CYP1A1 variant rs4646093 and Cancer susceptibility in Himachal Population

Project report submitted in partial fulfillment of the requirement for the degree

Of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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CERTIFICATE

I hereby declare that the work presented in this report entitled " **CYP1A1 variant rs4646093 and Cancer susceptibility in Himachal Population**" in partial fulfillment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology submitted in the department of Biotechnology & Bioinformatics**,** Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from July 2018 to May 2019 under the supervision of Dr.Harish Changotra, Associate Professor, Department of Biotechnology & Bioinformatics.

The matter embodied in the report has not been submitted for the award of any other degree or diploma.

KIRTI VARDHAN (151819)

VISHAKHA SAINI (151813)

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

Dr. Harish Changotra

Associate Professor,

Department of Biotechnology & Bioinformatics

Dated

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SUMMARY

Among varied disease, uncontrolled growth of cell termed as cancer has turned into a potent risk to identities all around. Cancerous cell growth is the secondary most commonplace disorder in Republic of India which is responsible for most mortality accounting about 0.3 million passing every year. That further can be owing to the poor comfort of prevention, recognizable proof and treatment of affliction, each sort of cancers are accounted for in Indian populace just as the diseases of skin, lungs, bosom, rectum, stomach, prostate, liver, cervix, throat, bladder, mouth and so on. The reason for such high rates of these abnormal growths could likewise be each internal (hereditary, changes, hormonal, poor resistant conditions) and external or ecological variables. The contribution of polymorphisms in substance metabolizing genes to overall cancer rates could vary wide between teams with differing factor frequencies and with variable level of malignant neoplastic disease exposure. Their effects are changed by interactions with one another and with other genes, significantly those associated with DNA repair mechanisms. Lung cancer remains a number one explanation for unwellness globally, with smoking being the most important single cause, clinical test enzymes, together with haemoprotein P450 family 1, taxon A, peptide one (CYP1A1), are concerned within the activation of carcinogens, like polycyclic aromatic hydrocarbons, to reactive intermediates that are capable of binding covalently to DNA to make DNA adducts, thus initiating the malignant neoplastic disease. The aim of this study was to check the association of *CYP1A1* gene polymorphism (rs4646093) with common cancers such as Head and Neck cancer, Lung cancer and cervical cancer in Himachal Pradesh population.

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CHAPTER- 1

INTRODUCTION

1.1 Overview

Cancer is a term that depicts a disease in which division of abnormal cells take place without control. These cells are capable of entrenching into other tissues. This disease can affect almost any part of the body. These cells often form a tumor. There are two kind of tumors i.e., benign and malignant form of tumors. Benign is a type which do not spread, or seized into contiguous threadwork and howbeit it is unfastened from the body so usually they do not move into the other parts of the body. The other kind is malignant tumor which encroaches nearby tissues and sometimes it spreads to distant areas of the body which is also termed as metastasizing. These are even dangerous also because they furnish and leap up to new tumors. There is a possibility of reoccurrence also and this could even possible after their detachment from the body. The advantageous thing about cancer is, its evaluation can be done in its early stages therefore, if it's treated as soon as possible then an individual can lead a life with the freedom from this disease. There is a great advancement in treatment that 80-85 % of those diagnosed with this diseased live beyond 5 years [1].

1.2 Scenario of cancer in India:-

There is an increase in cases of cancer every year in India. The maximum rates of oral, lung; gall bladder and cervical cancers are in India. The ICMR has of late anticipated that India is conceivably at more than 17 lakhs latest cases and more than 8 lakhs passing identified with this illness constantly till 2020[2].

1.3 Following are the reason behind different types of cancer:-

- 33% due to tobacco use and pan masala for lung and oral cancer mostly in men.
- 16% are due to obesity or overweight usually in breast, lungs, large intestine, and kidney especially in the metropolitan cities.
- 4% are due to genetic issues mostly like ovarian and breast cancer.
- 15% are because of infection due to various cancer-causing pathogens.
- 5% due to insufficient physical activity.
- 5% due to having poor dietary habits.
- 2% are consequences of exposure of UV lights.[1]

1.4 Lung Cancer

The lungs are a pair of an organ that makes a part of respiratory framework. The left lung is little as the heart consumes space on the left side and marginally not quite the same as one another. Left lung has two flags though right lung has three projections. Lung malignant growth fundamentally rises up out of the tissues of the lung, commonly from the cell covering air entry. The two reported types of lung cancers are- 1.Small cell lung cancer and 2. Non Small cell lung cancer. Moreover, Lung cancer is the second most cancerous cell growth which is recognized by the assessment of the cells under a magnifying lens. The noteworthy sub kinds of NSCLC are squamous cell carcinoma, huge cell carcinoma and adenocarcinoma. Lung cancer stretches out over a region through blood and lymphatic vessels and this process is known as metastasis. About 40% of individuals recently distinguished by the lung cancers as of now have metastasis to different regions of the body, examples are lymph hubs, liver, bone, cerebrum and adrenal organ.[3]

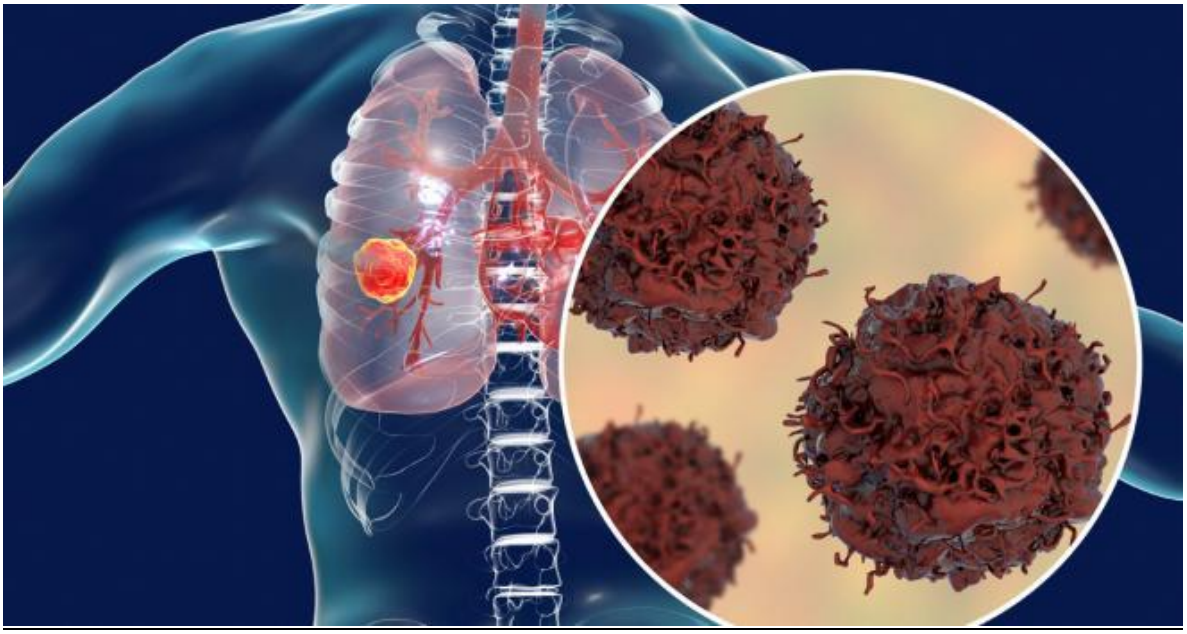


Figure 1: Depiction of lung cancer and its complications ("Lung Cancer: the facts", Pfizer Medical Team, Nov 18, 2018).

1.5 Scenario of lung cancer in India:-

- Males prevail with a Male: Female proportion of 4.5: 1 and this proportion fluctuate with the age and smoking status.
- This proportion increment continuously up to 51-60 years and after that remains constant.
- The distinct period of history of acquiring lung cancer in India is 54.6 years.
- Greater or equal to 65 years of age are suffering the most due to cancer in lungs.
- The smoker to non-smoker proportion is high up to 20.5 in different investigations.[3]

1.6 Head and Neck Cancer:-

It mostly begins in the parts such as squamous cells that lines the damp and mucosal stretch inside the oral and pharyngeal cancer. Squamous cell carcinoma of the head and neck usually derived from the squamous cell disease. Such growth of cancerous cells can be additionally isolated on the premise happening territory of the head and neck disease locales. [4]

- Paranasal sinuses and nasal hole: - These are small empty spaces inside the bones of head encompassing the nose.
- Salivary Organs: - the huge salivary organs lie in the floor of the mouth and enclosed by the jawbone. There is production of saliva from salivary organ.
- Oral cavity: - It includes the lips, the gums, and the front two third of the tongue, the coating inside the cheeks and lips, that sense the taste.
- Pharynx: - in layman language it is fundamentally throat which is unfilled cylinder long around 5 inches that get under at the back of the nose.
- Larynx: - It is likewise called voice box which is an indefinitely way conceived via ligament just underneath the pharynx in the neck. It involves the vocal strings too. It establish epiglottis which is a small bit of tissue and it keeps the nourishment from entering the air section as it moves spread the larynx.[4]

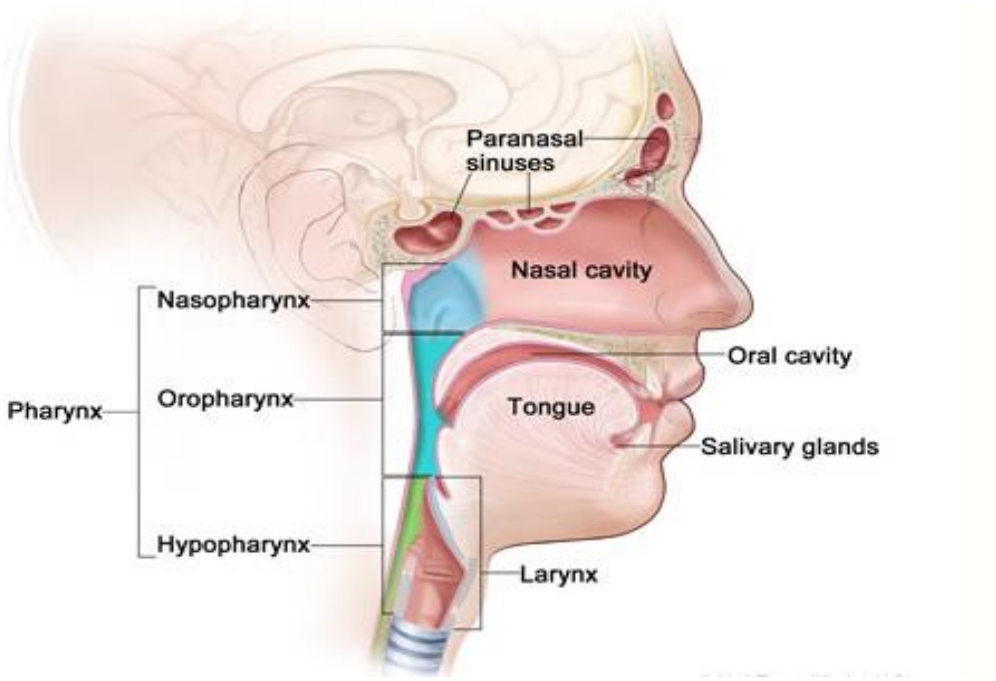


Figure 2: Representing the domains of the Head and Neck. (*"Head and Neck Cancers". NCI. March 29, 2017. Retrieved 17 September 2017).*

1.7 Reasons for head malignancy and neck cancer:-

Tobacco consumption and excessive drinking of alcohols both are the significant elements of neck and head carcinoma.

The harmful considerations which influence the cancer are:-

- **Preserved and saliferous foodstuff:** - During childhood there is a speculated constituent for cancer of nasopharyngeal area and it is because of pursuit of assertive saliferous foodstuff or preserved foodstuff.[5]
- **Consumption of betel liquid: -** This habit is significantly connected with the increased risk of this cancer.
- **Oral health: -** Poor oral hygiene may be the consequential risk factor for oral cavity cancer.[6]
- **Exposure to the harmful radiations :-** Harmful rays such as UV rays, X rays exposure to the head and neck area**,** for noncancerous conditions or malignancy is a hazard factor for the disease of the salivary organ.[7]
- **Occupational exposure-** Occupational exposure to asbestos, silica and hazardous chemicals might lead to cancers.

1.8 Symptoms of the Head and Neck Cancer:-

- Larynx: - There is a pain in mouth as well as in ear while swallowing.
- Salivary glands: -There is frequent pain and around the jawbone and enlargement under the chin or pain in the face that don’t goes away.
- Pharynx: -There is a difficulty in breathing and speaking, there is a pain during swallowing, continuous headaches and hearing problems are also there in this case.

- Oral pit: A ruddy white fix on the gums, bizarre seeping in the mouth and swelling of the jaw.
- Sinuses in par nasal and nasal hole: Chronic sinus infections would not respond with the treatment of antibiotics.[8]

1.9 Scenario of Head and Neck Cancer:-

- HNC is the third most prevalent cancer in India with 52067 passing's and 77003 cases analyzed in 2012 and the genuine frequency is considerably more than the reported cases as most of the cases go undiscovered.[9]
- In India, majority of cancers present as locally advanced Stage III/IV disease, as significant amount of efforts are focused on therapy and outcomes, thereby overlooking the need for early detection.[10]
- According to the study of Tata Memorial Hospital in Mumbai, India contributes about 57.5% cases of HNC globally. 80,000 cases are reported of oral cancer yearly.[11]

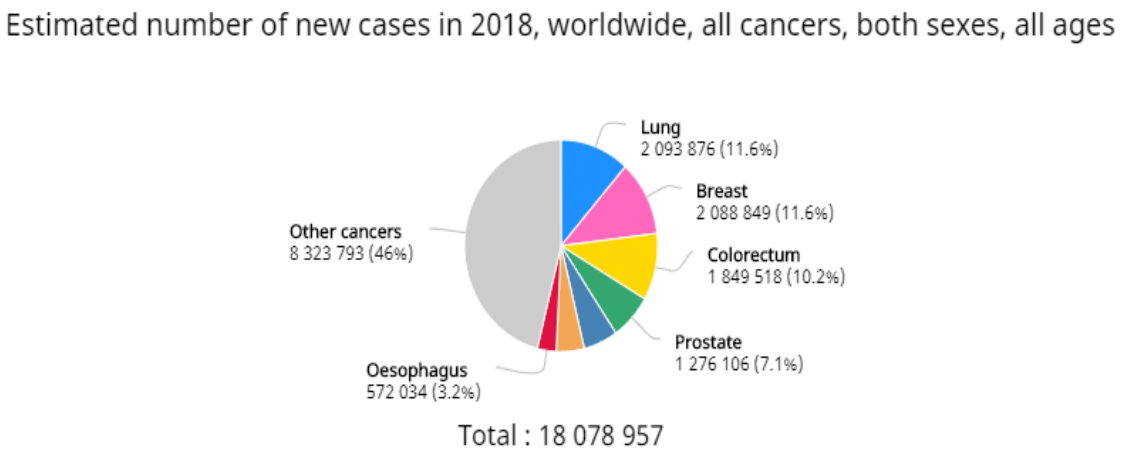


Figure 3: Latest number of new cases of cancer as of December 2018. Ref- GLOBOCAN 2018Graph production: Global Cancer Observatory (http://gco.iarc.fr/) International Agency for Research on Cancer, 2019.

1.10 CYP1A1 Gene:

CYP1A1 is a gene that encodes enzymes belonging to cytochrome P-450 super family. The enzymes belonging to this cytochrome P-450 family are monooxygenases which catalyze numerous responses engaged with medication digestion and combination of cholesterol, steroids and different lipids. It is limited in ER and its demeanor is inhibited by some polycyclic sweet-smelling hydrocarbons, some of which are found in tobacco smoke as it can process some PAHs to cancer causing intermediates and is related with risk of lung cancer [12]. It is present on Chromosome15 where its size is 6069 base pair with exon count: 7 and intron count: 6.

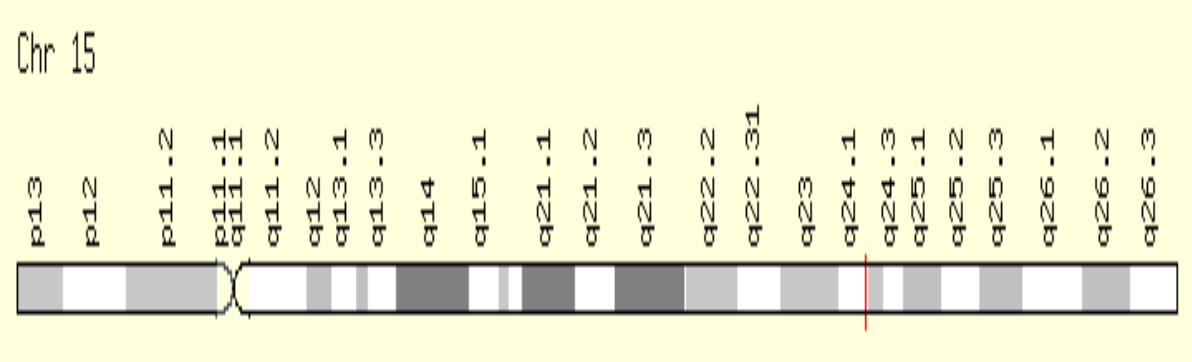


Figure 4: Location of CYP1A1 gene Ref- (https://www.genecards.org/cgi-bin/carddisp.pl?gene=CYP1A1)

CHAPTER-2

REVIEW OF LITERATURE

2.1 Cancer

All over the world, there are various things folks are facing and one in every of the matter is wealth of recent and difficult environmental problems every day. There is a rise in variety of patients of cancers globally that could be a burden on world and the reason behind the increment is the population's growth and the aging factor and yes of course the adoption of cancer causing factor such as smoking and excessive use of alcohol within a economically developed countries. Cancer is the most prominent disease after the cardiovascular disease which is the reason behind the maximum death in the world. Lung, breast, colo-rectal cancer's frequencies are higher in comparison to others and the cases of these cancers are increasing rapidly across the world. Non-communicable disease is the reason of the premature death and death due to this is the one of the highest in India. There are many disease which is not transmittable like heart related disease, diabetes, gasping ailments and malignancy which is chief concern of public health. Evidences of a growing body which we get from an experiment, ecological research and body burden indicates there is a connection between ecological factors and cancers. [13].

2.2 Contrasts between Normal and Cancer Cells:

Table 1: Difference between normal and cancerous cells.

Normal Cells	Cancer Cells
There is a vocation of cells in normal cells in comparison with cancer cells.	There is an aberrant growth in cancer cells and its nature is protruding.
There is a distinction of kinds of cells and normal cells that plays a specific function especially when it is mature.	Cancer cells do not active in functioning properly that's why it keeps dividing its cells eccentrically.
It possesses the capability for the detection of the signal so that it can weave the undesirable cells.	It does not have the competency to unmasking the signal to get rid from division of cells.
Normal matured cells provide the healthy environment so that normal cells can be protected from the damage or harmful division.	Diseased cells can change the encompassing typical cells, and veins that shape a territory called microenvironment to sustain developing tumor.
Normal matured cells supply nutrients as well as oxygen to their cell so that they can function properly and they can maintain a healthy environment within a body.	Malignancy cells prompt encompassing ordinary cells to shape veins, that are involved in oxygen supply and supplements to tumor for its development and furthermore expel squander items from tumors.
Normal cells possess the large cytoplasm and fine chromatin and it contains the single nucleus as well as single nucleolus.	Malignant cells possess small cytoplasm with coarse chromatin and it contains the multiple nucleus[14]

2.3 Evolution of Cancer

Driving cancerous cell growth advancement are stochastic physical cell changes in qualities that administers. It manages the different parts of human development control. The procedure administering the beginning and movement of cancerous growths are transformative ones in which regular determination follows up on the intrinsic or gained assorted variety of different physical clones, encouraging the outgrowth of those with some type of propagative favorable position.

Tumors develop inside contrasting physical conditions, every one of which forces its very own one of a kind imperative. For instance, shedding epithelia, for example, gut or skin 'shield' themselves against the development of seizable clones by censuring all descendants cells to terminal separation and passing. Diseases advance for a similar reason creatures appear to- we see just the victories, not the disappointments. This mutilates our factual perspectives on disease movement. Regardless of how uncommon the beginning and advancement of a cancerous cell growth or how powerful the counter malignancy treatment regulated. Advancement is a progressing procedure. As a neoplasm advances, grows and spreads, it faces moving specific weights. The heterogeneity and decent variety found in disease are remnants of a dynamic and stochastic developmental power that changes with varying substantial situations [15].

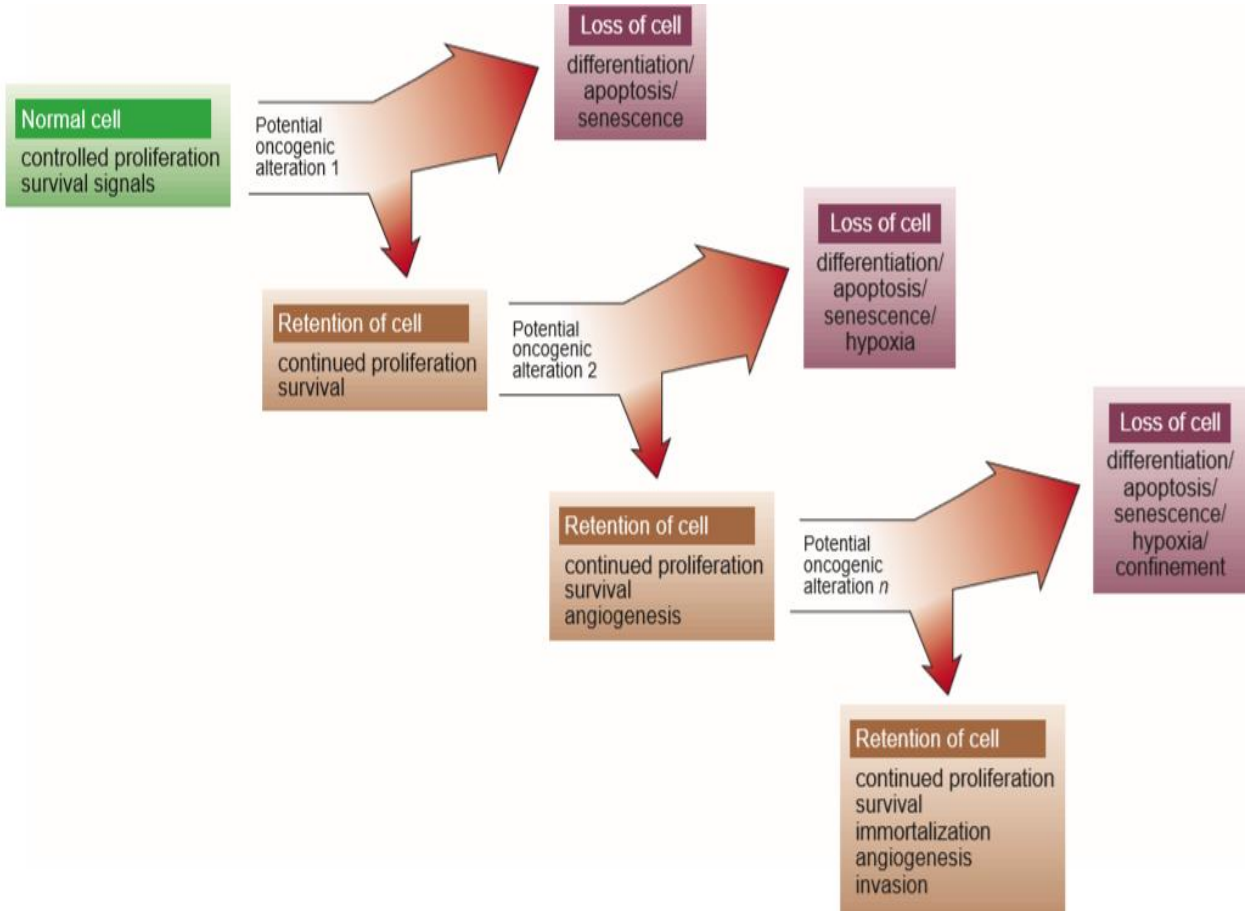


Figure 5: Role of cell cycle in prognosis of cancer. (Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature. 2001 May; 411(6835):342.)

2.4 Epidemiology

Epidemiology historically has relied on measures if external exposures in deciding the between the association between the exposure and unwellness. Recently, there has been increasing reliance on internal markers reflective internal dose or early stages of unwellness. Within the context of empirical studies of chronic unwellness during which there is a famed exposure disease- association, the questions arises whether or not the external exposure or the internal marker could be a higher predictor ultimate unwellness outcome. Here we have a tendency to describe some straight forward approaches to gauge the relative prognostic price of the interior marker (or biomarker, outlined within the most general sense), versus the exposure, similarly as their limitations, the issues of assessing the prognostic price of the internal marker are illustrated via two examples: (a) carcinogens, genetic outcome and cancer (b) amphibole, carcinoma and

asbestosis. We have a tendency to conclude that it is unlikely that empirical medical specialty can permit the full assessment of the prognostic price of genetics outcomes versus exposure for cancer in humans exposed to famed carcinogens within the close to future, though animal studies may offer vital complementary data. For amphibole, information up to now indicate the presence or absence of pneumoconiosis could be a higher predictor of carcinoma in associate In nursing exposed population than is that level of exposure to amphibole itself. In general the foremost helpful markers for predicting the chronic unwellness are ones that persists over time.[16]

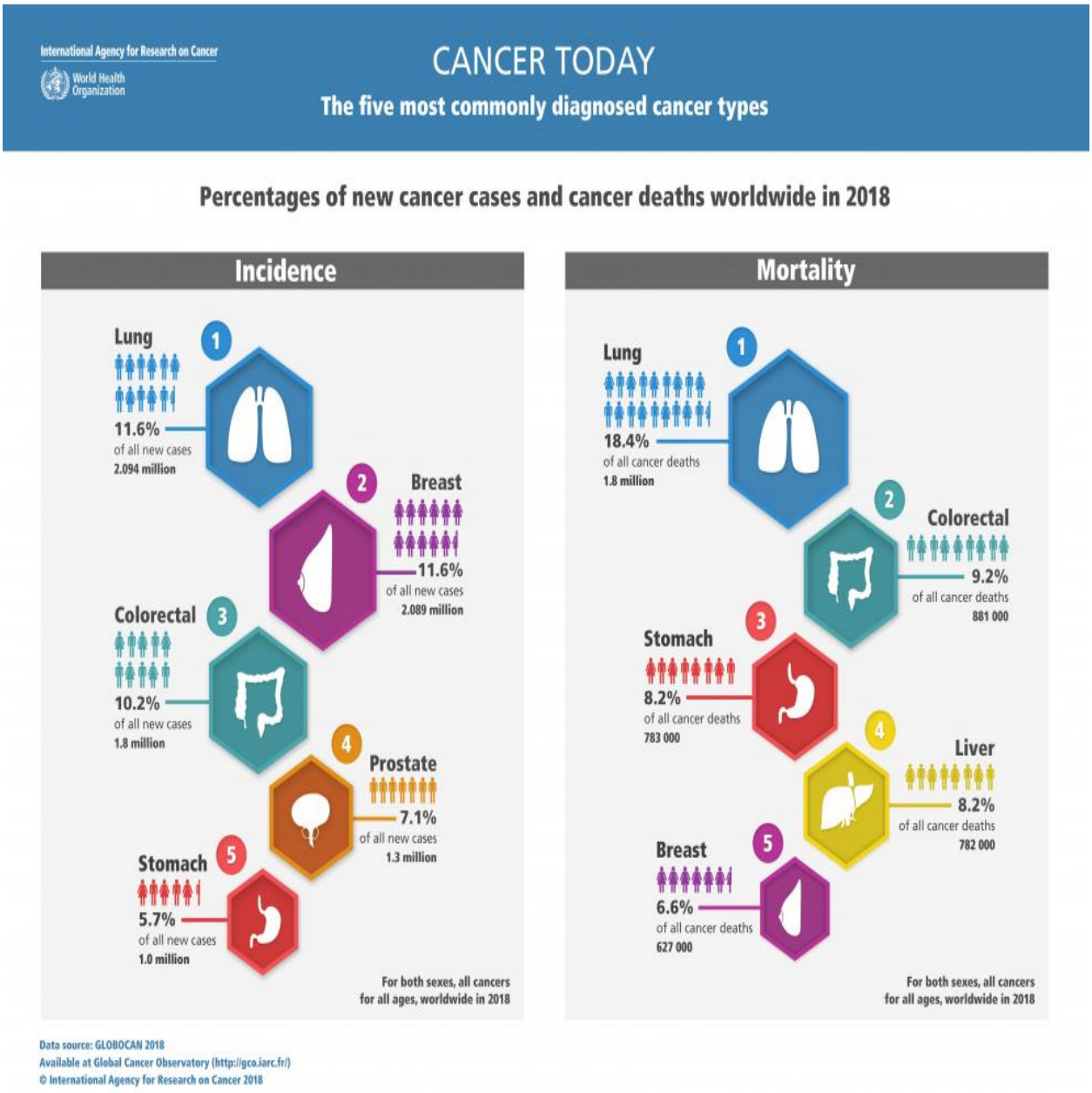


Figure 6: Cancer incidence and mortality for most common cancer. Ref, GLOBOCAN 2018Graph production: Global Cancer Observatory (http://gco.iarc.fr/) International Agency for Research on Cancer, 2019

2.5 Causes and causative agents

A solitary transformation is generally insufficient for the advancement of a threatening tumor. Different examinations propose that disease is advancement is a multistep procedure, which includes consecutive changes in a few qualities, for example, oncogenes, tumor-silencer qualities, micro-RNA qualities in malignant growth cells. Malignancy is for the most part brought about by changes in different qualities, for example, in oncogenes, tumor silencer qualities and microRNA qualities. These transformation are typically substantial changes, anyway germ line transformations can make an individual defenseless to heritable malignant growth [17].

The causing substances which tend to cause cancers in humans and these substances are present in the environment and lifestyle factors also influence the cause of cancers and these factors are:-

Pathogens: - The human digestive system contains several trillions of bacterial cells. In the correct situation, it shows up the wrong sorts of microbes can prompt ceaseless irritation, which can incline to malignant growth.

Prescribed drugs or medications: - Hormonal replacement therapy can stimulate cancer cells. Therapy which is gives to maintain estrogen level can lead to uterine or cancer in breast. Baring of few chemicals like formaldehyde etc can become the reasons of tumors in the body.

Consumption of tobacco: -Its use is the one of the biggest preventable cause of cancer of many types. There is a tremendous dangerous chemical which is present in tobacco smoking. It causes malignancy of kinds as oral, pharynx, larynx, and upper throat and can ultimately leads to kinds of leukemia.

Use of pesticides: - Epidemiologic proof on the connection between synthetic pesticides and malignant growth is explored. Numerous pesticides are cancer causing, as creosote and sulfallate while others eminently are the organ chlorines, DDT, chlordane are tumor advertiser. A few contaminants in business pesticide detail additionally may present a cancer causing agent hazard. In people, arsenic mixes and bugs sprays utilized occupationally have been delegated cancer-causing agents.

Hereditary or acquired disease: - In this genetic or acquired cancer growths are predominantly brought about by inheritable changes in the qualities. For instance, BRAC1 and BRAC2 qualities are in charge of acquired bosom malignancy and ovarian disease [18].

Hormonal changes: -It can likewise result being of development of disease. A typical is the adjustment in female hormones levels such as females with overabundance of estrogen levels are increasingly inclined to create uterine cancer [19].

Dysfunction due to immune system: -On the off chance that our insusceptible framework is not sufficiently able to shield our body from different pathogens, this can prompt a few cancer. [19].

Nutritious Plan: - Ill-advised eating regimen is one of the fundamental basis of disease persuasiveness in India. About 70% of colorectal malignancy cases are accepted to be because of imbalanced eating regimen. The job of eating routine towards malignant growth fluctuates incredibly indicated by the sort of the disease. According to the international relationship thinks about, overpowering positive relationship between dietary fat, red meat utilization [20].

Exposure to the radiation: -The significant wellsprings of radiations are radioactive mixes, bright UV, and high electromagnetic fields. The fundamental arrangement malignant growths actuated by presentation to the satisfactory, dosages of the cancer- causing radiations incorporate thyroid, skin, leukemia, lymphoma, lung and carcinomas in breast. In the created or creating nations, the radiations are additionally famous cancer-causing agents. About 10% malignant growth event is because of radiation impact both ionizing and non-ionizing. The most well-known wellspring of ionizing radiation introduction is Radon, which is a radioactive component. Radioactive cores of radon, radium, and uranium are observed to be related with an expanded danger of gastric disease in rodents. High danger of bosom malignant growth among young ladies at the adolescence is because of chest light of x-ray beams [20].

Many more cancer causing factors are like fungi toxicity, chemicals like Benzedrine and vinyl chloride, PAHs, dust particles , radiations comes from ultraviolet rays etc.[1]

2.6 Preventive measures taken to control cancers in India

- Scientist ought to make mindfulness among open about physical exercises, staying away from obesities, sound dietary works on, ecological exposures and training program.

- As it rightly said that “anticipation is superior to fix” the counteractive action techniques are significant in malignancy destruction. This methodology offers an extraordinary general wellbeing concern and economical long haul technique for disease control.

- Ecological contamination is a major issue and has turned into a test for us all as it in charge of the beginning of different sorts of malignant growths. Air contamination is the most remarkable reason for lung malignant growth in the metropolitan urban areas of India. The hurtful gases for example, CO, SO_2 delivered by ignition of powers in autos and a few mechanical procedures, individually, cause lung malignant growth, respiratory, stomach related, skin carcinomas. Cars that keep running on packed petroleum gas (CNG) ought to be supported at any rate in the metropolitan urban communities of the nation to maintain a strategic distance from air contamination.

- The utilization of CFCs, methyl halides, carbon tetrachloride and carbon tetra fluoride is the fundamental driver of the exhaustion of ozone layer, which shields us from the destructive UV- beams. The utilization of such synthetic compounds ought to be limited so as to diminish the frequency of skin malignant growth brought about by the hurtful impacts of UV-beams. The sewage released by a few ventures and districts is dirtying Indian water assets because of deficient water treatment plants; prompting different sorts of malignancies. In this manner, these squanders ought to be treated preceding their release to land or stream.

- According to the survey done tobacco is the most famous operator for diseases, which must be restricted to annihilate the predominance of tobacco related malignancies. India should give the most noteworthy need to tobacco control program because of its intense cancer-causing nature and it is claimed by the WHO. It has been anticipated that the restriction on tobacco use can counteract up to 30% malignancies in India. Liquor utilization is in charge of the event of colorectal disease. About 25% populace is

expending liquor in India, which must be limited or kept away from to destroy this ruin. Government needs to force a prohibition on the open clearance of liquor. Classes and general well- being camps ought to be led to make familiarity with alcoholic hurtful impacts among Indians. Radiations are quiet and genuine cancer-causing agents that reason various tumors and, henceforth, the procedures that diminish the presentation of individuals to these malignancies.

- India being one of the atomic power countries needs to manufacture safe outfitted atomic plants with more noteworthy insurance from the dangerous atomic radiations. Atomic reactors ought to be very much developed with great quality shields to give more insurance to the general population at work.[20]

2.7 Screening of Cancers

Screening programs are lucky for colon and cervical tumors, wherever future evacuation antecedents sores brought about exceedingly decrease in disease rate and mortality rate. Be that as it may, numerous sorts of tumors display a spread of heterogeneous practices and variable probability of movements and demise. Therefore screening for couple of malignancies may have most reduced effect on the mortality and ought to do extra damage than savvy. Malignant growth screening to make progress, it should principally finds tumors with fatal potential or antecedents early, bringing about restorative considerations that diminishes mortality and grimness. Extra delicate screening ways criminologist work littler and littler injuries, anyway this has not been amidst a practically identical decrease inside the occurrence of the obtrusive disease. Since the usage of screening for beyond any doubt malignant growth (for instances prostate and bosom cancer), a spike in frequency in set up and beginning time for tumors has been found, anyway a connection to decrease in malignancy explicit mortality has not been clear. It's troublesome to perceive what number of those mortality decreases are a direct result of screening and the manner in which many are because of improved medicines of tumors. In tumors with lower rate anyway high mortality (for instance exocrine organs disease), screening has focused on unsound populaces, anyway challenges equivalent to those for all inclusive community screening remaining, altogether with connection to discovering sores with hard to describe harmful potential (for instance, intraductal limb glycoprotein neoplasm). Amid this survey researcher

will in general represent considerably authority in the commitment of the screening ordinary and unsound populaces to over diagnosis, the results of over diagnosis of a lethargic disease through a comprehension of neoplasm heterogeneousness, the science of anyway malignancies of anyway advance and advancement, the atomic and cell alternatives of cell pathologic procedure and furthermore the elements of the communications of early sores with their enveloping tissue microenvironment.[21]

2.8 Treatment for Cancer

Cancer is mainly characterized on the basis of its malignancy and the amount of damage the organs are facing based upon this cancer is generally characterized into four stages and each stage has its own level of severity. Although, there are numerous amount of treatments for cancer around the globe but they are only efficiently effective if diagnosed in earlier stages. Even after expenditure of billions of dollars of research amount there is still no reported reason that why a cell become cancerous or malignant. Out of all the possible treatments the most effective ones are

1. Chemotherapy
2. Radiation therapy
3. Surgery
4. Targeted therapy

Cancer therapy is revolutionizing medical specialty. As indicated by the survey of the researchers, there are open door for a mix treatments upheld nanoparticles stages intended for the treatment, photo thermal therapeutic guide, photodynamic guide, radiation treatment and arrangement treatment. Malignancy nano medicine together with the immunotherapies offers the probability intensify development resistant reaction and to sharpen tumors to immunotherapies in an exceedingly sheltered and powerful way. In any case, portion constraining toxicities and low patient reaction rates remain significant difficulties inside the facility. Nonetheless, nanoparticles will be acclimated reprogrammed the immunological issue neoplasm microenvironment and to trigger general development invulnerability, synergizing with immunotherapies against cutting edge malignant growth. At long lasts the ends were that there are the approaches to upgrade

neoplasm and safe cell focusing on while lethality and invulnerable related antagonistic occasions and the researcher investigated the capability of theranostic nanoparticles for mix treatment [22]

2.9 Epigenome based malignant growth hazard forecast: reason, difficulties and openings:

Considerable proof has gathered proposing that the epigenome and specifically DNA methylation based tests meet these prerequisites. In any case, the advancement and execution of DNA methylations based hazard expectations tests present significant difficulties. Specifically, the cell type explicitness of DNA methylation and the broad cell heterogeneity of the effectively available surrogate cells that may contain the data important to less open tissues requires the utilization of novel techniques so as to represent these puzzling issues. Besides the commitment of established researchers with human services expert, policymakers and the open is required so as to recognize and address the hierarchical, moral social monetary and lawful difficulties related with the standard utilization of epigenetic testing.

The rate of disease is proceeding to rise and hazard custom made early demonstrative as well as essential counteractive action methodologies are critically required. The perfect hazard prescient test should incorporate the impacts of both hereditary and non genetic factors and intend to catch these impacts utilizing a methodology that is both organically steady and actually reproducible and get a score from effectively available natural examples that goes about as a surrogate for the organ being referred to, and empower the viability of hazard lessening measures to be checked.

DNA methylation based hazard forecasts models give novel chances to chance custom fitted screening and counteractive action of malignant growth. Usage of epigenome based hazard custom made screening and anticipation programs requires a few monetary, hierarchical, lawful, moral and social difficulties to be tended to notwithstanding the commitment of policymakers, medicinal services and general society. Epigenetic misprogramming is a fundamental segment of malignancy improvement. Multidisciplinary community inquire about is expected to defeat the logical difficulties related with the disclosures of DNA methylation tissues and creating novel explanatory strategies

2.10 Importance of this Examination

There are distinctive affiliations thinks about accessible in regards to cell cycle administrative qualities like p53, p73, BRAC-1, BRAC-2 and Mdm-2 which uncovers the relationship of hereditary variations of these qualities with the chance of growth in malignancies. Almost in maximum cases, there are restricted investigations accessible administrative SNPs. Subsequently there is a consideration that there is a relationship between *CYP1A1* SNPs with disease hazard. On the off chance that few alleles have higher recurrence in cases than in controls and at that point it might have job in carcinogenesis and in the event that high hazard in these SNPs which is accounted for, at that point they could be utilized as a biomarker for malignant growth patients.

The primary target for this investigation is to distinguish the job of administrative SNPs rs4646093 of *CYP1A1* quality in helplessness of lung and head and neck malignant growths in Himachal Pradesh populace.

2.11 Gene Introduction

CYP1A1 is a gene belonging to hemoprotein P450 family, a category of clinical test chemicals, its conjectured that inters individual differences in nucleotide within this gene has the capability to enact cancer-causing agents, and for instance, PAHs could prompt differential cancer-causing impact. But two variation polymorphisms are represented within the *CYP1A1* quality. The first could be T3801C base change in DNA; the second is an A2455G base change in, which ends up in an Ile to Val amino-corrosive amendment. These polymorphisms appear, by all accounts to be connected. Consistency in study results is missing, with typically distinctive outcomes among varied populaces. In Japanese populaces, each of the *CYP1A1* variation polymorphisms are connected with expanded respiratory organ malignancy probability was the first to report a relationship with polymorphisms with lung sickness hazard. Patients with respiratory organ malignancy had an in more than 2 overlay higher repeat of getting the homozygous variation genotype.

Among patients with epithelial cancer, the homozygous variation genotype was connected with expanded danger of making respiratory organ malignancy, notably at a lower combined portion of tobacco smoke. At low dimension of presentations to tobacco smoke, the possibilities

proportion for making respiratory organ cancers among those with homozygous variation genotype was seven. This expanded hazard was industrious, nonetheless of lesser greatness, at higher portion dimensions of tobacco smoke, proclaimed comparative findings. The Ile462Val polymorphism of *CYP1A1* has in addition been connected with respiratory organ sickness probability in Japanese populaces. Once more, the homozygous variation Val-Val genotype was connected with respiratory organ sickness at lower combination parts of tobacco smoke. One clarification set for the association with low portion level has been that at high portion levels, the vital compound is soaked, whereas at low dosages it isn't. the impact of hereditary inconstancy and differential catalyst movement are more and more evident at the lower coffin nail portion levels.

The gene *CYP2E1* is a discrete from the family of CYP phase 1 and it's engaged with enactment of amines of nitrogen. These genes i.e., *CYP2E1* and *CYP1A1* and *GSTM1* played out a populace grounded exposition- swayed and there were 341 lung malignancy samples and control number were 456, and it was evaluated that polymorphism of *CYP2E1* were related less, whereas *GSTM1* and *CYP1A1* may assume significant jobs in describing helplessness to the disease of lungs. An interdependent impact was repeatedly shown by the people linked by the genotypic variations in both *CYP1A1* AND *GSTM1* had an a lot greater danger of growth in malignancy of lungs in comparison to the genotypic variation in the *CYP1A1* variation. [27]

2.12 *CYP1A1*Gene and its polymorphisms

There are some haemoprotein P450 (CYP) heme-thiolate catalysts that take an interest within the detoxification, and incomprehensibly, the development of receptive intermediates of thousands of compounds that can hurt deoxyribonucleic acid, additionally as lipids and proteins. CYP expression may also have an effect on the assembly of the atoms got from archiodinoic corrosive, and adjusts various downstream flag transduction pathways. Such changes will be antecedents to the cancer. Ongoing examination in mice has modified the perception of the scientist regarding the operation of CYP1 enzymes. Scientist proposed two layered framework to anticipate associate degree generally speaking between individual danger of tumor genesis supported deoxyribonucleic acid variation in beyond any doubt early resistance CYP qualities, joined with polymorphism in various downstream qualities.[28].

SIGNIFICANT NOTCH:-

- Pharmacokinetic investigations of many have environmental toxicants in *CYP1A1* knockout mouse lines have demonstrate that *CYP1A1*, *CYP1A2*, *CYP1B1* can be valuable or hurtful- looking on their time explicit articulation. These new discoveries counsel that creature studies and human therapeutic forte examinations would potentially should returned to.

- Of the fifty seven human CYP in eighteen families, the individuals from CYP1 to CYP4 families process a huge number of Xenobiotics (and endogenous substrates) while all individuals from CYP5 family and better in the use of endogenous substrates in an exceedingly.

- There is an animating xenosensors relationship that is not all things considered surely known among CYPs, XME receptors that control CYP quality articulation and XRTs.

- The digestion of Xenobiotics by XMEs has been characterized into stage 1 clinical preliminary (functionalization) and clinical test (combination) responses. Haemoprotein P450 CYP (enzymes) includes 70-8-% of all stage 1 clinical preliminary XMEs.

- CYP protein will cause tumors commencement through the development of receptive intermediates (from exogenous and endogenous substrates). CYP also takes an interest in tumor commencement and movement through irritation, distinctive eicosanoid-interceded forms and other flag transduction pathways.

- Although initially thought to be responsible for the medication digestion for all intents and purposes totally inside the liver, it's right now been finished that everyone XMEs takes an interest in a few critical endogenous capacities, no doubt in each being cells and heaps of prokaryotes.

- A two layered is anticipated for the opportunity of creating disease because of a setting. To begin with, between individual varieties inside the in advance hPpM qualities, encoded by stage I qualities ought to effects a little (5%-15%) extent of any populace who have no critical polymorphism in downstream target qualities. Second, all

downstream focuses of stage I intervened receptive intermediates can have their own significant polymorphisms, continually bringing about a unimodal slope.

- Though oral prescription, a great deal normally utilize the vein framework (and first-pass disposal energy), we exhort the system lymphaticum can be a ton of important in conveying the appallingly hydrophobic oral PAHs and PHAHs to concentrate on tissues. This can be an especially pertinent to clinical medication, as oral introduction to those procarcinogens in creature models (extrapolation to people) is required.

- Albeit numerous high penetrance, dominantly inheritably (hPpM), attributes together with malignant growth are connected with fluctuated human CYP qualities, distinguishing proof of a choose procarcinogensis never achievable.[28]

2.13 Blends of Polymorphisms in various qualities: the case of DNA repair

SNPs present in DNA repair pathway genes can likewise add to varieties in DNA repair limit and subsequently helplessness to cancer-causing agents. Chemicals encoded by DNA repair qualities continually screen the genome repair harmed nucleotides deposits coming about because of natural and endogenous exposures. A noteworthy trouble that challenges epidemiologic examinations is that different proteins can perform comparative fix exercises. There has been as of late a move from the advancement and investigations of phenotypic tests of DNA repair ability to an attention on genotyping for recognized changes in DNA fix qualities. Scientist have examined the job of SNPs in three fix qualities (XPD-Lys751Gln, exon 23,

XRCC1-Arg399Gln, exon 10, and XRCC3-Thr241Met, exon 7) and their mix in adjusting the dimensions of massive DNA adducts in a populace test of a healthy people.[29]

2.14 Gene association with head and neck cancer:

The relationship of GSTM1 and CYP1A1 polymorphism and oral and pharyngeal malignant growths was evaluated through a meta-examination of distributed case- control study and a pooled investigation of both distributed and unpublished case –control ponders from the genetic susceptibility to environmental carcinogens database.

Since 1988, tobacco and liquor utilization have been perceived as free hazard factors for oral malignant growth. Epidemiologic investigations performed in all mainlands have discovered an expanded hazard in smokers and portion reaction association with every day of cigarettes and term of propensity.

An unnecessary utilization of mixed drinks has been related with oral and pharyngeal cancer cell growth, with relative dangers some of the time higher than those found for smokers. The hazard related to liquor increments with utilization, term, beginning age and type of liquor drink. At the point of joint utilization of liquor and tobacco was examined, the extraordinary dominant part of the writing proposes that the joint impact is multiplicative or, in any event, more noteworthy than added substance.

HPV is another conceivable factor for the etiology in the oral and pharyngeal disease; two late examinations detailed a higher danger of oral and pharyngeal of malignant growth related with HPV 16 and HPV18.[30]

2.15 Metabolic qualities and danger of oral and pharyngeal tumors:-

CYP1A1 and *GSTM1* are significant catalyst in the digestion of tobacco cancer-causing agent, which includes a harmony between the enactment steps intervened by the cytochrome P450 framework and detoxification steps including *GSTM1* that catalyze the change of the receptive electrophiles to inert, water solvent conjugates that can be effectively removed.

Past methodical audits, meta-examination and pooled investigations have announced a connection between *GSTM1* invalid genotype and then take a huge risk cancer, however the main report that stratified the investigation for malignancy site found significant contrast in hazard for oral and laryngeal tumors. No affiliation was found for the *CYP1A1* polymorphism in the last appraisal. Since various examples *GSTM1*and *CYP1A1* catalyst articulation have been appeared oral and pharyngeal epithelium in examination with laryngeal epithelium, scientist directed a pooled investigations to assess the connection between these polymorphisms and oral and pharyngeal tumors, the scientist investigated the consolidated impacts of polymorphisms in these two qualities alongside their association with smoking.[30]

2.16 *CYP1A1* gene polymorphism in Lung cancer:-

In spite of the fact that cigarette smoking is the prevailing danger for a few epithelial tumors, just a little part of people with tobacco introduction creates malignant growth. Through this audit, scientist will outline ongoing advances in hereditary pathways alter the tobacco- related disease. Underlining on hazard evaluation, scientist portrayed how hereditary varieties may help with foreseeing clinical result, for example, the normal history of malignant growth and treatment reaction. The estimations of hereditary helplessness by both genotypic and phenotypic examines are canvassed in the content. The hidden theory is that hereditary elements can furnish indubitable smokers more vulnerable to malignant growth in comparison of others. Hereditary modifications in basic administrative pathways may incline cells to carcinogenesis. These pathways incorporate guideline of Xenobiotics digestion; control of genomic solidness, including DNA fix components, cell-cycle checkpoints, apoptosis and telomere length; and control of micro environmental factors, for examples grid metalloproteinase, inflammation and development factors. What's more epigenetic occasions, for example, advertiser hyper methylation and loss of engraving, are likewise engaged with carcinogenesis. At long last, scientist presented various current worries that should be tended to as the energizing field of atomic disease the study of disease transmission propels quickly. [31]

2.17 Pathobiological Impacts and the pieces of Information to Etiology:-

In people, tobacco smoking prompts expanded adduct development in target tissue, for example the lung and in surrogate tissues for example the blood. The organically successful portion is every now and again alluded to as a middle biomarker of cancer. Ordinarily considered is the dimensions of cancer-causing agent DNA adducts, which speaks to the net impacts of dangerous metabolic actuation, absence to repairing of DNA or control instruments, absence of detoxification and absence of cell passing. Proof exists that cancer-causing agent DNA adduct levels are influenced by hereditary inclinations. A few sorts of studies show that cancer-causing agent DNA adducts is identified with malignant growth hazard. Case-control investigations of the lung demonstrate a positive relationship. Just a solitary imminent investigation has been led, and this one showed that more elevated amounts of blood adducts was related with later lung malignancy chance. The relationship of surrogate markers, for example, cancer causing agent

DNA adducts in blood to the objective organ has been incompletely considered, showing that blood level may reflect target organ levels, however this is not yet settled.

Biomarkers of mischief can extend from segregated early changes with or without impacts on capacity to occasions that obviously led to carcinogenesis and can be seen in disease cells. Chromosomal harm can be estimated utilizing established cytogenetic techniques, micronuclei development, Comet, fluorescent in situ hybridization, or PCR strategies surveying loss of heterozygosity where the last two techniques can be utilized for morphologically showing up cells. Utilization of changes in columnist qualities, for example, GPA or HRPT been utilized, however it is smarter to distinguish transformation rates in malignant growth weakness qualities, for example, p53. For p53, it has been accounted for that there is a portion reaction connection between tobacco smoking and p53 changes. Women's have more G to T transfusions than men for comparative dimensions of smoking, despite the fact that men have p53 transformation all the more generally. Curiously, given that the p53 mutational range for lung malignancy is comparative around the world, almost certainly, tobacco smoke is the significant determinant of lung p53 transformations around the world. Ongoing examinations have been concentrating on the relationship of smoking with hypermethylation of advertiser locales of tumor silencer qualities, which is seen in both present and previous smokers [32].

2.18 Single Nucleotide Polymorphisms (SNPs)

Polymorphism is defined as alteration in two or more than two nucleotides in a given DNA sequence. If we talk about SNP as its full form says single nucleotide Polymorphism it certainly depicts that here the alteration only occurs in one nucleotide in a given DNA sequence of more than one percent population. For instance if in a given sequence "ATGCC" instead of the first A the new or the mutant sequence has T then the resultant sequence will be "TTGCC".

Analysis of SNP is usually done with a bigger number of samples where we have to check the presence of mutant allele in a given population. After analysis if the SNP of given sequence is found to be associated in relationship to a given disease then this can help in finding out the root causes of the mutation that is ultimately leading to generation of and progression of the disease.

SNP's can also act as a biomarker which can help us in locating the other regions in a given city, state, country where the same mutation is responsible or can be responsible for a particular

disease. Enormous amount of study is still going on SNPs and the related mutation which can further be used in other application such as Forensics and identification of genetic mutation. So in our study we will be using SNP rs4646093 for the gene CYP1A1 to check its association with different common cancers (here Lung Cancer, Head and Neck Cancer and Cervical Cancer) and its susceptibility among the population of Himachal Pradesh.

SNPs under study:

rs4646093

Exons count: 7

Chromosome number- 15q24.1

2.19 SNP Genotyping: PCR-RFLP

Since our main motive is to check the susceptibility of gene CYP1A1 and its association to different common cancers which will be done by performing genotyping using the PCR-RFLP method. RFLP stands for Restriction Fragment length Polymorphism which is often used to find out the uniqueness in a given DNA sequence. This method of genotyping was invented by an English scientist named ALEC JEFFREYS in 1984. In order to perform this method of genotyping restriction endonuclease are the major key factors which helps in creating the banding pattern that we visualize over an agarose gel. Restriction endonucleases are the enzymes which cleaves the DNA on particular sites which are known as restriction sites. For different studies based on intraspecies or interspecies of different organism there is a possibility that the restriction endonuclease enzyme cleaves DNA at different sites even in same species. In order to overcome this limiting factor the DNA is amplified using pre-designed primers in order to get the amplified PCR product of the same size.To perform RFLP four major steps are involved which are enlisted respectively – first step is to design the set of primers that will amplify the DNA to one distinct size. Second step will be isolation of DNA from the desired sample organism. In the next step the isolated DNA will be amplified using the designed primers with the PCR. The final step will be the reaction of restriction endonuclease enzyme with the amplified PCR product. This can be further visualized on an agarose gel having EtBr with the help of electrophoresis.

CHAPTER 3

OBJECTIVES

1. Optimization of PCR-RFLP conditions for genotyping of selected SNP (rs4646093) of 3' untranslated region of the gene CYP1A1.
2. After genotyping the frequency of mutant allele that can be responsible for the association of *CYP1A1* gene with common cancers found in Himachal Pradesh Population.

CHAPTER 4

MATERIALS AND METHODS

1. POPULATION STUDY

Here we will be performing the study of total around 230 control samples which were healthy and not having any infection that might lead to cancer. Along with this 390samples of different cancer were collected and most of them matched on the basis of age and geographical distribution in localities of HP. Before collection of samples proper certification and signatures on consent letters were signed by the patients. All members signed a written agreement before drawing the blood sample and were provided with the knowledge about the project.

2. SAMPLE COLLECTION

Sample collection was done using fresh and sterile syringes from the patients and the collected blood was stored in vials containing EDTA in order to avoid clotting.

3. GENOMIC DNA ISOLATION

Genomic DNA was isolated from blood sample using modified inorganic methods.

- ***REAGENTS REQUIRED***

  - **Na_2EDTA (0.5M,pH 8.0)**

186.1g of Na_2EDTA was added to 800ml of distilled water which was stirred vigorously using a magnetic stirrer. Thereby, using 10M NaOH drop wise the pH was adjusted to 8.0. The salt was allowed to dissolve and final volume was made up to 1000ml.

  - **Tris (hydroxymethyl) aminomethane-chloride, Tris-Cl (1M,pH8.0)**

121.2g of Tris Base was dissolved in 800ml of distilled water. Using 1N HCl pH was adjusted to 8.0. Total volume brought up to 1000ml using distilled water in the end.

- **Tris-Cl (1M,pH 7.3)**

121.2g of Tris base was dissolved in 800ml of distilled water. Using 1N HCl pH was adjusted to 7.3. Total volume brought up to 1000ml using distilled water in the end.

- **Ammonium Chloride, NH_4Cl (1M)**

53.5g of ammonium chloride was dissolved in 800distilled water. Total volume brought up to 1000ml using distilled water in the end.

- **10% SDS**

10g of SDS was dissolved in 70ml of distilled water. Solution was heated at 68°C for proper mixing. Total volume brought up to 1000ml using distilled water in the end.

- **Red Blood Cell Lysis Buffer**

Composition: Tris 10mM, pH – 8.0, EDTA 1mM; NH4Cl 125mM,pH 8.0

i. EDTA (0.5M) 2ml

ii. Tris (1M, ph-8.0) 10ml

iii. NH4Cl (1M) 125ml

Mix the above reagent in distilled water to obtain final volume of 1L.

- **Tris – EDTA (TE) buffer (pH 8.0)**

Composition: Tris 10Mm, EDTA 1mM, pH 8.0

i. EDTA (0.5M) 2ml

ii. Tris (1M , pH8.0) 10ml

Final volume was raised up to 1Land pH was adjusted to 8.0

❖ **Tris – EDTA (TE) buffer (pH7.3)**

Composition: Tris 10Mm, EDTA 1Mm, pH7.3

i. EDTA(0.5M) 2ml

ii. Tris (1M, pH 7.3) 10ml

Final volume was raised up to 1L.

Ammonium Acetate (7.5M)

28.9g of ammonium acetate was dissolved to 20 distilled water. Total volume brought up to 50ml using water in the end.

➢ ***Procedure***

- 300µl blood sample with 900µl RBC lysis buffer was added in an autoclaved centrifuge tube and later incubated at room temperature for 40-60 minutes until the mixture turns glittery red.
- Perform centrifuge for 1 minute at a speed of 13000 rpm to get whitish pellets of WBC.
- After centrifugation the supernatant was discarded using a pipette and the leftover pellet was suspended in 300 µl of TE buffer optimized at pH 8.0. This later can be mixed using a vortex machine in order to mix the pellet in suspension. After this 20µl of 10% SDS was added to the tube and incubated at 56°C for 30 min in water bath.
- After incubation 150µl of ammonium acetate (7.5M) was added and mixed using a vortex machine. Later again centrifugation was done in order to separate the proteins as pellet, at a speed of 13,000 RPM for 15 minutes.
- In a fresh sterile centrifuge tubes the clear supernatant was transferred and to this chilled absolute ethyl alcohol cooled down by placing it at -20 degree of refrigeration. The final mixture was again rocked mildly for separation of gDNA.

- This precipitated gDNA was again centrifuged at 13000 rpm for 10 min in order to obtain pellets. This was consecutively washed in 150µl of 70% ethyl alcohol and air dried at RT.
- After complete drying off of the ethanol 100µl of TE Buffer (pH 7.3) was added and incubated at 65°C for 10 minutes. The finally obtained dissolved DNA can later be stored at -20°C for further use.

QUANTIFICATION OF DNA

After DNA isolation, quantification and quality analysis are unavoidable to ascertain the accurate quantity and purity of DNA sample for future analysis. This is a significant step for carrying out restriction digestion or performing different techniques like PCR and RAPDs.

There are most commonly used methods for quantifying the amount of nucleic acid

(i) Gel electrophoresis of aliquot of DNA sample with standard DNA.

(ii) Spectrophoto metric determination

(iii) Flour metric determination

(iv) DNA quantification using Micro Drop.

➢ DNA QUANTIFICATION USING MICRODROP

The Micro Drop spectrophotometer works as a confinement system that holds up 1-2µl of sample depriving the usage of cuvettes and capillaries. Merging fiber optics technology and surface tension, the sample is placed between two optical surfaces that define the path lengths in vertical direction. The framework utilizes shorter path lengths, which result in broad range of nucleic acid concentration measurement, therefore eliminating the need of making aliquots. Preparation of next sample only requires wiping of both vertically placed optical surfaces with simple laboratory wipe. Total time required for completion of whole process is near about ~30 sec. We used Micro Drop spectrophotometer for quantification of our nucleic acid.

Materials required for this process are:

1. Sample [nucleic acid]
2. Water or buffer in which sample is dissolved [TE buffer pH 7.3 in this case]
3. Micro Drop spectrophotometer.

PROCEDURE TO USE MICRODROP

1. Pipetting 2-3 µl of demonized water on the optical surfaces for cleaning of the micro spectrophotometer sample retention plate. First row of the retention plate serve as blank and they are loaded with 1µl of TE buffer. There are 8 rows in total thus making 16 spots but two of them work as blank so at a time 14 samples can be quantified.
2. The plate is placed in the Micro Drop.
3. Micro Drop software was opened and nucleic acids module was selected.
4. Access the program by clicking on 'RUN'. Once the measurement is done the plate will eject out automatically clean the plate with demonized water.
5. The 260/280 ratio and concentration of the sample are shown on the software in tabular manner.

POLYMERASE CHAIN REACTION

A revolutionary method developed by Kari Mullis in 1980s.It is a methodical technique in molecular biology for amplification of single or few copies of piece of DNA. First step is to design a primer that is two short DNA sequences which can bind to the start and end of the DNA target.

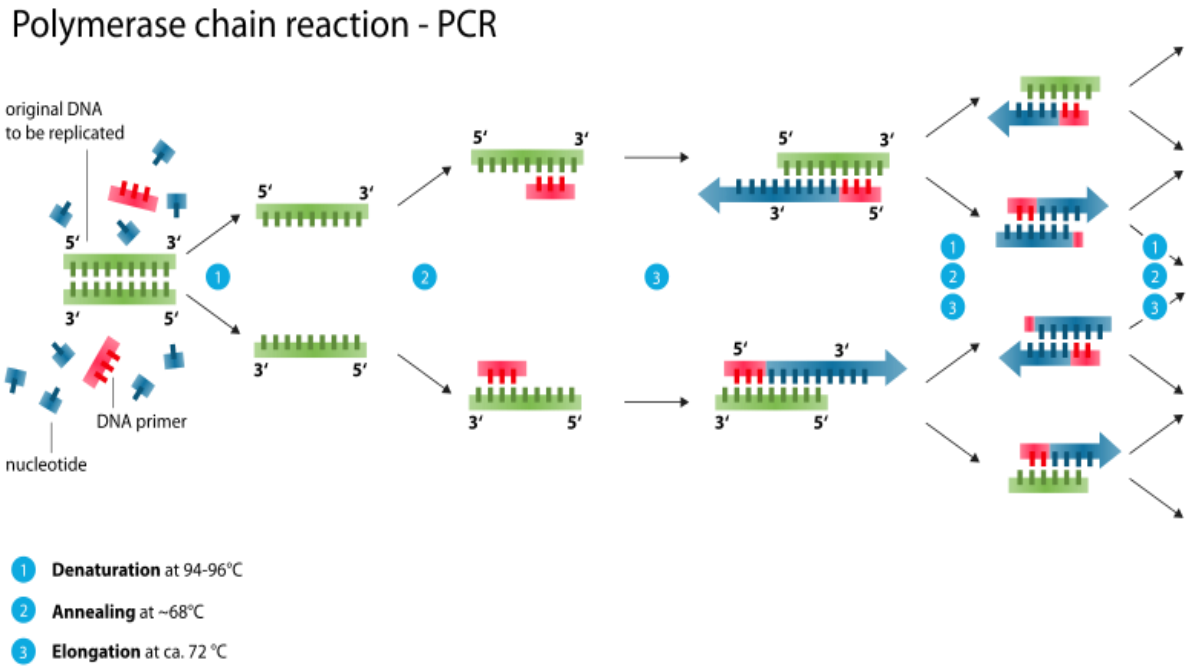
To carryout PCR reaction we require following:

1. Primer
2. Free nucleotides
3. Enzymes- DNA polymerase

4. Template strand

Rest of the process is carried out in PCR machine where machine automatically increases and decreases the temperature of sample in automatic and programmed steps.

Figure 7: Diagrammatic view of PCR



1. PRIMER DESIGNING

The primers were retrieved from the research paper we followed to carry out our project. After retrieval the set of primers were re-checked using BLAST from NCBI.

Table 2: Primer obtained for *CYP1A1* gene

Sequence (5'>3')	Primer	Length (bp)	Tm
Forward primer	CAGTGAAGAGGTGTAGCCGC	20	61.4°C
Reverse primer	TAGGAGTCTTGTCTCATGCC	20	57.3°C
Product length		340	
Restriction enzyme	MSP1		

PRIMER RECONSTITUTION

To make volume of 100µM, following amount of nuclease free water is added.

Table 3: Reverse and forward primers after reconstitution

PRIMERS	**NFW(µl)**
4646093F	276.1
4646093R	285.6

PCR

OPTIMIZATION

GRADIENT PCR

It is a technique that is precisely used for the empirical determination of optimal annealing temperature for both set of primers used in further amplification reactions.

Table 4: Gradient PCR conditions

Reaction components	Per reaction volume (µl)
Master mix	5.4 µl
DNA template	1 µl
Annealing temperatures	57°C, 58°C, 59°C, 60°C, 61°C, 62°C
Forward primer	.2 µl
Reverse primer	.2 µl
Nuclease free water	5.2 µl
Total	12 µl

Table 5: PCR components used for DNA amplification

Reaction components	Per reaction volume (µl)
Master mix	5.4
DNA template	1
Forward primer	.2
Reverse primer	.2
Nuclease free water	5.2
Total	12

Table 6: PCR cycling conditions for rs4646093

Steps	Temperature (°C)	Time	Cycle/s
Initial Denaturation	94	3 min	1
Denaturation	94	35 sec	
Annealing	61	35 sec	
Extension	72	1 min	32
Final extension	72	5 min	1
Hold	°C 4	∞	-

Genotyping PCR-RFLP

A process of determining small genetic differences that can lead to major changes in phenotypes, including both physical differences that make every individual unique and pathological changes which can cause diseases. PCR product for rs4646093 was digested with restriction enzyme MSP1.

Table 7: Reaction conditions for RFLP

Reaction components	Reaction volume for rs4646093
Nuclease free water	3.4 µl
Cut smart buffer	1.5 µl
Enzyme (1 unit) MSP1	0.1 µl
PCR product	10 µl
Total	15 µl

Analysis of digested PCR Product:

- Banding pattern was studied using Agarose gel electrophoresis containing EtBr.
- A 100bp marker ladder was used.
- 15 µl of amplified and digested product was loaded into the wells.
- Run time of gel was 40-50 minutes in 1X TAER buffer at 100volts.
- Bands were finally visualized using gel Doc system.

Table 8: Various genotypes with their banding patterns

Genotype	Band size (bp)
CC (WW)	340+200
TC(WM)	340+200+140
TT(MM)	340

Statistical analysis

After performing the experimentation on around 625 samples it is necessary to determine the association of *CYP1A1* gene polymorphism and its susceptibility with common cancers. For performing this we have used the tool MedCalc for the calculation of. Odd ratio and the confidence interval. In order to calculate the frequency of mutant allele (which is "C" here) we have also used Hardy Weinberg equation.

CHAPTER 5

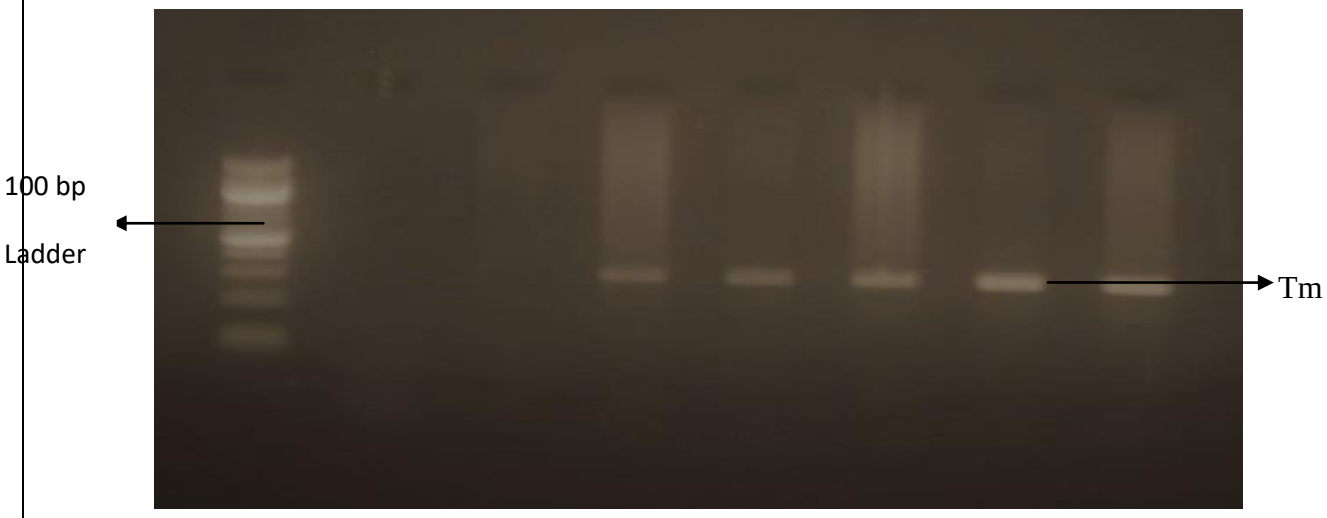
Result and discussion

GRADIENT PCR GEL RESULT FOR rs4646093

Figure 8: visualization of gradient PCR

L T1 T2 T3 T4 T5 T6 T7

56°C 57°C 58°C 59°C 60°C 61°C 62°C



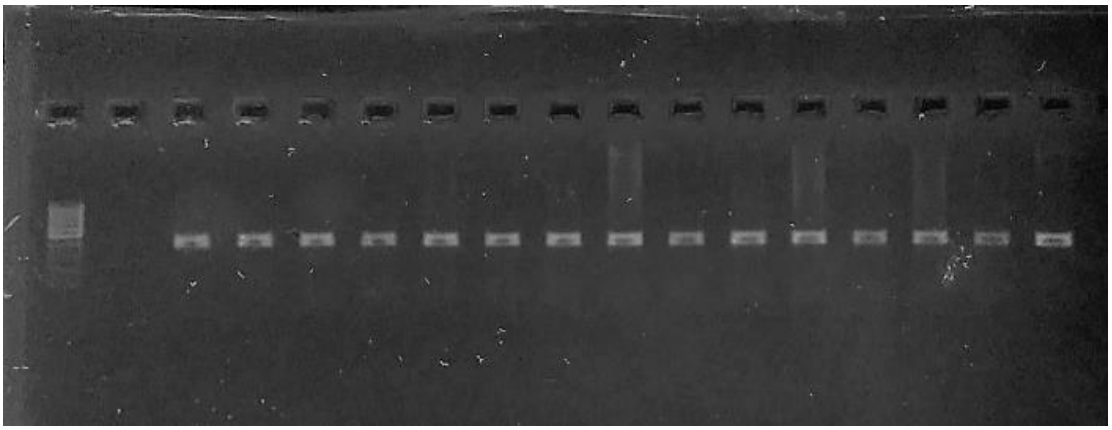
Lane1: Ladder (100 bp) **Lane 2**: sample at 56°C **Lane 3**: sample at 57°C

Lane 4: Sample at 58°C **Lane 5**: Sample at 59°C **Lane 6**: Sample at 60°C

Lane 7: Sample at 61°C **Lane 8**: Sample at 62°C

Figure 9: Agarose Gel Electrophoresis (1.5%w/v) showing the amplification of PCR products.

L -ve 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



Afterwards restriction digestion has been carried of amplified PCR product through restriction digestion enzyme MSP1.

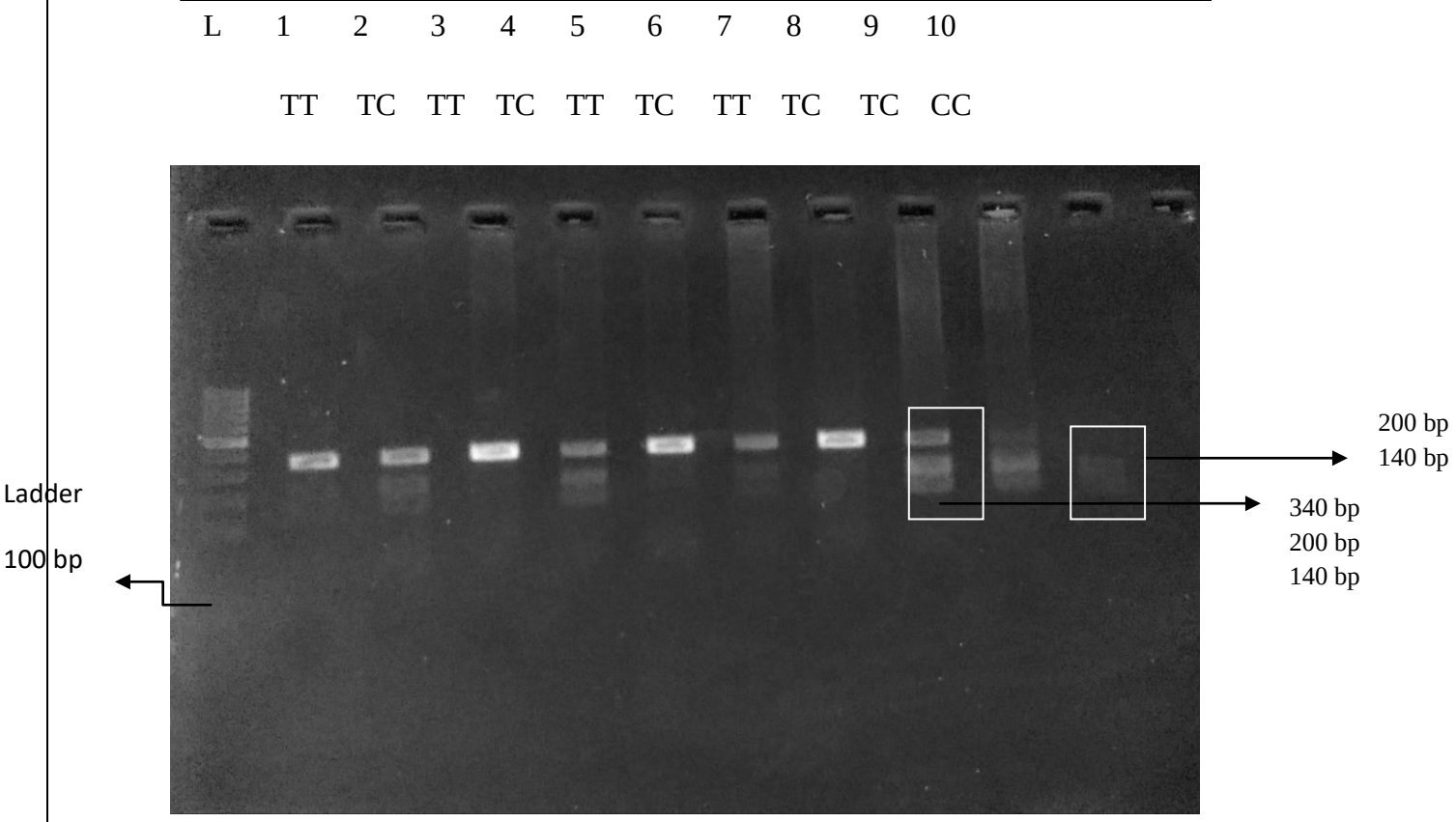
Lane 1: Ladder **Lane 2**: negative sample **Lane 3**: sample1 **Lane 4**: Sample 2

Lane 5: Sample 3 **Lane 5**: Sample 4 **Lane 6**: Sample 5 **Lane 7**: Sample 6

Lane 8: Sample 7 **Lane 9:** Sample 8 **Lane 10:** Sample 9 **Lane 11:** Sample 10

Lane 12: Sample 11 **Lane 13**: Sample 12 **Lane** 14: Sample 13 **Lane15**: Sample 14

Figure 10: Visualization of samples after genotyping of different samples of cancer



Lane 1: Ladder (100 bp) **Lane 2**: sample 1 (340bp) **Lane 3**: sample 2 (340+200+140bp) **Lane 4**: sample 3 (340bp) **Lane 5**: sample 4 (340+200+140bp) **Lane 6**: sample 5 (340bp) **Lane 7**: sample 6(340+200+140 bp) **Lane 8**: sample 7 (340bp) **Lane 9**: sample 8 (340+200+140bp) **Lane 10**: sample 9 (340+200+140 bp) **Lane 11**: sample 10 (200+140bp)

RESULT ANALYSIS

rs4646093

A total of 184 control samples and along with them 107 samples of head and neck cancer, 151 samples of lung cancer and 89 samples of cervical cancer were analyzed for rs4646093 in the gene CYP1A1. After the genotyping of CYP1A1 gene it was found that "C" allele was present in ~37% of the total cases in head and neck cancer and around 44% in the control samples. Similarly in case of lung cancer the frequency of mutant allele was found to be ~26% and in case of cervical cancer the frequency was ~29%.

Table 8: Genotypic analysis of rs4646093 for head and neck Cancer

Genotype	HNC n/N [%]	Control n/N [%]	Odds ratio	Confidence Interval	p-Value
TT	67/107 (62.6%)	149/184(80.9%)		Ref	
CT	40/107(37.3%)	36/184(19.5%)	**2.4710**	**1.4477-4.2177**	**0.0009**
CC	0/107(0%)	1/184(0.5%)	0.7383	0.0297-18.3587	0.8532
T	107/107(100%)	183/184(99.4%)		Ref	
C	40/107 (37.3%)	37/184(44%)	**2.4710**	**1.4477-4.2177**	**0.0009**

Table 9: Genotypic analysis of rs4646093 for Lung Cancer

Genotype	Lung Cancer n/N [%]	Control n/N [%]	Odds ratio	Confidence Interval	p-Value
TT	112/151(74.1)	149/184(80.9%)		Ref	
CT	39/151(25.8)	36/184(19.5%)	1.4412	0.8611-2.4122	0.1643
CC	0/151(0)	1/184(0.5%)	0.4430	0.179-10.9762	0.8532
C	39/151(25.8%)	183/184(99.4%)	1.4412	0.8611-2.4122	0.1643
T	151/151(100%)	37/184(44%)		Ref	

Table 10: Genotypic analysis of rs4646093 for Cervical Cancer

Genotype	Cervical n/N [%]	Control n/N [%]	Odds ratio	Confidence Interval	p-Value
TT	63/89(70.7%)	149/184(80.9%)		Ref	
TC	26/89(29.2%)	36/184(19.5%)	1.7081	0.9524-3.0624	0.0724
CC	0/89(0%)	1/184(0.5%)	0.7848	0.315-19.5260	0.8825
C	26/89(29.2%)	183/184(99.4%)	1.7081	0.9524-3.0624	0.0724
T	89/89(100%)	37/184(44%)		Ref	

From the above observations after the net analysis we can see that allelic frequencies as calculated from HWE for different type of cancer for SNP rs4646093 where “p” is the frequency of wild type allele and “q” is the frequency of mutant type allele.

So for Cervical cancer p=0.8539, q=0.1461

For Head and Neck cancer p=0.8131, q=0.1869.

And, for lung cancer p=0.8907, q=0.1291.

Since, only one mutant was found in control sample and after calculation was done the carrier frequency found to be 1 in 7.32 which is around 13.66%. The resultant statistical analysis as done with the help of MEdcalc tool for rs4646093 for different type of cancers are listed as-

For Head and neck cancer: for “CT”, OR=2.4710, CI=1.4477 to 4.2177, p=0.0009; for “CC” OR=0.7383, CI=0.0297 to 18.3587, p=0.8532.

For Lung cancer: for “CT”, OR=1.4412, CU=0.8611 to 2.4122, p=0.1643; for “CC”, OR=0.4430, CI=0.179 to 10.9762, p=0.6191.

For Cervical cancer: for “CT”, OR=1.7081, CI=0.9524 to 3.0624, p=0.0724; for “CC”, OR=0.7848, CI=0.3152 to 19.5260, p=0.8825.

Conclusions and Future prospects

After performing the genotyping in association with the SNP rs4646093 to check its susceptibility with association to common cancer in Himachal Pradesh population, the results show that no association with the case samples of different cancers were found with the variant rs4646093 in the local population of the state with respect to the different common cancers found such as lung, head and neck and cervical cancer. This data can further be used to check the range of mutation that the local population of Himachal Pradesh has and with greater sample number maybe the chances of occurrence of allelic frequency might turn out with greater number. Further this data can also be used in order to take references for checking the association of same SNP rs4646093 with the gene CYP1A1 in different population in different states of the country.

APPENDIX

GLASSWARE AND INSTRUMENTS

Glassware

• Storage tubes

• Micropipette tips

• Measuring cylinder

Storage bottles

INSTRUMENTS

• PCR vial stand

• Pipette

• PCR

• Minicentrifuging machine

• Weighing balance

• Autoclave

•PCR cabinet

• Vortexingmachine

• Centrifuge machine

• Gel electrophoresis setup

• Gel Dock

REFERENCES

1. Ingole SP, Kakde AU, Bonde PB. "*A review on statistics of cancer in India*", J Environ SciToxicol Food Technol, vol.10, pp.107-6, July. 2016.
2. Jemal A, DVM, Bray F, Center M, Ferlay J, Ward E, Forman D. " *Global CancerStatistics ‖"*Ca Cancer J Clin, Vol. 61(2), pp. 69–90,2011.
3. NICPR, feb, 2019.
4. Head and neck cancer, national cancer institute.
5. Mimi CY, Yuan JM. *"Epidemiology of nasopharyngeal carcinoma"*, In Seminars in cancer biology, Academic Press, Vol. 12, No. 6, pp. 421-429, Dec 2002.
6. Guha N, Boffetta P, WünschFilho V, ElufNeto J, Shangina O, Zaridze D, Curado MP, Koifman S, Matos E, Menezes A, Szeszenia-Dabrowska N. *"Oral health and risk of squamous cell carcinoma of the head and neck and esophagus: results of two multicentric case-control studies*", American journal of epidemiology, vol.166(10),pp. 1159-73, Aug.2007.
7. Preston-Martin S, Thomas DC, White SC, Cohen, *" Prior Exposure to Medical and Dental X-rays Related to Tumors of the Parotid Gland"*, JNCI: Journal of the National Cancer Institute,vol.80(12),pp.943-9, Aug.1988.
8. Chan JK, Yip TT, Tsang WY, Poon YF, Wong CS, Ma VW. *"Specific association of Epstein-Barr virus with lymphoepithelial carcinoma among tumors and tumor like lesions of the salivary gland",* Archives of pathology & laboratory medicine,Vol.118(10),pp.994-7,Oct.1994
9. Poddar A, Aranha RR, Muthukaliannan GK, Nachimuthu R, Jayaraj R. *"Head and neck cancer risk factors in India: protocol for systematic review and meta-analysis*". BMJ open,Vol.8(8),pp.020-014, Aug .1998.
10. Shah SB, Sharma S, D'Cruz AK. *"Head and neck oncology. The Indian scenario",* South Asian journal of cancer,Vol.5(3),pp.104, Jul.2016.
11. Yancik R. Cancer burden in the aged. *"An epidemiologic and demographic overview. Cancer: Interdisciplinary International"* Journal of the American Cancer Society. Vol.80(7),pp.1273-83,Oct.1997.

12. Lewis DF, Watson E, Lake BG. *"Evolution of the cytochrome P450 superfamily: sequence alignments and pharmacogenetics",*Mutation Research/Reviews in Mutation Research. Vol.410(3),pp.245-70, Jun.1988.
13. Ingole SP, Kakde AU, Bonde PB. *"A review on statistics of cancer in India",* J Environ SciToxicol Food Technol, Vol.(10)pp.107-6,2016.
14. Croce CM "*Oncogenes and cancer".* New England journal of medicine, Vol.31,pp.358(5)-502-11,Jan.2008.
15. Evan GI, Vousden KH. "*Proliferation, cell cycle and apoptosis in cancer".* Nature, Vol.411(6835),pp.342,May.2001.
16. Suruda A, Schulte P, Boeniger M, Hayes RB, Livingston GK, Steenland K, Stewart P, Herrick R, Douthit D, Fingerhut MA. *"Cytogenetic effects of formaldehyde exposure in students of mortuary science".* Cancer Epidemiology and Prevention Biomarkers, Vol.2(5),pp.453-60,Sep.1993.
17. Suruda A, Schulte P, Boeniger M, Hayes RB, Livingston GK, Steenland K, Stewart P, Herrick R, Douthit D, Fingerhut MA. *Cytogenetic effects of formaldehyde exposure in students of mortuary science.* Cancer Epidemiology and Prevention Biomarkers,Vol.2,pp.453-60,Sep.1993
18. Clapp RW1, Jacobs MM, Loechler EL; *"Environmental and occupational causes of cancer: new evidence* 2005-2007"; Rev Environ Health. Vol.23,pp.1-37,2008.
19. Ames BN, Gold LS, Willett WC. *The causes and prevention of cancer*. Proceedings of the National Academy of Sciences, Vol.92(12),pp.5258-65,Jun.1995
20. A. J. Phillips., *"The Epidemiology of Cancer",* Vol.15,pp.4447,1969.
21. Kozłowski M, Marciak-Kozłowska J. *Cancer Tumor Life: Biological & Physical Aspects.* Nova Science; 2012.
22. Ali I, Wani WA, Saleem K. *Cancer Scenario in India with Future Perspectives*. Cancer therapy,Vol.8, Jan.2011.
23. Nam J, Son S, Park KS, Zou W, Shea LD, Moon JJ. *Cancer nanomedicine for combination cancer immunotherapy*. Nature Reviews Materials. Vol 26, pp.1, Apr.2019
24. Mallath MK, Taylor DG, Badwe RA, Rath GK, Shanta V, Pramesh CS, Digumarti R, Sebastian P, Borthakur BB, Kalwar A, Kapoor S. *The growing burden of cancer in India: epidemiology and social context.* The Lancet Oncology, Vol 5,pp.205-12,May.2014.

25. Kerbel RS. "*Expression of multi-cytokine resistance and multi-growth factor independence in advanced stage metastatic cancer. Cancer melanoma as a paradigm*." The American journal of pathology1,Vol.41,pp.519,Sep.1992
26. Widschwendter M, Jones A, Evans I, Reisel D, Dillner J, Sundström K, Steyerberg EW, Vergouwe Y, Wegwarth O, Rebitschek FG, Siebert U,*"Epigenome-based cancer risk prediction: rationale, opportunities and challenges*". Nature reviews Clinical oncology,Vol.15,pp.292, May.2018.
27. Morgan DO. "*Principles of CDK regulation*." Nature,Vol.374,pp.131,Mar.1995
28. Weinberg RA. Tumor suppressor genes. Science. 1991 Nov 22;254(5035):1138-46.
29. Wu X, Zhao H, Suk R, Christiani DC, *"Genetic susceptibility to tobacco-related cancer"* Oncogene.Vol.23,pp.6500, Aug.2004.
30. Nebert DW, Dalton TP. "*The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis."* Nature Reviews Cancer. Vol.6,pp.947,Dec.2006
31. Vineis P. "*Individual susceptibility to carcinogens"*,Oncogene. Vol.23,pp.6477,Aug.2004.
32. Varela-Lema L, Taioli E, Ruano-Ravina A, Barros-Dios JM, Anantharaman D, Benhamou S, Boccia S, Bhisey RA, Cadoni G, Capoluongo E, Chen CJ. "*Meta-analysis and pooled analysis of GSTM1 and CYP1A1 polymorphisms and oral and pharyngeal cancers: a HuGE-GSEC review",* Genetics in medicine, Vol.10,pp.369,Jun.2008
33. Steenland K, Tucker J, Salvan A. "*Problems in assessing the relative predictive value of internal markers versus external exposure in chronic disease epidemiology*", Cancer Epidemiology and Prevention Biomarkers. Vol.;2,pp.487-91,Sep.1993.
34. Shields PG. "*Molecular epidemiology of smoking and lung cancer*",Oncogene,Vol.21,pp.6870, Oct.2002.
35. Preskill C, Weidhaas JB. "*SNPs in microRNA binding sites as prognostic and predictive cancer biomarkers"*, Critical Reviews™ in Oncogenesis. Vol.18,2013.
36. Rasmussen HB. Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) and gel electrophoresis-valuable tool for genotyping and genetic fingerprinting. InGel electrophoresis-principles and basics 2012 Apr 4. InTechopen.