In the reported work of NQO1 cDNA in 10 human colon carcinoma cell lines a nucleotide change C609T was discovered which resulted in P187S mutation. This mutation led to the decline NQO1 activity (Traver *et al*., 1992).

Table 2.2: Polymorphic forms of NQO1

Patient	Allele	NQO1 Enzyme activity
Normal	*NQO1*1/*1*	Normal enzyme activity
Hetezygotes	*NQO1*1/*2*	Intermediate between normal and mutant
Homozygotes	*NQO1*2*	Negligible activity

(Nebert et al., 2002)

Literature reflects that P187S is the most frequent and prominent type of amino acid change. It is a substitution at nucleotide position 609 of cDNA from C to T. This manipulation of the nucleotide in an amino acid change from proline to serine at position 187. This leads to some loss of enzyme activity because of the instability in the resulted protein. P187S alteration is termed as NQO1*2. The heterozygotes (NQO1*1/*2 or C/T) have an intermediate activity between the wild type (NQO1*1/*1 or C/C) and homozygous SNP. The homozygous variant genotype is represented by NQO1*2/*2 or T/T and this mutation results in the complete loss of the enzyme activity. Since the enzyme completely loses its ability to function the Homozygous (T/T) has been reported to show elevated risk of many types of cancers like colon cancer, lung cancer, etc., high chances of benzene poisoning (Kolesar *et al*., 2011; Yu H *et al.*, 2012; Zhou *et al*., 2012).

Another SNP found in NQO1 is Arg139Trp . In this case, there is a nucleotide alteration from arginine to tryptophan at amino acid position number 139. It is denoted by NQO1*3 allele. This SNP is known to be associated with acute leukemia risk but on a very small scale *(Hairong et al., 2017).*

The frequencies of NQO1 allele varies in different ethnic population. A study has been shown below in the table.

Table 2.3: Frequency of NQO1 genotypes in population studies

Ethnic groups	**NQO1*1/1**	**NQO1*1/*2**	**NQO1*2/*2**	**N**	**Frequency of NQO1*2 allele**
Non-Hispanic White	64 (56.1%)	45 (39.5%)	5 (4.4%)	114	0.22
Mexican – American	52 (32.3%)	84 (52.2%)	25(15.5%)	161	0.369
African-American	83 (61.0%)	46 (33.8%)	7 (5.2%)	136	0.23
Asian^{n}	37 (31.4%)	57 (48.3%)	24 (20.3%)	118	0.45

2.3.5. Association of NQO1 with Cancer

Being an efficient cytoprotective gene NQO1 is found in all the cell types. It has been seen that the low level of enzyme activity makes the system more susceptible to cancers. But it has also been seen that not only the low levels of enzyme activity but also increased levels or overexpression of NQO1 makes it sensitive to increased risk of cancers (*Jaiswal 2000*). The table below shows the rate of overexpression of NQO1 and evidences linked to different cancers.

Table 2.3: NQO1 protein expression rates in different forms of cancer

Cancers	**Number of cancer patients**	**NQO1 protein expression rates (%)**	**Refrences**
Breast cancer	176	84.70	Yang et al., 2014
Serous ovarian cancer	160	85.60	Cui et al., 2015
Small cell lung cancer	115	70.43	Cui et al., 2014
Squamous cell carcinoma of the uterine cervix	177	80.23	Ma y et al.,2014
Pancreatic ductal adenocarcinoma	126	83.30	Ji M et al., 2017
Gastric adenocarcinoma	203	75.86	Lin L et al., 2014

The reported work concluded in the above table concludes that high level of NQO1 is linked to decline in survival of cancer patients and poor prognosis. Observation of overall survival time of cholangiocarcinoma patients suggested a potential use of NQO1 as a tumor marker (Buranrat *et al.*, 2010). Literature reflects that up regulation of NQO1 has a major role in cancer development and significantly reduces the survival time of the patient affected by cancer. But possibilities to use NQO1 as a biomarker have also been seen. Moreover, it can also be used as a therapeutic target for cancer treatment and drugs.

2.3.6. Gaps in study

As the cases of Lung Cancer are increasing rapidly and NQO1 being an important phase II detoxification gene, needs to be studied to find out its association with clinical outcomes of lung cancer patients undergoing platinum based chemotherapy. To our knowledge this study have not been carried out in North Indian population regarding the association of *rs 1800566* polymorphism in NQO1 gene. Therefore, identification of polymorphism of detoxification gene in understanding its correlation to survival in lung cancer patients and susceptibility to lung cancer would yield insights how the gene polymorphism adversely or favourably influence the chemotherapy outcomes in the north Indian population.

CHAPTER 3

Aims and Objectives

AIMS AND OBJECTIVES

- ❖ To investigate the effect of NQO1 gene polymorphism on protein structure and function, and whether these polymorphism contribute in disease or not.

- ❖ To predict the clinical outcomes of Advanced Lung Cancer Patients having polymorphism *rs 1800566* in NQO1 gene treated with Platinum Based Doublet Chemotherapy.

CHAPTER 4

Materials and Protocols

MATERIALS AND PROTOCOLS

197 cases of Lung Cancer were enrolled for the present study from the department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. Institute Ethics Committee of PGIMER has analyzed and approved the study. From all the patients or their representatives informed written consent was taken. In other words, the newly diagnosed patients with Lung Cancer were enrolled for present study. On the basis of histopathology all the desired patients were diagnosed with SCLC and NSCLC. For this study patients with some cancer background were eliminated while the gender, histology, age, smoking and TNM stage were considered as the important parameters. In addition 131 individuals with zero signs of any form of cancer normally visiting to the hospital for checkups were considered as the control group. Sex, smoking, age were taken as parameters for control group to ensure that there is no possible risk for Lung Cancer among control population. A trained interviewer filled a complete set of questions including demographic and smoking information for each case and control. Smoking of cigarette and beedi were reported from the smokers. From smoking reports pack years were calculated using [(cigarettes or beedis per day/ 20) * years smoked]. Other medical data of Cancer cases like clinical staging, histology, primary tumor size, metastasis and lymph node involvement were collected from the medical records of the hospital. From each individual in the study population approximately 3-5 ml of venous blood was taken.

4.1. Isolation of DNA From Peripheral Blood

Isolation of genomic DNA was done using Protein K digestion, phenol/chloroform extraction and ethanol precipitation method from whole blood sample of controls & cases. By using equal volume of washing buffer (320mM sucrose; 1.5% Triton X-100; 20mM TrisHCl, pH-8.0; 5mM $MgCl_2$) blood was washed to thoroughly separate out red blood cells. The WBC pellet obtained was lysed using lysis buffer (400mM Tris-HCl, pH-8.0; 200mM NaCl; 80mM EDTA; 1% SDS; 100 µg/ml proteinase Phenol:Chloroform:Isoamyl alcohol (25:24:1) solution K). The resultant then obtained was deproteinized using Phenol:Chloroform:Isoamyl alcohol (25:24:1) solution. Chloroform:Isoamyl alcohol (24:1) solution was then added to remove polysaccharides and remaining protein followed by the addition of equal volume isoamyl alcohol for the precipitation of genomic DNA. In the end, the resulted DNA was dissolved in 200µL of TrisEDTA buffer.

4.2. Preparation of Buffers & Reagents

Washing Buffer

Table 4.1: Stock and Working concentration of Washing Buffer

Stock concentration	Working concentration
1M Sucrose	320 mM Sucrose
100% Triton X-100	1% Triton X-100
100m M Magnesium Chloride	5mM Magnesium Chloride
100m M Tris-HCl (p H=8.0)	10mM Tris-HCl pH (8.0)

Lysis Buffer

Table 4.2: Stock and Working concentration of Lysis Buffer

Stock concentration	Working concentration
1M Tris-Cl buffer (pH 8)	400 mM Tris-Cl buffer (pH 8)
10% SDS	1% SDS
0.5M EDTA (pH 8)	60 mM EDTA (pH 8)
5M NaCl	150 mM NaCl

5X TBE buffer

5X TBE buffer was prepared by dissolving 54g of Tris base, 27.5g of boric acid and 20 ml 0.5M EDTA. Volume was made up to 1000 ml by distilled water.

6X loading dye

6X loading dye was prepared by dissolving 0.25% Bromophenol blue (0.05 g), 0.25% Xylene cyanol (0.05 g), 40% sucrose (8 g). Make up the final volume of 20 ml with TE buffer.

4.3. DNA Quantification

DNA quantification was done to check the quality of DNA with Thermo Scientific Nano drop Spectrophotometer. 1 µl of sample is needed to check the quality without any exposure to cuvettes as hence is considered as a better method than Visible Spectrophotometer. Path length is determined vertical orientation by placing the samples between two optical surfaces. This methods allows the path length to change in real time for a sample. This significantly removes the need of make dilutions and is more convenient method.

Procedure:

- Cleaned the Nano drop using 1µl of Deionized water.
- Opened the Nano drop software and select Nucleic acid module.
- Loaded 1µl of TE buffer to set blank measurement.
- Loaded 1µl of DNA sample and selected 'measure' to take the reading.
- Concentration and purity of sample were calculated automatically.

DNA concentration can also be calculated as follows:

DNA concentration (µg/mL) = O.D. at 260 nm X 50 X Dilution factor (Standard DNA sample O.D. = 1 at 50 µg/mL concentration) Quality/purity of DNA = $\boldsymbol{O.D.\ at\ 260\ nm/O.D.\ at\ 280nm}$ NOTE: A ratio of ~1.8 indicates purity of DNA; a ratio of ~2.0 is generally accepted as pure for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants.

4.4. Genotyping by Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP)

4.4.1. Amplification of NQO1 by PCR

The polymerase chain reaction (PCR) is an enzymatic process commonly used for detecting desirable genes within a DNA sample. Oligonucleotide primers are short DNA fragments used for the process of amplification. The sequences of these oligonucleotides are complementary to the target regions of the specific gene. By this method, single DNA molecule can be amplified and single-copy genes can be extracted out of complex mixtures of genomic sequences hence making it a sensitive assay. The SNP C609T of NQO1 gene was amplified by PCR method.

The table below shows a detailed information Primers used in PCR amplification of NQO1 SNPs, Amplified product size & Restriction Enzyme (Table: 4.3) and reagents used in PCR (Table: 4.4)

Requirements

- 10X PCR Buffer
- BSA
- Forward Primer
- Reverse Primer
- dNTPs
- Tag DNA polymerase
- Water
- DNA sample

Table 4.3: Primers used in PCR amplification of NQO1 SNPs, Amplified product size & Restriction Enzyme

Gene	**Amino Acid substitution**	**Primer used**	**PCR product size & Restriction enzyme**
NQO1 rs 1800566	C609T	Forward primer : TCCTCAGAGTGGCATTCT Reverse Primer: TCTCCTTCATCCTGTACCTCT	230 bp & Hinf1

Table 4.4: Reaction Mixture for PCR carried out for NQO1 rs 1800566

Reagent	**Stock Concentration**	**Final Reaction Concentration**	**Quantity Used**
Additive I BSA	1000 µg/ml	100 µg/ml	33 µl
PCR Buffer (Mg Conc.)	10 X 25mM	1 X 1.5 mM total	33 µl
NQO1 rs 1800566 (Forward primer)	10 µM	0.5 µM	16.50 µl
NQO1 rs 1800566	10 µM	0.5 µM	16.50 µl

(Reverse primer)			
Taq Polymerase	2.0 U/ µl	1.5 mM	3.52 µl
dNTP	10mM each	0.2 mM each	6.60 µl
PCR Grade Water	-	-	130.48 µl
DNA Template	100 ng/10 µl	200ng	2 µl

Table 4.5: Cycling Profile of PCR for NQO1 rs 1800566

Steps	Temperature	Time
Initial Denaturation	95 ^oC	5 min
Denaturation	94 ^oC	30 seconds
Annealing	55 ^oC	45 seconds
Polymerization	72 ^oC	30 seconds
Final Extension	72 ^oC	5 min

Note : The above reaction was carried out for 30 cycles

4.4.2. Restriction Digestion of NQO1 SNPs

PCR products obtained were used to cleave the DNA amplicons by the restriction digestion technique. 10µL of the PCR product was taken to which 0.2 units of restriction enzyme was added for the digestion of the PCR product. The reaction was incubated for 16 hours at 37ºC. After the incubation the restriction pattern was noted by Polyacrylamide gel electrophoresis method. The enzyme used was:

Hinf1: *The* source microorganism from which this enzyme has been isolated is *Haemophilus influenzae. This enzyme was used for the digestion of amplicons obtained from the amplification of NQO1 rs 1800566. The enzyme was used along with NEB4 Buffer (10X) which helps in maintaining the homeostatic conditions with respect to ions within the mixture.*

5’...G↓ANTC...3’
3’...CTNA↓G...5’

4.4.3. Native-Polyacrylamide gel electrophoresis (N-PAGE)

PAGE gel is widely used method to determine the resolution of DNA molecule by 1bp. PAGE gel is a mixture of acrylamide and bis-acrylamide and is considered as a highly sensitive method. The monomeric acrylamide molecules are linked together which are cross linked by the bis-acrylamide molecules. These two are mixed in a fixed ratio so that pore size of the gel can be determined. Further polymerization of N-PAGE is done by adding APS (ammonium per sulphate) followed by TEMED (N, N, N, N-tetramethylethylenediamine). The basis of N-PAGE is the amount of acrylamide present in the gel and the pore size varies according to the amount of acrylamide & bisacrylamide added to the gel. For C609T polymorphic variant 10% gel was used for resolution of DNA fragments. After running the gel vertically, the gel was stained by ethidium bromide to visualize the bands and to study the cutting pattern. The table 4.6 below shows the composition of N-PAGE gel.

Table 4.6: Composition for N-PAGE gel for NQO1 rs 1800566

Gel %	**30% Acrylamide**	**Distilled water**	**5X TBE**	**10% APS**	**TEMED**
10% (*NQO1 rs 1800566)*	4.0 mL	5.6mL	2.4mL	200µL	10 µL

4.5. Computational analysis of NQO1 C609T polymorphic variant. (Abdelraheem *et al*., 2016).

The computational analysis was done using various bioinformatics tools that are described below. These tools determine the effect of C609T polymorphic variant on the structure & function of proteins. Also, it determines the contribution of the SNP in causing any kind of disease or not. The software used for this analysis are:

- **SIFT (Separating Intolerant from Tolerant) -** Prediction of structural Impact of nsSNP on protein. **(http://sift.jcvi.org).**
- **PROVEAN -** Prediction of functional effect of amino acid substitution. **(http://provean.jcvi.org).**
- **POLYPHEN2 (Polymorphism Phenotyping v2) -** Prediction of deleterious SNP. **(http://genetics.bwh.harvard.edu/pph2/).**
- **I-MUTANT** - Analysis of nsSNP impact on protein stability. **(http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/IMutant3.0.cgi).**

- **MuPro -** Prediction of protein stability on the basis of energy change. **(http://www.ics.uci.edu/~baldig/mutation.html).**
- **Phd-SNP-** Predict whether the mutation is disease related or neutral. **(http://snps.biofold.org/phd-snp/phd-snp.html).**
- **SNP & GO -** Predicts the mutation related to disease or not. **(http://snps-and-go.biocomp.unibo.it/snps-and-go/).**
- **ELASPIC-** Effects of mutations on protein folding and protein-protein interactions. **(http://elaspic.kimlab.org/many/).**
- **PANTHER -** Predicting the deleterious SNP. **(http://pantherdb.org/tools/csnpScoreForm.jsp).**
- **MutPred -** Prediction of harmful mutation. **(http://mutdb.org/mutpred)**
- **Mutation 3D-** To study the spatial arrangement of amino acid substitution on protein models and structures. **(http://mutation3d.org/index.shtml)**
- **Genemania -** Investigation of Interaction gene with other genes. **(http://www.genemania.org/).**

4.6. Statistical Analysis

Genotype frequency of NQO1 gene variant was calculated for controls and cases by using Hardy Weinberg Equilibrium theory (p2+q2+2pq=1; where p is the frequency of wild type allele, q is the frequency of variant allele). The homozygous wild type CC *rs1800566* variant was used as reference. Association between different parameters like smoking, pack years, histology, TNM staging with the risk of lung cancer was checked by using logistic regression method. Adjusted Odd's ration (OR) with 95% Confidence Intervals (CI) and significant values ($p<0.05$) were calculated by using logistic regression method. Also, overall survival was estimated by using unadjusted univariate method (Kaplan Meier) and multivariate analysis was done by Cox proportional hazard model. All the statistical analysis was performed with Medcalc version 14.8.1 (Medcalc Software, Ostend, Belgium).

CHAPTER 5

Results

RESULTS

5.1. Genotyping

Isolation of DNA was done from the peripheral blood. The obtained DNA from peripheral blood was conserved as a stock of the individual's DNA and some part of the DNA was diluted to a concentration of 100ng/µl. This diluted DNA was further used as in template for the amplification reactions in different volumes by PCR method. Designed primers correlating to the segment of the gene to be amplified were used to obtain the desired segment of the gene by the amplification method. The temperature for PCR was optimized at temperature 55^0 C. The desired segments of chromosomal DNA and were run on 0.8% agarose gel.

Figure 5.1 : Representative example of the PCR product of NQO1 gene with amplicon size 230 bp Lane 1 : 100 bp Ladder ; Lane 2 : 2,3,4,5 : PCR products of amplification reaction.

After obtaining these PCR products, they were digested with their correlated enzyme which has an ability to cleaving the PCR products at a specific region being related to a single nucleotide sequence and enzyme. After 16 hours of incubation the digestion reactions were examined by Native PAGE method. Samples were run at 10% polyacrylamide gel for NQO1. The gel was then stained in ethidium bromide and after staining the bands were visualized clearly on the gel under UV.

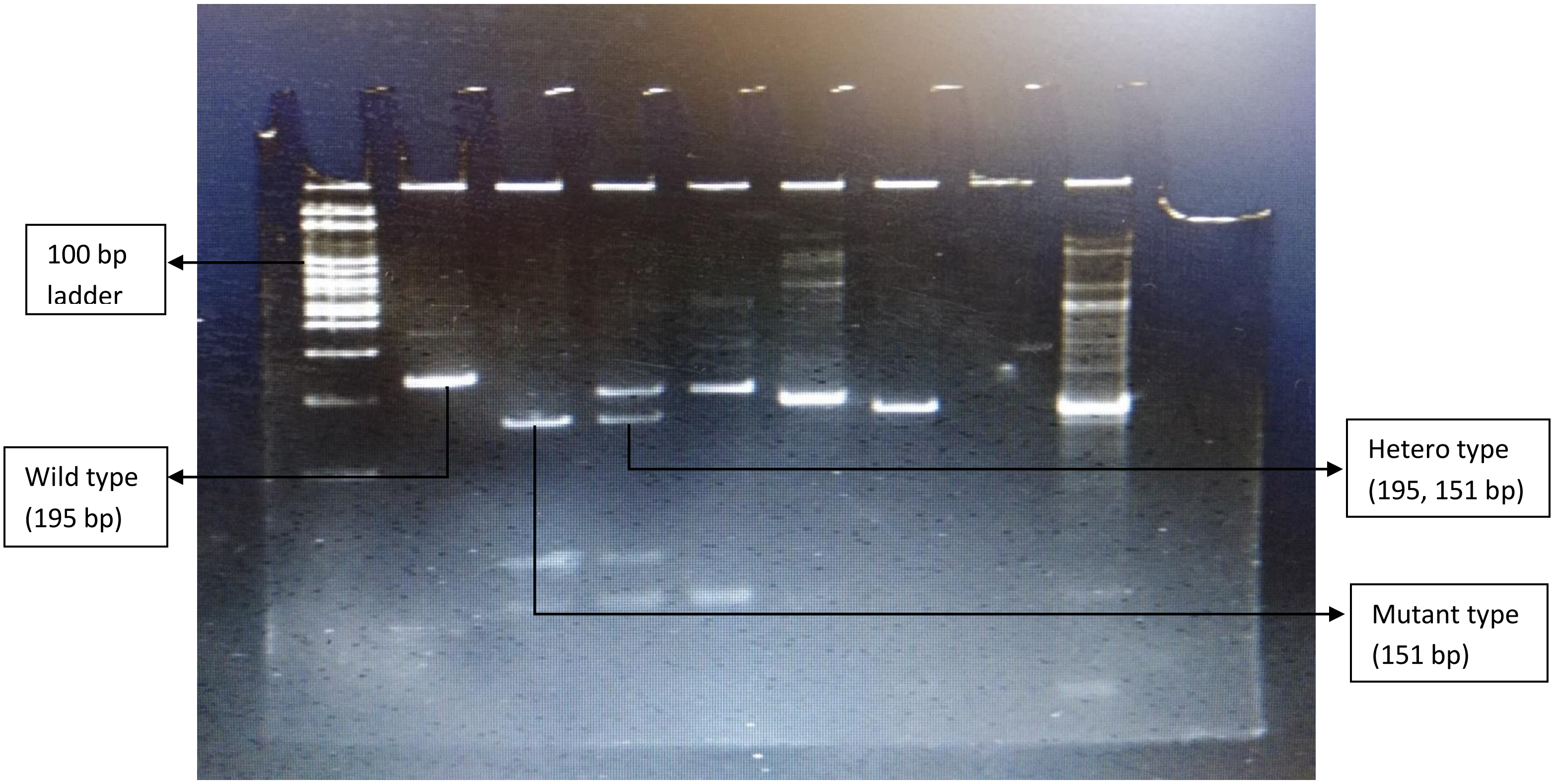


Figure 5.2 : Representative example for the restriction digestion by Native PAGE method of PCR product for NQO1 gene showcasing the digested products (wild : 195 bp ; heterozygote : 195 bp , 151 bp ; mutant : 151 bp).

(Lane 1: 100 bp Ladder)

(Lane 2, 5, 6, 7: wild; Lane 3: mutant; Lane 4: Heterozygote)

5.2. Epidemiology for NQO1 rs 1800566 polymorphism

Table 5.1 : Distribution of demographic characteristics for NQO1 rs 1800566 amongst cases and controls			
VARIABLE	**CASES, n (%)** **N = 197**	**CONTROLS, n (%)** **N = 131**	**p – value**
Age (years) Mean ± SD Range	 57.37 ± 10.85 29 – 80	 52.79 ± 10.78 32 – 83	0.0002
Gender Male Female	 166 (84.26) 31 (15.73)	 127 (96.94) 4 (3.03)	
Smoking Status Smokers Non – smokers	 157 (79.69) 36 (18.27)	 100 (75.75) 31 (23.48)	
Pack years Mean + SD	 25.34 ± 32.97	 20.01 ± 21.52	0.1034
Histological types SCLC ADCC SQCC	 68 (34.51) 58 (29.44) 63 (31.97)		
TNM Staging I II III IV Unclassified	 3 (1.52) 4 (2.03) 83 (42.13) 82 (41.62) 26 (13.19)		
Tumor Size T1 T2	 6 (3.04) 22 (11.16)		

T3 T4 Tx	38 (19.28) 99 (50.25) 2 (1.01)		
Lymph Node Involvement N0 N1 N2 N3 N4 Unknown	 19 (9.64) 12 (6.09) 86 (43.65) 47 (23.85) 3 (1.52) 30 (15.22)		
Metastasis M0 M1 Unknown	 95 (48.22) 74 (37.56) 28 (14.21)		
KPS 90-100 80-70 < 60 Unknown	 62 (31.4) 94 (47.71) 21 (10.65) 21 (10.65)		
ECOG 0 1 2 3 Unknown	 30 (15.22) 53 (26.90) 75 (38.07) 18 (9.13) 22 (11.16)		

The above table represents the demographic characteristics of the study group that includes age, gender, smoking years, pack years, histological subtypes, TNM staging, KPS and ECOG. The study contains 197 lung cancer cases and 131 controls. The mean age of cases and control subjects came out to be 57.37 ± 10.85 and 52.79 ± 10.78 respectively. The cases study consists of 166 (84.26) males and 31 (15.73) females while the controls study consists of 127 (96.94) males and 4 (3.03) females. A notable difference in the number of males and females. It also seen in the study that in cases there were 79.69 smokers and 18.27 non-smokers whereas in controls there were 75.75 smokers and 23.48 nonsmokers. Moreover, the study reveals that in cases pack years for smokers are remarkably higher (25.34 ± 32.97) than pack years for smokers in controls (20.01 ± 21.52). It may be concluded from the statistics shown in the table that smoking is the most important parameter for analyzing the risk towards lung cancer. From all 197 lung cancer cases 68 (34.51) were SCLC, 58 (29.44) ADCC & 63 (31.97) SQCC. The table also shows TNM stage data of 197 patients (Stage I: 3, Stage II: 4, Stage III: 83, Stage IV: 82) and 26 (13.19) cases were left unclassified.

Table 5.2 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls and its susceptibility towards lung cancer

GENE	**Cases, n (%)** **N = 197**	**CONTROLS, n (%)** **N = 131**	**Adjusted OR** **(95% CI)**
NQO1 rs 1800566			
CC	85 (43.14)	65 (49.61)	1.00 (Reference)
CT	99 (50.25)	57 (43.5)	1.20 (0.73 – 1.98)
TT	13 (6.60)	9 (6.87)	1.14 (0.43 – 3.00)
CT + TT vs CC	112 (56.85)	66 (50.3)	1.23 (0.76 – 1.99)
T Allele	125 (31.72)	75 (28.62)	
C Allele	269 (68.27)	187 (71.37)	
Minor allele frequency	0.32	0.29	

The genotypes of cases and controls were identified for *NQO1 rs 1800566* by PCR – RFLP method. Out of 197 cases studied (43.14%) were identified to be homozygous wild type (CC) genotype, (50.25%) were identified as heterozygous genotype (CT) & (6.60%) of the individuals were homozygous mutant genotype (TT). Whereas, out of 131 controls, (49.61%) were identified as homozygous wild type (CC) genotype, (43.5%) were identified as heterozygous genotype (CT) & (6.87%) of the individuals were homozygous mutant genotype (TT). As the number of percentage of homozygous mutant genotype (TT) is low we merged heterozygous and mutant genotype in a single genotype and then compared it to the homozygous wild genotype taken as reference genotype.

The CT genotype with reference to CC genotype of NQO1 gene polymorphism reveals risk of lung cancer but was found to statistically insignificant (OR = 1.20 ; 95% CI = 0.73 – 1.98 ; p = 0.45). For the mutant (TT) genotype, the risk remains same but lesser than the CT genotype (OR = 1.14; 95% CI = 0.43 – 3.00 ; p = 0.78) whereas for the combined heterozygous (CT) and the homozygous (TT) variant genotype when compared with reference genotype (CC), increased statistical values were seen but no such association of Lung Cancer with combined genotype can be concluded (OR =1.23 ; 95% CI = 0.76 – 1.99 ; p = 0.38).

Table 5.3 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of histological subtypes of lung cancer

NQO1 rs 1800566 **(SCLC)**	**Cases, n (%) N = 68 (34.34)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	30 (44.11)	65 (49.61)	1.00 (Reference)	
CT	33 (49)	57 (43.5)	1.40 (0.66 – 2.96)	0.37
TT	5 (7.4)	9 (6.87)	1.09 (0.23 – 5.00)	0.91
CT+TT vs CC	38 (56)	66 (50.3)	1.38 (0.66 – 2.87)	0.38
NQO1 rs 1800566 **(ADCC)**	**Cases, n (%) N = 58 (29.3)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	20 (34.5)	65 (49.61)	1.00 (Reference)	
CT	32 (55.2)	57 (43.5)	1.26 (0.61 – 2.58)	0.52
TT	6 (10.34)	9 (6.87)	1.91 (0.57 – 6.38)	0.29
CT+TT vs CC	38 (66)	66 (50.3)	1.41 (0.72 – 2.78)	0.31

NQO1 rs 1800566 **(SQCC)**	**Cases, n (%) N = 63 (32)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	31 (49.2)	65 (49.61)	1.00 (Reference)	
CT	31 (49.2)	57 (43.5)	1.17 (0.62 – 2.24)	0.61
TT	1 (2)	9 (6.87)	0.25 (0.02 – 2.15)	0.20
CT+TT vs CC	32 (51)	66 (50.3)	1.08 (0.57 – 2.04)	0.80

In the present study, it was seen that 34.34% of the cases were diagnosed with SCLC, 29.3% of the cases were diagnosed with ADCC and 32% of the cases were diagnosed with SQCC. On the basis of genotype further calculation revealed that for homozygous wild genotype the SCLC, ADCC, SQCC had 44.11%, 34.5%, and 49.2% of the individuals respectively. For heterozygote genotype the SCLC, ADCC, SQCC were found to have 49%, 55.2, and 49.2% of the individuals respectively and for mutant genotype 7.4%, 10.34%, 2% respectively.

The statistical values are shown in the table above that were retrieved for the association of NQO1 C/T polymorphism with Lung cancer. On calculating the data on the basis of histology of lung cancer it was observed that individuals with homozygote mutant genotype having Adenocarcinoma shows high statistical value with risk of lung cancer (OR = 1.91; 95% CI = 0.57 – 6.38; p = 0.29). The combination of heterozygous and mutant form having Adenocarcinoma portrays risk of lung cancer (OR = 1.41; 95% CI = 0.72 – 2.78; p = 0.31). Although there were no significant values on statistical calculation and so high risk of association cannot be concluded by the data.

Table 5.4 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of smoking habits of patients				
NQO1 rs 1800566 (Smokers)	**Cases, n (%)** **N = 157(80.5)**	**CONTROLS, n (%)** **N = 100 (76.3)**	**Adjusted OR** **(95% CI)**	**p – value**
CC	68 (43.31)	53 (53)	1.00 (Reference)	
CT	78 (49.68)	41 (41)	1.39 (0.80 - 2.42)	0.23
TT	11 (7.00)	6 (6)	1.17 (0.67 - 2.05)	0.56
CT+TT vs CC	89 (56.68)	47 (47)	1.41 (0.83 - 2.40)	0.20
NQO1 rs 1800566 (Heavy Smokers)	**Cases, n (%)** **N = 65(41.4)**	**CONTROLS, n (%)** **N = 44 (44)**	**Adjusted OR** **(95% CI)**	**p – value**
CC	28 (38.64)	24 (54.54)	1.00 (Reference)	
CT	33 (50.76)	18 (40.90)	1.49 (0.60 - 3.71)	0.38
TT	4 (6.15)	2 (4.54)	1.49 (0.55 - 4.03)	0.42
CT+TT vs CC	37 (56.92)	20 (45.45)	1.30 (0.55 - 3.06)	0.53
NQO1 rs 1800566 (Light Smokers)	**Cases, n (%)** **N = 78 (49.6)**	**CONTROLS, n (%)** **N = 56 (56)**	**Adjusted OR** **(95% CI)**	**p – value**
CC	34 (43.58)	29 (51.78)	1.00 (Reference)	
CT	39 (50)	23 (41.07)	1.52 (0.72 - 3.21)	0.26
TT	5 (6.41)	4 (7.14)	1.02 (0.50 - 2.08)	0.94
CT+TT vs CC	44 (56.41)	27 (48.21)	1.41 (0.69 - 2.87)	0.33
NQO1 rs 1800566 (Non Smokers)	**Cases, n (%)** **N = 36 (18.4)**	**CONTROLS, n (%)** **N = 31 (23.6)**	**Adjusted OR** **(95% CI)**	**p – value**
CC	15 (41.66)	12 (38.70)	1.00 (Reference)	

CT	19 (52.77)	16 (51.61)	0.77 (0.25 - 2.39)	0.66
TT	2 (5.55)	3 (9.67)	0.515 (0.14 - 1.87)	0.31
CT+TT vs CC	21 (58.33)	19 (61.29)	0.68 (0.22 - 2.08)	0.50

The individuals enrolled for the epidemiological study were classified as Smokers & Non Smokers to confirm the association between the risks of developing lung cancer from cigarette smoking. Cases studied revealed 80.5% of the individuals as Smokers and 18.4% of the individuals as non-Smokers. On the other side in controls study 76.3% were found out to be Smokers while 23.6% non-smokers. In addition on the basis of pack years we classified Smokers into Heavy smokers and Light Smokers. Pack years can be defined as the no of cigarettes smoked by an individual in one day multiplied by the total number of years smoked. For individuals with pack years > & equal to 25 were classified as Heavy smokers while individuals with pack years below 25 were classified as Light smokers. Heavy smokers and light smokers came out to be 41.4% & 49.6% respectively for cases whereas 44% & 55% as heavy and light respectively for controls.

Cigarette smoking portrays a cumulative effect on the polymorphism of NQO1 towards lung cancer. Although there were no significant values shown on statistical calculation but high OR values were seen in smokers than in non-smokers. The highest OR value was seen in heterozygous genotype of light smokers (OR = 1.52; 95% CI = 0.72 - 3.21; p = 0.26). Since there were no significant values seen in the data we can say that there's no high risk associated with lung cancer.

Table 5.5 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of Tumor stage of patients

NQO1 rs 1800566 (Stage III)	**Cases, n (%)**	**CONTROLS, n (%)**	**Adjusted OR**	**p – value**
	N = 98 (50.2)	**N = 131**	**(95% CI)**	
CC	37 (37.75)	65 (49.61)	1.00 (Reference)	
CT	53 (54.08)	57 (43.5)	1.68 (0.90 - 3.14)	0.10
TT	8 (8.16)	9 (6.87)	1.31	0.34

			(0.74 - 2.34)	
CT+TT vs CC	61 (62.24)	66 (50.3)	1.71 (0.93 - 3.13)	0.07
NQO1 rs 1800566 (Stage IV)	**Cases, n (%) N = 97 (49.7)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	45 (46.39)	65 (49.61)	1.00 (Reference)	
CT	47 (48.45)	57 (43.5)	1.29 (0.70 - 2.37)	0.40
TT	5 (5.15)	9 (6.87)	0.89 (0.45 - 1.74)	0.74
CT+TT vs CC	52 (53.60)	66 (50.3)	1.25 (0.69 - 2.26)	0.45

We did statistical analysis on the basis of the tumor stage of patients. Stage III and Stage IV were only considered for this study as there were very less individuals in Stage I and Stage II. On stratification it was found that 50.2% of the individuals were diagnosed with Stage III and 49.7% with Stage IV. The combined genotype of heterozygous and homozygous mutant type on comparison with wild type contributes 62.24% of the individuals for Stage III while 53.60% of the individuals for stage IV.

The present shows that there were no significant values obtained. The combined genotype of heterozygous and homozygous mutant type on comparison with wild type gave a value near significant value (OR = 1.71 ; 95% CI = 0.93 – 3.13 ; p = 0.078). It was seen that the Stage III has lesser p values than Stage IV. Since, no significant values were obtained in the above data there's no high risk associated with occurence of lung cancer.

Table 5.6 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of Lymph node of patients

***NQO1 rs 1800566* (Lymph Node 0)**	**Cases, n (%) N = 49 (25.12)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	20 (40.8)	65 (49.61)	1.00 (Reference)	

CT	26 (53.06)	57 (43.5)	1.61 (0.72 - 3.59)	0.23
TT	3 (6.12)	9 (6.87)	1.13 (0.53 - 2.39)	0.74
CT+TT vs CC	29 (59.18)	66 (50.3)	1.62 (0.75 - 3.52)	0.21
NQO1 rs 1800566 **(Lymph Node N1 + N2 + N3 + N4)**	**Cases, n (%) N = 178 (91.28)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	74 (41.5)	65 (49.61)	1.00 (Reference)	
CT	92 (51.6)	57 (43.5)	1.34 (0.80 - 2.23)	0.25
TT	12 (13.14)	9 (6.87)	1.04 (0.63 - 1.73)	0.85
CT+TT vs CC	104 (58.4)	66 (50.3)	1.32 (0.80 - 2.16)	0.27

Statistical analysis was done on the basis of the lymph nodes of lung cancer patients and association of *NQO1 CT* polymorphism was checked with risk of occurrence of lung cancer. The lymph node data was classified into Lymph node 0 and Lymph node N1+N2+N3+N4. On stratification it was observed that 25.12% patients were diagnosed with Lymph node 0 and 91.28% of the patients with lymph node N1+N2+N3+N4. For lymph node 0 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 40.8%, 53.06%, 6.12% respectively whereas for lymph node N1+N2+N3+N4 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 41.5%, 51.6%, 13.14% respectively.

The combined genotype for both lymph node 0 and N1+N2+N3+N4 were found to be 59.18% & 58.4% respectively. The combined genotype heterozygous and homozygous mutant genotype of lymph node 0 showed the least p value but was not found to be significant (OR = 1.6291 ; 95% CI = 0.75 – 3.52 ; p = 0.21). Since, no significant values were seen in the data hence we can say that there’s no high risk associated with lung cancer.

Table 5.7 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of Tumor size of patients				
***NQO1 rs 1800566* (Tumor Size Tx + T1 + T2)**	**Cases, n (%) N = 60 (30.76)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	21 (35)	65 (49.61)	1.00 (Reference)	
CT	36 (60)	57 (43.5)	2.45 (1.15 - 5.21)	0.01
TT	3 (5)	9 (6.87)	1.15 (0.54 - 2.47)	0.70
CT+TT vs CC	39 (65)	66 (50.3)	2.30 (1.09 - 4.83)	0.02
***NQO1 rs 1800566* (Tumor Size T3 + T4)**	**Cases, n (%) N = 167 (85.64)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	73 (43.71)	65 (49.61)	1.00 (Reference)	
CT	82 (49.10)	57 (43.5)	1.17 (0.69 - 1.96)	0.54
TT	12 (7.18)	9 (6.87)	1.05 (0.63 - 1.73)	0.84
CT+TT vs CC	94 (56.28)	66 (50.3)	1.17 (0.71 - 1.93)	0.51

Statistical analysis was done on the basis of the Tumor size of lung cancer patients and association of *NQO1 CT* polymorphism was checked with risk of occurrence of lung cancer. The Tumor size data was classified into Tumor size (Tx + T1 + T2) and Tumor size (T3+T4). On stratification it was observed that 30.76% patients were diagnosed with Tumor size (Tx + T1 + T2) and 85.64% of the patients with Tumor size T3+T4). For Tumor size (Tx + T1 + T2) the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 35%, 60%, 5% respectively whereas for Tumor size T3+T4 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 43.71%, 49.10%, 7.18% respectively.

The combined genotype for both Tumor size (Tx + T1 + T2) and Tumor size (T3+T4) were found to be 65% & 56.28% respectively. The study showcased that heterozygous genotype for tumor size (Tx + T1 + T2) has a significant p value indicating a threefold risk associated with lung cancer (OR = 2.45; 95% CI = 1.15 – 5.21; p = 0.019). Also, for combined genotype heterozygous and homozygous mutant genotype for tumor size (Tx + T1 + T2) significant p value was seen (OR = 2.30; 95% CI = 1.09 - 4.83; p = 0.02). From significant p values it can said that Tumor size (Tx + T1 + T2) has a higher risk associated with lung cancer than the tumor size (T3+T4)

Table 5.8 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of Metastasis of patients

***NQO1 rs 1800566* (Metastasis M0)**	**Cases, n (%) N = 110 (56.41)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	44 (40)	65 (49.61)	1.00 (Reference)	
CT	58 (52.72)	57 (43.5)	1.44 (0.79 - 2.59)	0.22
TT	8 (7.27)	9 (6.87)	1.17 (0.67 - 2.05)	0.56
CT+TT vs CC	66 (60)	66 (50.3)	1.45 (0.82 - 2.56)	0.20
***NQO1 rs 1800566* (Metastasis M1)**	**Cases, n (%) N = 89 (45.64)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	42 (47.19)	65 (49.61)	1.00 (Reference)	
CT	42 (47.19)	57 (43.5)	1.27 (0.68 - 2.38)	0.44
TT	5 (5.61)	9 (6.87)	0.91 (0.46 - 1.79)	0.79
CT+TT vs CC	47 (52.80)	66 (50.3)	1.23	0.48

			(0.67 - 2.27)	

Statistical analysis was done on the basis of the Metastasis of lung cancer patients and association of *NQO1 CT* polymorphism was checked with risk of occurrence of lung cancer. The lymph node data was classified into Metastasis 0 and Metastasis 1. On stratification it was observed that 56.41% patients were diagnosed with Metastasis 0 and 45.64% of the patients with Metastasis 1. For Metastasis 0 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 40%, 58%, 8% respectively whereas for Metastasis 1 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 47.19%, 47.19%, 5.61% respectively.

The combined genotype for both Metastasis 0 and Metastasis 1 were found to be 60% & 52.80% respectively. The combined genotype heterozygous and homozygous mutant genotype of Metastasis 0 showed the least p value but was not found to be significant (OR = 1.45; 95% CI = 0.82 – 2.56; p = 0.20). Since, no significant values were seen in the data hence we can say that there's no high risk associated with lung cancer.

Table 5.9 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and controls on the basis of KPS of patient

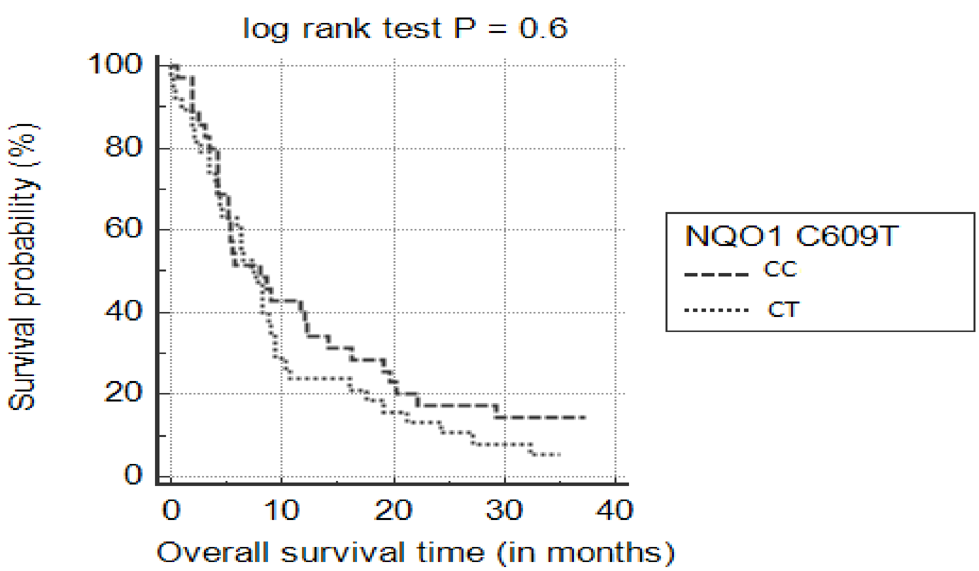
***NQO1 rs 1800566* (KPS 90-100)**	**Cases, n (%) N = 82 (42.05)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	36 (43.9)	65 (49.61)	1.00 (Reference)	
CT	40 (48.7)	57 (43.5)	1.10 (0.59 - 2.05)	0.76
TT	6 (7.31)	9 (6.87)	1.13 (0.64 - 2.00)	0.66
CT+TT vs CC	46 (56)	66 (50.3)	1.12 (0.61 - 2.05)	0.69
***NQO1 rs 1800566* (KPS 80-70)**	**Cases, n (%) N = 113 (57.94)**	**CONTROLS, n (%) N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	48 (42.4)	65 (49.61)	1.00 (Reference)	
CT	56 (49.5)	57 (43.5)	1.45	0.21

			(0.80 - 2.61)	
TT	9 (7.96)	9 (6.87)	1.19 (0.68 - 2.09)	0.53
CT+TT vs CC	65 (57.5)	66 (50.3)	1.46 (0.83 - 2.59)	0.18
NQO1 rs 1800566 **(KPS <60)**	**Cases, n (%)** **N = 40 (20.51)**	**CONTROLS, n (%)** **N = 131**	**Adjusted OR (95% CI)**	**p – value**
CC	17 (42)	65 (49.61)	1.00 (Reference)	
CT	21 (52.5)	57 (43.5)	1.74 (0.77 - 3.95)	0.18
TT	2 (5)	9 (6.87)	1.16 (0.49 - 2.72)	0.72
CT+TT vs CC	23 (57.5)	66 (50.3)	1.70 (0.76 - 3.79)	0.18

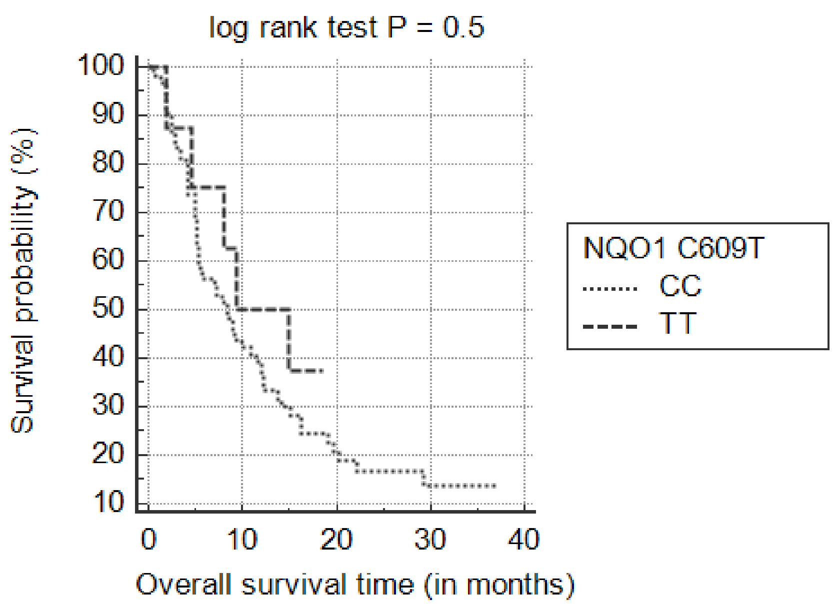
Statistical analysis was done on the basis of the KPS of lung cancer patients and association of *NQO1 CT* polymorphism was checked with risk of occurrence of lung cancer. The KPS data was classified into KPS 90-100, KPS 70-80, KPS <60. KPS is a parameter to measure the chances of a cancer patient to perform ordinary task. Patients with KPS 90-100 are considered to be in a good condition, KPS 70-80 are considered to be in a bad condition whereas KPS <60 means that the ability of any patient to perform tasks is poor. On stratification it was observed that 42.05% patients had KPS between 90-100, 57.94% patients had KPS between 70-80 and 20.51 patients had KPS <60. For KPS 90-100 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 43.9%, 48.7 %, 7.31 % respectively, for KPS 70-80 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 42.4%, 49.5%, 7.96% respectively whereas for KPS <60 the wild genotype, heterozygous genotype and homozygous mutant genotype came out to be 42%, 52.5 %, 5 % respectively.

The combined genotype for KPS 90-100, 70-80, <60 were found to be 56%, 57.5% & 57.5% respectively. Since, no significant values were seen in the data hence we can say that there's no high risk associated with lung cancer.

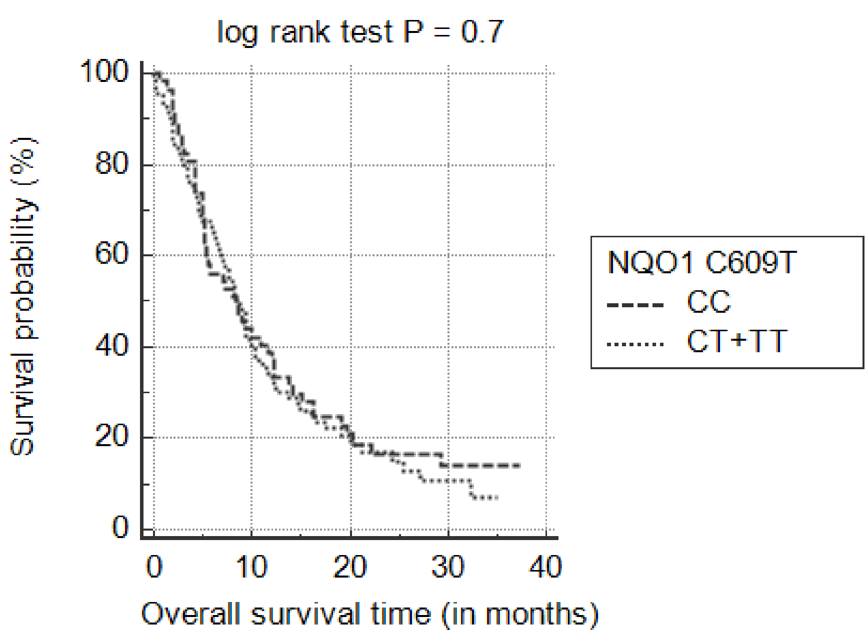
Table 5.10 : Genotypic frequency distribution of NQO1 rs 1800566 among patients and their associated death risk along with overall survival in lung cancer patients					
***NQO1 rs 1800566* (DEAD ALIVE)**	**MEDIAN**	**HR (95% CI)**	**Log P**	**HR (95% CI)**	**P value**
CC	8.40	1.00 (Reference)		1.00 (Reference)	
CT	8.26	1.11 0.76 - 1.61	P = 0.57	1.27 0.83 – 1.94	0.25
TT	11.53	0.73 0.34 – 1.56	P = 0.46	0.68 0.41 – 1.14	0.15
CT+TT vs CC	8.3	1.06 0.73 – 1.53	P = 0.73	1.22 0.80 – 1.85	0.33



Graph 5.1: Kaplan survival analysis of wild and hetero type



Graph 5.2 : Kaplan survival analysis of wild and mutant type



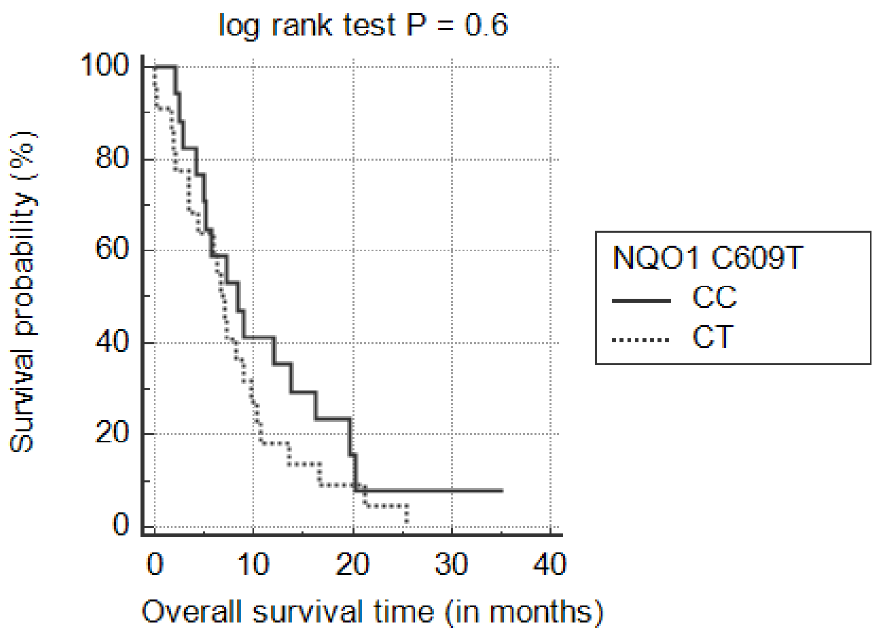
Graph 5.3 : Kaplan survival analysis of wild and hetero+ mutant type

Analysis of overall survival was performed for 195 lung cancer patients by using statistics. Analysis was first done by using univariate Kaplan-Meir and was then adjusted by multivariate Cox hazard proportional analysis for multi parameters including age, gender, smoking status, histology, pack years and KPS whereas genotype was taken as an independent parameter. The wild type genotype was taken as the reference and it was seen that for heterozygous genotype the overall survival months were less than that of homozygous mutant genotype (8.26 vs 11.53; HR = 1.11; 95% CI = 0.76 to 1.61; log P = 0.57). therefore, it can said that since the hazard ratio of heterozygous genotype is more so the patient has less overall survival time as compared to mutant genotype. On applying multivariate Cox hazard proportional analysis heterozygous genotype was again found to have high hazard ratio which means that heterozygous genotype was the least overall survival time. Since, there were no significant values found on Cox analysis so it can be concluded that there is no direct high risk associated with lung cancer.

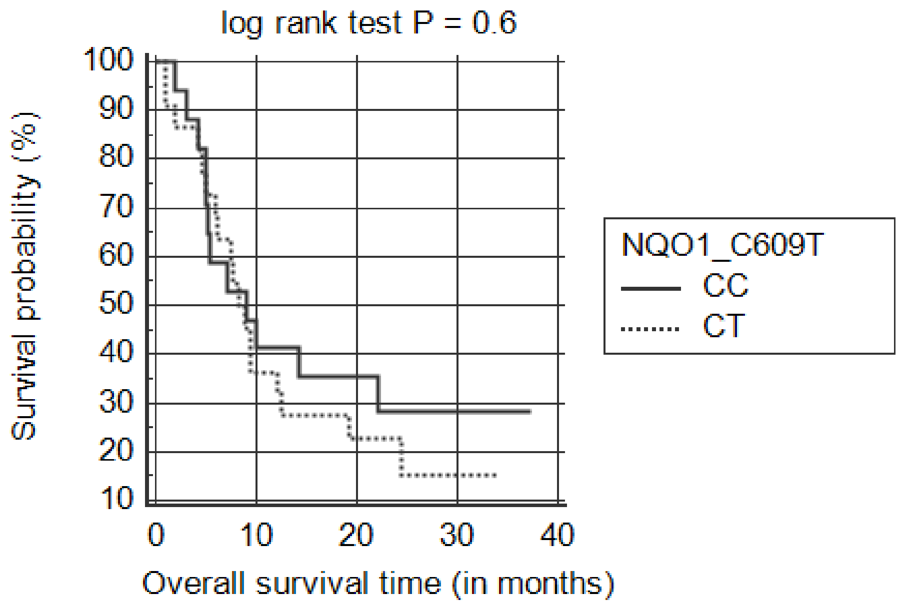
Table 5.11 : Genotypic frequency distribution of NQO1 rs 1800566 among patients on the basis of histology and their associated death risk along with overall survival in lung cancer patients

NQO1 rs 1800566 (SCLC)	**MEDIAN**	**HR (95% CI)**	**Log P**	**HR (95% CI)**	**P value**
CC	8.4	1.00 (Reference)		1.00 (Reference)	
CT	6.76	1.54 0.80 – 2.96	0.15	3.55 1.43 – 8.84	**0.0066**
TT	11.53	0.84 0.20 – 3.37	0.81	1.86 0.68- 5.04	0.22
CT+TT vs CC	7.13	1.43 0.75 – 2.71	0.27	3.79 1.54 – 9.31	**0.0038**
NQO1 rs 1800566 (ADCC)	**MEDIAN**	**HR (95% CI)**	**Log P**	**HR (95% CI)**	**P value**
CC	9.13	1.00 (Reference)		1.00 (Reference)	
CT	8.3	1.23 0.60 – 2.53	0.56	0.86 0.37 – 1.99	0.73
TT	9.36	1.03 0.28 – 3.71	0.95	0.58 0.16 – 2.04	0.40
CT+TT vs CC	8.80	1.21 0.60 – 2.43	0.58	0.84 0.35 – 1.99	0.70
NQO1 rs 1800566	**MEDIAN**	**HR (95% CI)**	**Log P**	**HR (95% CI)**	**P value**

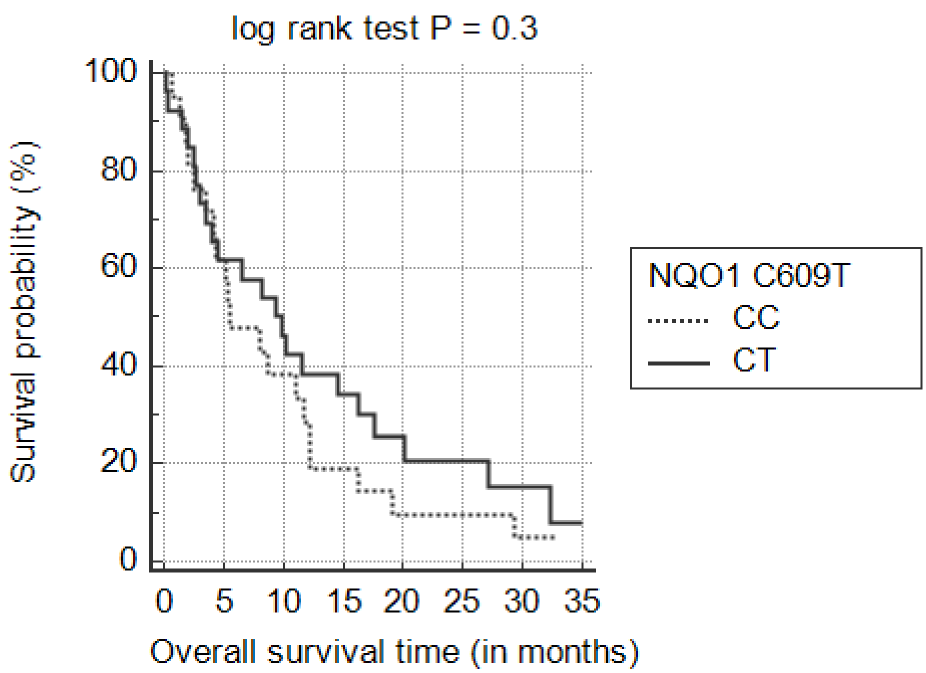
(SQCC)					
CC	5.56	1.00 (Reference)		1.00 (Reference)	
CT	9.33	0.72 0.38 – 1.35	0.30	0.86 0.43 – 1.72	0.68
TT	8.06	1.16 0.13 – 10.08	0.87	1.35 0.45 – 4.08	0.59
CT+TT vs CC	9.33	0.73 0.39 - 1.36	0.31	0.87 0.44 – 1.72	0.69



Graph 5.4 : Kaplan survival analysis of SCLC histological form



Graph 5.5 : Kaplan survival analysis of ADCC histological form



Graph 5.6 : Kaplan survival analysis of SQCC histological form

Overall survival time was analyzed on the basis of different histological forms of lung cancer including Adenocarcinoma, Squamous cell lung cancer, and Small cell lung cancer. Analysis was first done by using univariate Kaplan-Meir and was then adjusted by multivariate Cox hazard proportional analysis for multi parameters including age, gender, smoking status, histology, pack years and KPS whereas genotype was taken as an independent parameter. The wild type genotype was taken as the reference and for SCLC it was seen that heterozygous genotype has less overall survival months as compared to mutant genotype (6.76 vs 11.53; HR = 1.54 ; 95% CI = 0.80 – 2.96 ; log P = 0.15). Heterozygous genotype has the highest hazard ratio suggesting that the patients with heterozygous genotype have very less survival time. On the other hand for SCLC when multivariate Cox analysis was done it was seen heterozygous genotype has the highest hazard ratio with significant p value (HR =3.55 ; 95% CI = 1.43 – 8.84 ; P = **0.0066**) and also for the combined genotype heterozygous and homozygous mutant a significant value was seen (HR = 3.79 ; 95% CI = 1.54 – 9.31 ; P = **0.0038**).

For ADCC, least overall survival time was seen by heterozygous genotype when compared to the homozygous mutant genotype (8.3 vs 9.36; HR = 1.23; 95% CI = 0. 60 – 2.53; log P = 0.56). On applying multivariate Cox analysis no significant values were seen. For SQCC, least overall survival time was seen by the homozygous mutant type when compared to the heterozygous genotype (8.06 vs 9.33; HR = 1.16; 95% CI = 0.13 – 10.08; log P = 0.87). On applying multivariate Cox analysis no significant values were seen.

It can be concluded from the above data SCLC came out to be highly associated with the risk of lung cancer. For heterozygous genotype and combined heterozygous & homozygous mutant genotype significant values were observed confirming very less survival time in case of SCLC and direct association with the occurence of lung cancer.

4.3. Computational Analysis

Table 5.12: Protein Sequence and Protein ID of NQO1

GENE	NQO1
SNP	rs1800566
PROTEIN ID	NP_000894
AA CHANGE	pro187ser
PROTEIN SEQUENCE	>sp|P15559|NQO1_HUMAN NAD(P)H dehydrogenase [quinone] 1 OS=Homo sapiens OX=9606 GN=NQO1 PE=1 SV=1 MVGRRALIVLAHSERTSFNYAMKEAAAAALKKKGWEVVESDLYAMNFNPIISRKDITGKL KDPANFQYPAESVLAYKEGHLSPDIVAEQKKLEAADLVIFQFPLQWFGVPAILKGWFERV FIGEFAYTYAAMYDKGPFRSKKAVLSITTGGSGSMYSLQGIHGDMNVILWPIQSGILHFC GFQVLEPQLTYSIGHTPADARIQILEGWKKRLENIWDETPLYFAPSSLFDLNFQAGFLMK KEVQDEEKNKKFGLSVGHHLGKSIPTDNQIKARK

Table 5.13 : Analysis by SIFT Software

SIFT			
AMINO ACID	**PREDICTION (CUTOFF 0.05)**	**SCORE**	**MEDIAN**
P	TOLERATED (>0.05)	1	2.66
S	TOLERATED	0.07	

Table 5.14 : Analysis by PROVEAN Software

PROVEAN	
PROVEAN SCORE	**PREDICTION (CUTOFF = -2.5)**
-7.392	DELETERIOUS

Table 5.15 : Analysis by I-MUTANT Software

I MUTANT				
SVM2 Prediction Effect		SVM2 Prediction Effect		DDG Value Prediction
DECREASE	RI = 4	DECREASE	RI = 5	-1.36Kcal/mol

Table 5.16 : Analysis by PhD-SNP Software

PhD-SNP	
EFFECT	**RELIABILITY INDEX**
Neutral: Neural Polymorphism	5

Table 5.17 : Analysis by MUPRO Software

	MUPRO
DELTA G	**STABILITY OF PROTEIN STRUCTURE**
-0.85352751	DECREASE

Table 5.18 : Analysis by SNPs&GO Software

SNPs&GO	
EFFECT	**RELIABILITY INDEX**
Neutral: Neural Polymorphism	6

Table 5.19 : Analysis by PANTHER Software

PANTHER		
Substitution	**Preservation Time**	**Message**
P187S	2	probably benign

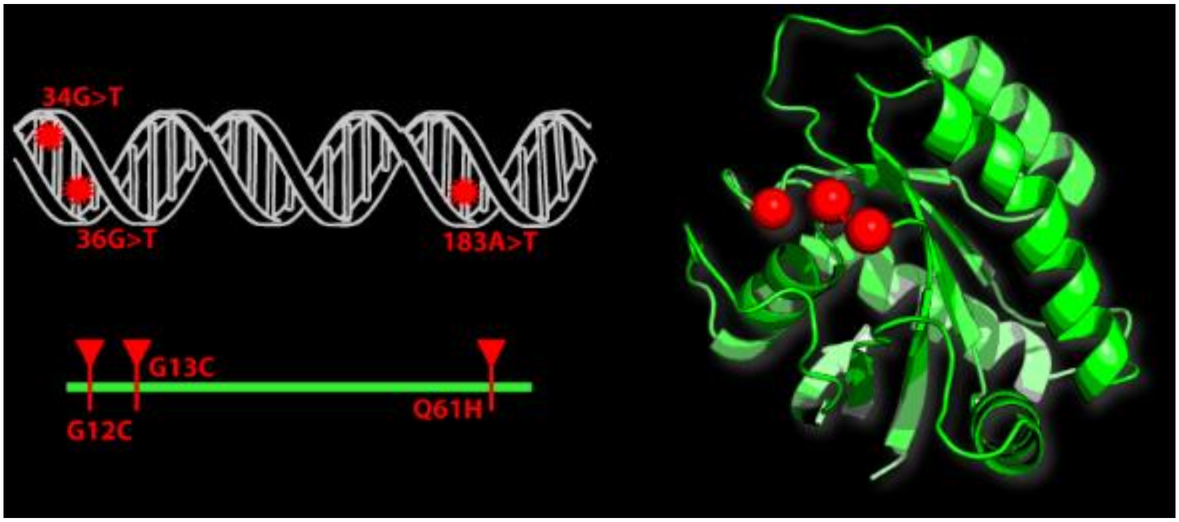


Figure 5.3: 3D representation of NQO1 mutations

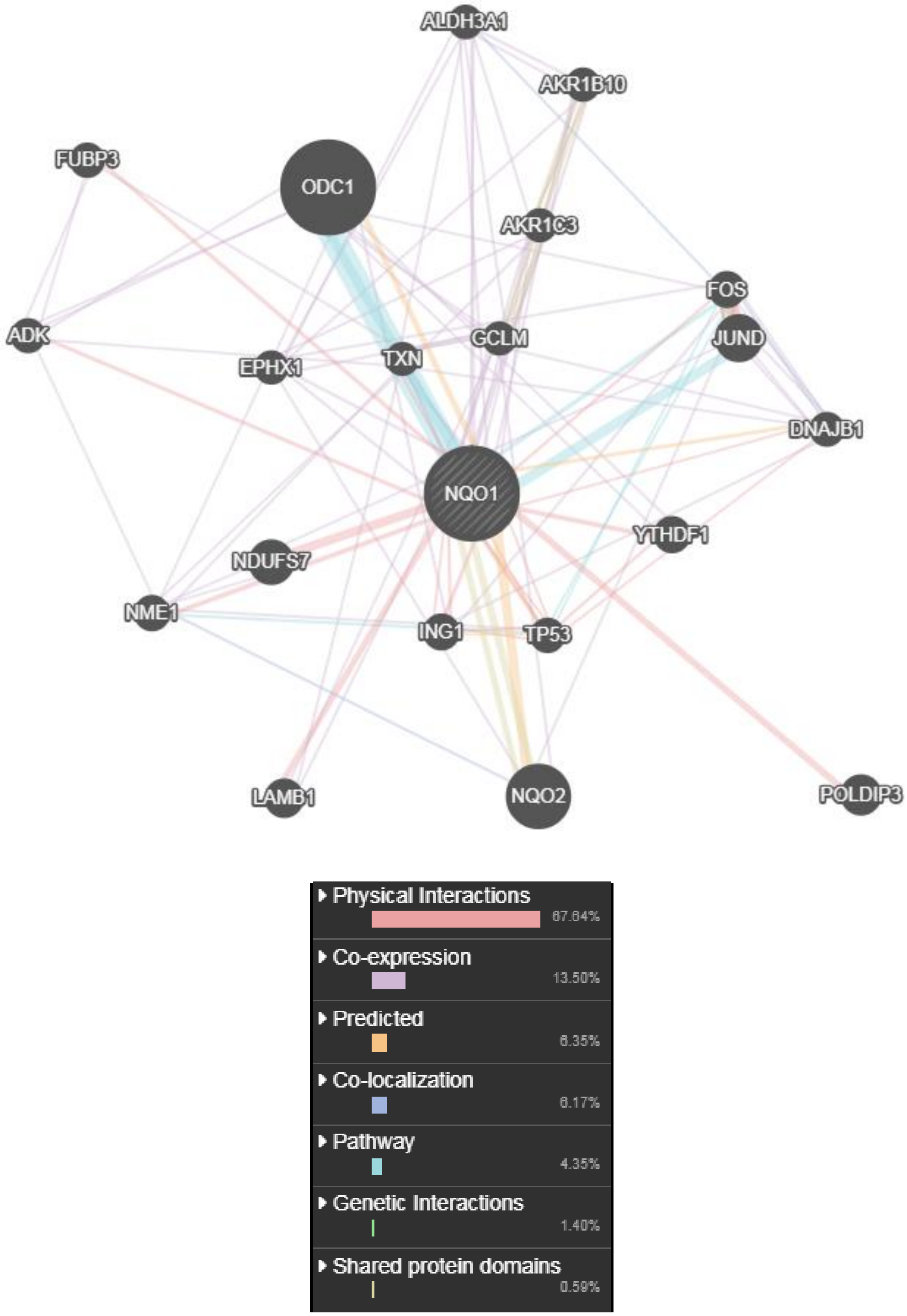


Figure 5.4: 3D representation of NQO1 mutations

From the above analysis for *rs1800566* it can be seen that in SIFT analysis the predicted score value is greater than 0.05 (cut off value) and so the mutation can be tolerated. On the other hand, on PROVEAN analysis the predicted score came out to be -7.39 (cut off -2.5) and it resulted in a deleterious effect of NQO1 mutation. On further analysis by I-MUTANT & MUPRO decreased effect of the mutation has been seen. PhD-SNP and SNPs&GO showcased neutral polymorphisms whereas PANTHER software showed a slight damage due to the mutation. This concludes that *rs1800566* came out to be less damaging and disease causing.

Discussions

DISCUSSIONS

Our results regarding the polymorphism in the variant of NQO1 with the risk of lung cancer matches with a study done in differential susceptibility to lung cancer based on smoking behavior of an individual. This study reported that an overall association between *NQO1* genotype and lung cancer risk was not supported in 1900 individuals enrolled for the study. They published that it's the intensity of the smoking that was more useful in predicting risk of lung cancer rather than the duration of smoking. There can be a rapid increase of risk in current smokers than in former smokers as the smoking intensity increases. (Lian *et al*., 2001).

In another study, a slight increased risk of lung cancer associated with CT and TT variant NQO1 genotypes was seen in Caucasians. Their results depicted that in both cases and controls the allele frequencies were found to be in Hardy–Weinberg equilibrium. Furthermore, on stratifying the data on the basis of age to find the association between NQO1 and lung cancer risk in Caucasians population, they found out effectively higher risk in women and younger population when compared to men. In conclusion, they suggested that NQO1 C609T polymorphism is associated with risk of lung cancer among younger population, women and never smokers (Saldivara *et al*., 2005).

Statistics of another study in Japanese population suggested that there was an inverse association seen between NQO1 C609T polymorphism and risk of lung cancer. Also, in Caucasians and Native Hawains statistically an inverse association was found out and moreover, the number of variant allele in these ethnic groups were less to conclude any results. It was also reported that a significant ethnic variation in the predominance of NQO1 was seen. In Japanese population mutant NQO1 was twice than that in Caucasian population. In, conclusion inverse association between NQO1 polymorphism and lung cancer and difference in allele frequency portrays a lower risk of lung cancer among Japanese as compared to Caucasians (Chen *et al*., 1999).

In a Korean-ethnic population, it was reported an association between NQO1 C609T polymorphism and chemotherapy response in advanced NSCLC patients. They reported a crucial role of NQO1 C609T polymorphism in the medical conditions of NSCLC patients after medical surgery and platinum based chemotherapy. It can be concluded from this study that NQO1 c609T polymorphisms is a potential molecular marker for advanced non-small cell lung cancer as it is associated with risk of NSLC as well medical status of the patient after platinum based chemotherapy (Tian *et al.*, 2014).

There are not many evidences depicting the direct association between the NQO1 polymorphism and risk of lung cancer. Since, NQO1 plays an important role in the detoxification. Polymorphism of NQO1 is considered to have a high association with the risk of lung cancer. But the reported studies have suggested that NQO1polymorphism is not highly associated with the risk of lung cancer. Also, the results vary depending upon the ethnicity. As seen Caucasians have more risk associated with NQO1 polymorphism than other ethnic population.

Conclusions

CONCLUSIONS

In conclusion, evidences suggest that NQO1 polymorphism do not support association with high risk of lung cancer. In 195 cases and 131 controls, mutant genotypes were found to be less. Also, on statistical analysis significant values were not found which could suggest a high risk of lung cancer from NQO1 C609T polymorphism. Since, NQO1 plays an important role in the detoxification. Polymorphism of NQO1 is considered to have a high association with the risk of lung cancer. But the reported studies have suggested that NQO1polymorphism is not highly associated with the risk of lung cancer. Also, the results vary depending upon the ethnicity. As seen Caucasians have more risk associated with NQO1 polymorphism than other ethnic population. The case control sample size for the study taken was less and hence it for better documentation of the role of NQO1 larger size study should be done.

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