Determination of the Association of Head and Neck Cancer with NOTCHI Gene Polymorphism in Pakistani Population

By

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Cancer

Cancer - Diagnosis

Retinoblastoma.

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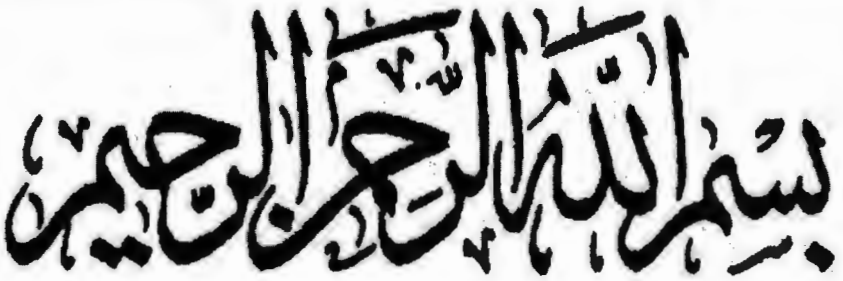
بسم الله الرحمن الرحيم

In the name of Allah,
The most Gracious, The most merciful.

"And say: My Lord increase me in knowledge."

(Qur'an, Ta-Ha 20:114)

"My Lord, put my heart at peace for me, make my task easy for me, and loose a knot from my tongue so they may understand my speech."

(Qur'an, 20:25-2

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Miss. Kiran Noreen and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for MS degree in Biotechnology.

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A thesis is submitted to Department of Bioinformatics and Biotechnology, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Biotechnology.

Dedication

DEDICATION to ALLAH

Say, *"Indeed, my prayer, my rites of sacrifice, my living and my dying are for Allah, Lord of the worlds"*

(Qur'an 6:163)

Dedication to PARENTS

You are both special in every way,
Encouraging me more and more each passing day.

You both are the reason why I'm so strong,
With you two at the helm not a thing could go wrong.

You've both helped me through many trials and tribulations,
You've made things better in every situation.

Thank you both for always being there,
And showing me that you truly care.

Words could never explain how I feel about you,
But I hope you know that I truly love you two!

DECLARATION

I hereby declare that the work present in this thesis is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Dated: _____

Kiran Noreen

Kiran

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My first word of thanks to the One Who taught words to Adam, Who blessed man with knowledge, Who is the sublime without Whose *"Kun"* nothing is possible.

Secondly my humblest thanks from the core of my heart to my beloved prophet HAZRAT MUHAMMAD (S.A.W.W) who is the eternal fountain of knowledge and guidance for the whole mankind.

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I pray to ALLAH Almighty to give me the strength and resources to serve world to the best of my efforts and wisdom not to neglect humanity. AMEEN

LIST OF ABBREVIATIONS

HNC	Head and Neck Cancer
TNM	tumor, node, metastasis
HPV	human papilloma virus
MDM2	Mouse double minute 2 homolog
ATM	Ataxia telangiectasia mutated
ATR	Ataxia Telangiectasia Rad 3
CDK	Cyclin Dependent Kinases
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Rb	Retinoblastoma
EGF	Epidermal growth factor
NICD	NOTCH intracellular domain
NECD	NOTCH extracellular domain
TAD	Transcriptional activation domain
PEST	proline, glutamic acid, serine and threonine residues.
HD	heterodimerization domain
PTB	phosphotyrosine binding domain
CI	Confidence Interval
SNPs	Single Nucleotide Polymorphism
PCR	Polymerase Chain Reaction
TARMS	Tetra Amplification Refractory Mutation System

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Abstract

Head and neck cancer are the malignant that appears in the tissue, organ and mucous membrane of head and neck. It is stated as the seventh most common malignancy in the world. According to the reports, about 900,000 novel cases and 350,000 deaths have been estimated every year worldwide. It has been reported more in Males than females with a fluctuating ratio from 2:1 to 4:1 and it occurs mostly in men at the age of 50 or above 50. In Pakistan, Head and Neck cancer is almost one-tenth of all cancers in females and approximately one-fifth of all the cancer in male population. Multiple risk factors have been identified to develop the risk of HNC including modifiable (tobacco smoking, alcohol consumption, diet) and non-modifiable risk factors (family history, age, gender). Diverse genomic modifications can lead to the development of head and neck cancer. A tumor suppressor gene, NOTCH1 gene has been considered as one of the key innovative finding in causing head and neck carcinoma. It is located on chromosome number 9 consisting of 34 exons. Frame shift, synonymous, missense and nonsense mutations have been detected in EGF like repeats domain, playing an important role in ligand binding that ultimately activates the NOTCH1. This study was designed to analyze the significant association of NOTCH1 gene (rs139994842) with head and neck cancer in selected Pakistani population. DNA was extracted from all 100 HNC and 50 control samples. Tetra-ARMS PCR was performed to genotype NOTCH1 polymorphism. Among all three genotypes, two genotypes GG and GA were associated and third genotype AA was not associated in this study. NOTCH1 C1083 rs139994842 polymorphism was found higher in HNC patients than controls. Calculated p-value of NOTCH1 was significant (0.000) and allelic frequency of G was 0.71% and frequency of A allele was 0.58%. These findings showed that in our selected population, NOTCH1 gene rs139994842 shows significant association with Head and neck cancer.

Chapter 1

Head and neck cancer are the malignant that appears in the tissue, organ and mucous membrane of head and neck. Head and neck cancer is not a single term.it is actually the combination of wide variety of tumor types which includes craniofacial bones, soft tissues, skin, mucosal membrane, salivary glands. Head and neck is used to define all carcinoma originating from the epithelium lining the Sino nasal tract, oral cavity, oropharynx, hypo pharynx, larynx, throat, lips, mouth and nose.

1.1 Occurrence of Head and Neck cancer

Head and Neck Cancer are stated as the seventh most common malignancy in the world. According to the reports, about 900,000 novel cases and 350,000 deaths have been estimated every year worldwide (Ferlay *et al.,* 2010) (Parkin *et al.,* 2005) (Silveira *et al.,*2012).

Figures indicated that in Europe, there were new 250,000 cases and 63,500 reported deaths with Head and Neck cancer in 2012 (Gatta *et al.,* 2015). In the United States, it has been estimated that about 62,000 Americans carrying this malignancy and approximately 13,000 dying every year (Siegel *et al.,* 2016). In 2007 95,660 new cases were observed (Jemal *et al.,* 2007). In 2009 97,000 new cases and 11,000 deaths were reported in USA. It occurs mostly in men at the age of 50s or 60s, however the frequency among younger are increasing day by day (Jemal., 2009).

Head and neck cancer have been reported more in Males than females with a fluctuating ratio from 2:1 to 4:1. It have been reported that in the states of central and eastern Europe, France, Spain, Hong Kong, Italy, Brazil, United States and Indian subcontinent, the occurrence rate exceeds 20 per 100,000 in males. The research has revealed that the incidence of pharyngeal and laryngeal cancer is fifty percent higher in African American men than women (DeSantis *et al.,* 2013). Asian literature has presented that the cancer of Mouth and tongue are more common in Indian subcontinent than the other types and the Nasopharynx have been reported as the widely spreading cancer in Hong Kong (Bray *et al.,* 2008) (Lambert *et al.,*2011).

Australian literature has reviewed that in Australia, head and neck cancer is becoming a serious and developing disease. The frequency of HNC is reported more in males than females. In males the occurrence seems to be 12.3 per 100,000 (Australian Institute of Health and Welfare and Australasian Association of Cancer Registries, 2008). The Oropharynx cancer is dominant in Australia. Its frequency is increased in the time period of 2002-2011 from 23% to 28%. It contributes 2–3% of all the cancers in Australia (Park *et al.*,2011). The indigenous Australian have high frequency of head and neck carcinoma (Cunningham *et al.*, 2008) as it has been reported that rectal, colon, prostate, testis, breast, brain tumor lymphoma and leukemia have low rate of occurrence when compared with the frequency of Oropharyngeal carcinoma, thus Head and Neck Cancer having high rate of incidence among original Australians (Zhang *et al.*, 2011).

1.1.2 Occurrence in Pakistan

In the field of technologies, multi-disciplinary treatment approaches and recent surgical novelties, these innovations carry the rapid changes and perfections. However regardless of these innovations, the ratio for Head & Neck Carcinoma illustrated that patients have not considerably upgraded much regarding the innovative treatment aspect (Carvalho *et al.*, 2004).

It is the most shocking situation for South-Asian countries especially in Pakistani population where Head and neck carcinoma seems to be one of the most publically common carcinoma. HNC is reported to be the second most highly elevated carcinoma in both male and female Pakistani population (Jemal *et al.*, 2011). The highest occurrence rate of oral cancer is observed in the region of Karachi (Chaudhry *et al.*, 2008).

As for as the occurrence ratio in Pakistan is estimated, Head and neck cancers is demonstrated as almost one-tenth of all cancers in females and approximately one-fifth of all the cancer in male population (Bhurgri *et al.*, 2006).

1.2 Symptoms of Head and Neck cancer

Head and cancer can lead to the development of abnormal patches or ulcer creating open sores in mouth and throat, pain in mouth, unusual bleeding, sore throat, mass created in the neck, discomfort during swallowing, appearance of dark patches in the mouth cavity, change in voice, Earache, overlapping of speech, complications in breathing or rising size of lymph nodes, weight loss. If it remains undiagnosed it can spread to other part of body such as lungs. It will have worse prognosis and can become fatal. The survival rate is five years in most cases after diagnosis. A substantial illness and death rate have been reported in this disease (Leemans *et al.,* 2011).

1.3 Types of Head and Neck cancer

Head and Neck cancer is divided in to the following types:

- Oral Cavity cancer
- Nasopharynx cancer
- Oropharynx cancer
- Hypopharynx cancer
- Larynx cancer
- Paranasal Sinuses cancer

1.3.1 Oral Cavity Cancer

Oral cavity malignant tumor shows strong relationship with the consumption of alcohol and tobacco. It comprises the region of inner lip, tongue, palate and floor of mouth. Appearance of the tumor in this particular area is mostly treated with the surgery named as Maxillectomy, Mandibulectomy, Glossectomy.

1.3.2 Nasopharynx Cancer

The particular area where the Eustachian tubes along with the nasal cavities join the upper portion of the throat known as the nasopharynx. Nasopharyngeal carcinoma is known to be appearing in this nasopharynx region.

1.3.3 Oropharynx Cancer

Oropharyngeal tumor demonstrates the strong relationship with human papilloma virus infection. Human papilloma virus is recognized as the strong causative agent in causing head and neck cancer. Patient with oropharynx tumor are more at risk to develop second head and neck tumor. The base of the tongue, soft palate and tonsils made up the middle portion of the throat known as oropharynx. Oropharyngeal carcinoma arises in this region (Krishnatreya *et al.,* 2013).

1.3.4 Hypopharynx Cancer

At diagnoses, hypopharynx cancer mostly appears at an advanced stage. Hypo pharyngeal carcinoma tends to appear in the area of hypopharynx which constitute the posterior pharyngeal wall, post cricoid and pyriform sinuses.

1.3.5 Larynx Cancer

This tumor illustrates the strong relationship with the tobacco consumption as well. Larynx is also known as voice box. Laryngeal cancer arises in this larynx region which is subdivided in to glottis, sub glottis and supraglottis.

1.3.6 Paranasal Sinuses Cancer

The tumor arises in the paranasal sinuses is subdivided in to maxillary sinus, frontal sinuses, sphenoid and ethmoid system. The most common sinonasal sinuses tumor is maxillary sinus (Krishnatreya *et al.,* 2013).

1.4 Classification of Head and neck cancer

Head and neck cancer are classified as

- Squamous Cell Carcinoma
- Adenocarcinoma

The tumor that appears in the area of both skin and mucous membrane term as squamous cell carcinoma. This carcinoma has been reported to arise in more than 90% of head and neck cancer cases. Researches evaluate that squamous cell carcinoma is considering as susceptible more to arise in males having smoking and alcohol consumption history with the age above 40 years. Adenocarcinoma are the tumor arises in the region of epithelial tissues with glandular characteristics are characterized as adenocarcinomas. Several head and neck cancers are reported to be adenocarcinomas (Haines., 2013).

1.5 Staging of Head and Neck cancer

The TNM staging system is used to categorize the disease of head and neck cancer. TNM is the abbreviation of tumor, node, and metastasis. It permits the physicians to classify the region of head and neck carcinoma for analyzing the status and grade of the disease, diagnoses and management. The TNM staging is used to allocate the stages I, II, III, or IV to the patient thus describing the disease status of the patient. Stage I, II is designated as an early stage disease and III, IV as an advanced disease stage.

1.6 Risk factors of Head and Neck cancer

In Head and Neck cancer, alteration of the DNA in the cell is influenced by the diversity of factor. Both the environmental and genetic factor also plays an important role in HNC. Smoking and alcohol are major risk factor for the establishment of this malignancy (Haddad *et al.*, 2008). Although all the conditions either separately or in combination play an important role in the development of HNC, these conditions include smoking, carcinogen exposure, alcohol consumption, infectious agents, diet, oral hygiene, preexisting medical condition or having another disease and family history.

Risk factors classified as

- Modifiable
- Non modifiable

1.6.1 Modifiable risk factors

1.6.1.1 Tobacco Smoking

One the noticeable factor in causing Head and Neck cancer is tobacco smoking and it is associated with duration, amount and strength of smoking. Inhibit smoking doesn't eliminate but can decrease the risk of cancer development (Schlecht *et al.*, 1999). The gene toxic effects of toxin present in tobacco smoke such as nitrosamines and polycyclic hydrocarbons are considered as the major threat.

Recent researches have shown that tobacco smoking is associated with high incidental rate of Head and Neck cancer (Blot *et al.*, 1988) (Basu *et al.*, 2008). It has been reported that the risk of developing HNC in smoker patient is 5 to 10 times higher than nonsmokers (John *et al.*, 2014). Smokeless tobacco and areca nut are the main factors contributing toward the occurrence of HNC in south Asian countries (Graham., 1977) (Dayal *et al.*, 1978). Zarda, snuff, mashiri, khaini, mava, paan are the other types of smokeless tobacco

which are reported to be commonly used in India, Bangladesh, Pakistan, Sri Lanka, Thailand, Taiwan, Malaysia and China (Gupta *et al.,* 2002).

In Asian countries, betel quid is predicted to be another risk factor in causing oral cancer. Betel quid is prepared by combining various ingredients together such as seeds of betel, tobacco, pan, betel leaf, catechu and slaked lime (Proia *et al.,* 2006) (Warnakulasuriya *et al.,* 2002). It has been reported that betel quid is continuously consumed by 10% of world population (Gupta *et al.,* 2004).

Oral sub mucous fibrosis is a chronic disorder of aero digestive tract causing stiffness of the oral mucosa (Angadi *et al.,*2011). One of the carcinogen known as areca nut which have shown association with this disease (Gupta *et al.,* 1998). In western countries this disease is rare because areca nut is not commonly use. In Indian population areca nut is very popular where approximately 5 million people suffer from the disorder of oral sub mucous fibroses.

The reports have demonstrated that the tobacco consumption in India is increasing day by day where 8.4 million deaths are expected to occur due to tobacco usage by the year 2020 (Murray *et al.,* 1996). Head and Neck cancer in south Asian countries are more observed in people with worse socio economic status (Agarwal *et al.,* 2011). Thus rise in the rate of tobacco consumption is increasing the disease burden on the society

In Australian population, tobacco smoking is contributing to the 70% of larynx carcinoma (Australian Institute of Health and Welfare, 2004). One of the studies has demonstrated that cannabis smoke containing high level of carcinogen than the cigarette smoke. It can play role in causing head and neck cancer (Wu *et al.,* 1988). The study reveals that in Australian remote communities, people are more turning toward the use of cannabis smoke due to having limitations on alcohol consumption (Lee *et al.,* 2009).

In the report of European Union, it has been estimated that cigarette smoking is the factor causing head and neck carcinoma especially oral cancer in almost 30% women and 60% men (Bray *et al.,* 2002).

Research illustrates that the patients who smoke have shown TP53 alteration as compared who do not smoke (Brennan *et al.,* 1995). Although the straight forward association have been difficult to create as obviously not every patient who develop HNC through smoking attains TP53 mutation. It is indicating the individual differences in carcinogen metabolizing enzymes which may transform cancer risk. Researchers have main attention on genetics polymorphism in those enzymes that initiate pro-carcinogen and detoxify carcinogen (Ho *et al.,* 2007). However, the relationship may not be direct, the risk may have linked to the complex relations among exposure, activation, detoxification and repair of DNA damage.

1.6.1.2 Heavy alcohol consumption

Another important risk factor in the development of HNC is high alcohol consumption (Sturgis *et al.,* 2004). Alcohol itself is not a direct carcinogen. It metabolizes acetaldehyde and establishes DNA adducts that alter DNA synthesis and repair (Brooks *et al.,* 2005).

It has been researched that the active metabolite of alcohol such as acetaldehyde are carcinogenic and they are capable to cause head and neck carcinoma. A large number of the patients have been reported in which alcohol individually act as a risk factor. People consuming alcohol heavily have five times greater risk of developing HNC as compared to those who do not consume (Pelucchi *et al.,* 2008). In the developed countries, the consumption of alcohol is increasing heavily day by day specially the western countries. In 2008, one of the study conducted in Australian population have demonstrated that out of 180 countries worldwide, Australia is on 30th number for consuming high level of alcohol (Victoria Department of Health, 2008).

1.6.1.3 Infection with human papilloma virus HPV

The third most important risk factor in causing head and neck cancer is human papilloma virus. HPV infection play role about 25% in causing HNC (Termine *et al.,* 2008) (Kreimer *et al.,* 2005) (Dayyani *et al.,* 2010). HPV is considered as an important causative agent in causing head and neck cancer with the high prevalence rate up to 50% (Cruz *et al.,* 1996).

The most affected areas are the base of tongue and tonsils. HPV strains are mostly reported to cause infection in lesion of tongue. These infections are diagnosed to cause oral cancer about 16%, oropharyngeal carcinoma with the ratio up to 30.9% and in larynx tumors up to 16.6%. The facts and figures have demonstrated that in Asia, the ratio of HPV prevalence in oral cancer is higher up to 33% as compared to Europe and North America where the prevalence rate is reported up to 16% (Kreimer *et al.,* 2005).

100 different types of HPV exist in which 15 different types are researched to contain tumorigenesis prospective (Muñoz *et al.,* 2003). HPV 16 is considered as the major threat in causing infections. In some cases, HPV 16 is reported to cause multiple infection along with HPV 18. These infections have been reported in 41% of the patients (Erikson *et al.,* 2009).

A meaningful research has been conducted to evaluate the role of HPV in causing head and neck cancer. It has been demonstrated that HPV is a sexually transmitted disease. The definite carcinogenic passageway of the virus plays an important role in initiating HNC. HPV positive is a significant predictive factor in causing HNC than HPV negative (Ang *et al.,* 2010) (Posner *et al.,* 2011) however the survival rate of head and neck cancer patient with HPV positive is longer than HPV negative (Goon *et al.,* 2009).

HPV infection accounts for increasing incidents (Bisht *et al.*, 2011). The incidence of HPV is increasing every year both in male and female patient. The ratio is stated up to 1.42% in males while in female patients, 1.04% have been reported (Hocking *et al.,* 2011). The modes of transmission of HPV in HNC have not been exclusively determined, however the risk may be increase with sexual behavior (D'Souza *et al.,* 2007).

The occurrence of HPV in causing head and neck cancer is increasing every year in Australia due to change in sexual behavior particularly oral sex (Hocking *et al.,* 2011). The link between HPV and HNC has been discovered in the research study by the detection of HPV DNA and HPV specific antibodies in oral sample of HNC patient (D'Souza *et al.,* 2007). In national immunization programme, the HPV vaccination have yet not been

introduced and it have not researched so far that vaccination can prevent HPV strain infections in head and neck cancer or not.

1.6.1.4 Poor oral hygienic condition

Poor oral hygienic condition that can lead to periodontal diseases due to infection with the release of cytokines and other inflammatory mediators and the outcome of these reactions against inflammation can lead to the progress of cancer. It has been reported that the poor dental hygiene can act as the causative agent in oral carcinoma (Zheng *et al.*, 1990) as teeth loss can lead to the growth of oral cancer.

1.6.1.5 Sunlight exposure and carcinogenic contact

People having continuous exposure to sunlight and working in direct contact with carcinogenic substances, which ultimately contribute to the development of oral cavity cancer. Lip cancer is the common subtype of oral cavity cancer. Due to continuous exposure of lip mucous lining with the sun rays, it has been demonstrated that lip carcinoma is the result of direct exposure to the ultraviolet solar radiations in majority of cases (Perea-Milla López *et al.*, 2003) (Sugarman *et al.*, 2002). The current reports have demonstrated that mostly males having outdoor work and working under nonstop sunlight exposure, lip tumor is occurring three times higher in males than females (PereaMilla López *et al.*, 2003) (Ariyawardana *et al.*, 2013).

It has been revealed that ratio of lip carcinoma is higher in white population as compared to non-white particularly in Australia and Canada (Warnakulasuriya *et al.*, 2009). In Australian population, about 50% of the head and neck cancer have been reported as lip carcinoma (Sugarman *et al.*, 2002).

1.6.2 Non Modifiable risk factors

1.6.2.1 Age

The risk of head and neck cancer is reported more in middle aged patients. The recent reports have demonstrated that the risk of developing head and neck cancer in patients having age 40 years or above is 98% in Europe (Mehanna *et al.,* 2010). It has been observed that the ages of the head and neck cancer patients having infection with the HPV strains are diagnosed mostly between 40 to 60 years (D'Souza *et al.,* 2007). In 2005 the particular study was conducted about Australian population, in which the approximate age of HPV associated HNC male patients were 59.8 years and female patients were observed 63.6 years (Hocking *et al.,* 2011).

1.6.2.2 Gender

The study has revealed that due to the heavy usage of alcohol and cigarette smoking, the occurrence ratio of head and neck carcinoma is five times higher in males than females. The fact and figures from 1995 to 2006 have demonstrated that the ratio of developing head and neck carcinoma among males have increased from 7.6% to 17.7% (Chen *et al.,* 2008). In west, the consumption rate of tobacco smoking, betel nut and alcohol is increasing day by day in females as compared to past so the risk of developing head and neck cancer is becoming higher (Marcu *et al.,* 2009).

1.6.2.3 Family history

The genetic hereditary factor is an important risk factor in increasing the risk of disease transmission among the family members. The ratio of developing head and neck cancer is higher in male patient with the smoking associated family cancer history (Turati *et al.,* 2013). The reports have revealed that in those patients with family history of head and neck cancer, the risk of developing oral cavity carcinoma is reported higher up to 3.8%. The

genomic polymorphism link with the carcinogens digestion is reflected as the major threat (Canova *et al.,* 2009).

1.7 Genetic variation of Head and Neck cancer

In the complicated pathway, gathering of the diverse genomic modifications can lead to the development of head and neck cancer. Detection of the oncogenes overexpression, alterations, modifications, deletions and de novo promotor methylation of tumor suppressor genes have been recognized in the progress of head and neck carcinoma. Due to exposure to the collective carcinogens, majority of the alterations identified at the early stages of mutagenesis.

1.7.1 Mutation in Tumor suppressor genes and proteins

Normally tumor suppressor genes act as transducers of negative regulatory pathway (Weinberg., 1991). These tumor suppressor genes normally function by maintaining cell cycle regulation and cause cell cycle apoptosis. These genes can be modified or alter in their roles by numerous mutations such as non-synonymous, frame shift, missense, deletion, synonymous, point mutations or upon binding with viral proteins (Weinberg., 1991) (Levine., 1993). These genes have been exclusively research and identified in causing head and neck carcinoma. In a normal pathway, the tumor suppressor genes perform its function by minimizing the tumor growth, by slowing down or altering cell cycle progress. Mostly in the case of head and neck carcinoma, the function of tumor suppressor genes to suppress the uncontrolled division of cells have been lost (Lane., 1992).

1.7.1.1 P53

P53 is a tumor suppressor protein. For the protection of genetic strength and stability, it has an important function to perform in the pathway of cell cycle. The main function of P53 is the regulation of DNA repair and apoptosis (Vogelstein *et al.,* 2000).

The position of tumor suppressor gene P53 is normally on the short arm of chromosome 17 at 13.1 gene locus. It carries 11 Exonic regions. They function by encoding a 393 amino acid phosphor protein that is p53 protein, when DNA is damaged, p53 respond immediately by arresting the cell cycle. It acts by regulating the repair pathway and by initiating apoptosis of the impaired cells to restore the DNA damage. TP53 gene involve in the regulation of various biochemical pathways which includes the metabolism of the cells (Boren *et al.*, 2012) and to silent the non-coding RNA (Leonova *et al.*, 2013). TP53 expressions thus regulate the mechanism of life and death (Cho *et al.*, 2012) (Polager *et al.*, 2009).

Mouse double minute 2 homolog MDM2 is involved in the degradation of P53. MDM2 is an E3 ubiquitin ligase which perform its function by binding to P53. CDKN2A gene encode P14 protein, which in turn protect P53 degradation by inhibiting the expression of MDM2 (Sherr *et al.*, 2012). When DNA is damaged, ATM and ATR which are Ataxia telangiectasia mutated and Ataxia Telangiectasia Rad 3 pathways are activated. These pathways phosphorylate CHKI and CHK2. CHKI and CHK2 are the cell cycle check point kinases. Activation of these kinases lead to the p53 activation. P53 in turn regulate various proteins ultimately leading to the cell cycle arrest and apoptosis.

TP53 mutation is diagnosed to be the most common detected mutation in the progress of Head and Neck cancer which is reported in 50-80% HNC cases (Agrawal *et al.*, 2011) (Poeta *et al.*, 2007). After Ovarian and Lung cancer, HNC is considered as the third major TP53 mutation carrying carcinoma (Kandoth *et al.*, 2013). TP53 mutations can cause inhibition or loss of tumor suppressor function (Morton *et al.*, 2010).

The major mutation reported are the missense mutations in the region of DNA binding domain. Mutations can affect the functionality of whole gene by leading toward the inactivation or overexpression of MDM2. It can result in loss or deletion of P14 gene as well (Vogelstein *et al.*, 2000). Thus leading toward the dysregulation of the DNA repair pathway and p53 becomes incapable to repair the damaged DNA or to induce apoptosis in

cells (RaybaudDiogene *et al.,* 1996) thus the cells replicate with the damaged DNA resulting into tumor malignancy (Stricker *et al.*, 2007).

1.7.1.2 Retinoblastoma

Retinoblastoma is one of the tumor suppressor protein, which is involve in the regulation of cell cycle. It is located at chromosome position 13q14 (Buchkovich., 1989). Retinoblastoma perform its function by monitoring the expression of various genes through G1 phase. The main pathway involves binding of Retinoblastoma with E2F transcription factor and thus leading toward its inhibition. E2F transcription factors mainly function to regulate the S phase expression and proliferation of cell. Retinoblastoma are phosphorylated by the action of cyclin D1/CDK4/CDK6 and as the result these complexes induce the activation of E2F transcription factor. Cyclin D1/CDK4/CDK6 multiplexes are actually activated in response to some mutagenic indications. CDKN2A gene encode P16 protein. P16 induce inactivation of these cyclin D1/CDK4/CDK6. P16 and P21 do not allow these complexes to phosphorylate Retinoblastoma after binding to them thus lead to prevent the S phase progression. IHC studies determine that Rb abnormalities has been demonstrated about 6% to 74% that can lead toward head and neck carcinomas associated with poor survival rate.

1.7.1.3 P21 and P27

The chromosomal location of p21 and p27 genes have been identified at 6p21 and 14q32 chromosome position. In normal pathway, these genes produce proteins, which are activated by p53 gene. Upon activation of these proteins, they induce cell cycle arrest. Mutation in these genes have been reported to cause an unchecked cell cycle progression and can lead toward the malignancies (Rasmussen *et al.,* 1993).

1.7.1.4 NOTCH1

1.7.1.4.1 Structure

TAN1 was the previous name of NOTCH1 gene. In 1917, Thomas Hunt Morgan illustrates TAN1 in the fruit fly drosophila melanogaster strain where notches look outward on their wings. In 1980, the first genomic study and sequencing of NOTCH1 gene product was carried out. The NOTCH1 gene functional gene product is NOTCH1 which is a protein in nature.

1.7.1.4.2 Location

In length, the notch1 functional RNA transcript is 9,371 base pair. It is located on chromosome number 9. It contains 51,418 base pair DNA. It comprises 34 exons (Cayo et al., 2008).

1.7.1.4.3 Receptor and Ligand family

The notch gene pathways basically comprise of four transmembrane receptors which are NOTCH1–4. The notch1 gene basically encode NOTCH1 transmembrane receptor. It also contains two ligand families. The ligand families are Jagged 1, 2 and Delta like 1, 3 and 4. The notch1 gene delivers the guideline instructions for producing a protein known as NOTCH1 protein receptor. NOTCH1 receptor is the member of the NOTCH receptor family. As the notch1 gene function as oncogene and also as a tumor suppressor gene so the NOTCH1 protein receptor produce by it perform many diverse functions. A critical role of the transmembrane receptor is demonstrated in various cell and tissue development (Cayo *et al.,* 2008). Having tumor suppressor property, notch1 gene suppress the cells from growing and dividing in an uncontrolled manner. Mutations in them leads to the development of cancer.

Being an Oncogenes, NOTCH1 protein stimulate cell division and survival normally. But when mutation occur or over expressed, they encode the proteins which outcome in the

form of abnormal cell growth or malignancies. NOTCH signaling play an important role in specializing cells into certain well define cell types. These specific cell types perform various functions in embryonic and adult tissue development, proliferation, cellular differentiation, apoptosis and in cell fate decision (Ranganathan *et al.*, 2011).

1.7.1.4.4 Domains

1.7.1.4.4.1 Receptor Domains

In general, largely there are two domains of NOTCH1 protein

- NOTCH1 Extracellular domain
- NOTCH1 Intracellular Domain

The NOTCH1 extracellular domain comprises 36 EGF-like repeats. These repeats are also known as cysteine containing tied motifs. The structure of EGF-like repeats are well demonstrated that each repeat consists of six cysteine residues, these preserved residues join to form three disulfide bonds. This characteristic is considered very important in ligand binding. Three LN (Notch/Lin12) repeats are also located within this extracellular domain. These LN repeats are illustrated to be rich in cysteine. The function of these repeats is to inhibit the signal when the ligand is absent (Cayo *et al.*, 2008).

The NOTCH1 intracellular domain structure demonstrated that it contains six Ankyrin repeats. These Ankyrin repeats are considered very important in creating protein-protein contact and interactions. The intracellular domain (NICD) also contains sub domains such as TAD which is transcriptional activation domain and PEST domain. PEST is considered to be rich in proline, glutamic acid, serine and threonine residues. The function of PEST is illustrated that it regulates the protein strength and stability but in negative way. Transcriptional activation domain (TAD) along with the Ankyrin repeats which are located within NICD are present in NOTCH1 and NOTCH2 receptors. However, NOTCH3 and 4 do not contain Transcriptional activation domain TAD. NOTCH1 intracellular domain also

contain N1 and N2. Both are reported as nuclear localization signals. Another domain is located within NICD known as RAM23 domain (Cayo *et al.,* 2008).

The place where NOTCH1 receptor specifically create interaction with notch ligand are located within the extracellular amino terminus. NOTCH1 intracellular fragment domain, transmembrane domain, NOTCH1 extracellular fragment domain are located at the carboxyl terminus of Notch receptor. NOTCH1 extracellular fragment domain comprise of 36 EGF like repeats. The NOTCH1 extracellular domain are bounded by hetero dimerization domain towards the carboxyl terminus.

1.7.1.4.4.2 Ligand Domains

The notch ligand consists of an extracellular domain involve in ligand binding. A conserved module is located on N terminal region. Another domain known as DSL (delta/serrate/Lag-2) domain is present near N terminus. Jagged 1, Jagged 2 and DII1 ligands contain tandem EGF like repeats. DOS (Delta and OSM11) domain are created by these tandem repeats. Although both notch ligand and NOTCH1 receptor carries EGF like repeats. Jagged 1 and Jagged 2 contain cysteine rich domain. The notch ligand domain DSL and DOS considered critical in ligand binding to its NOTCH1 receptor (Kopan *et al.,* 2009). The DOS domain presence or absence are considered critical in evaluation of notch ligand structural range and diversity.

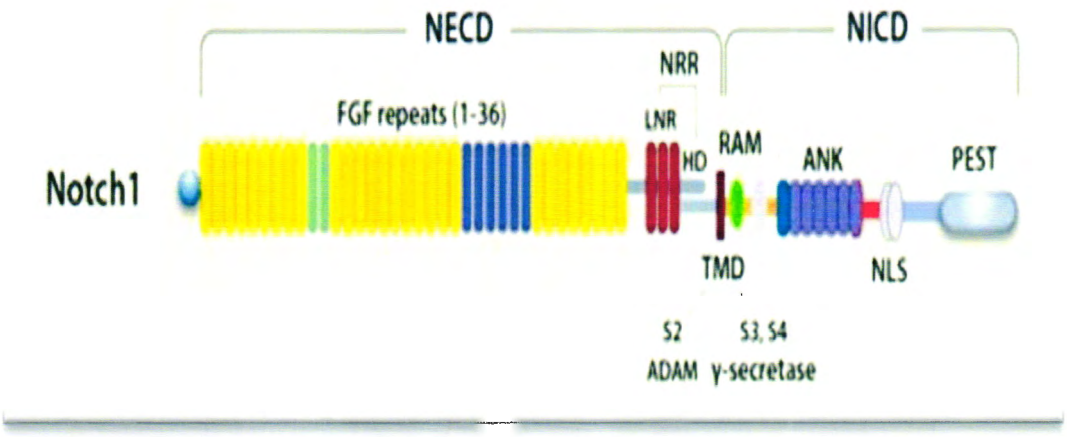

Figure 1 Schematic Representation of NOTCH1 Domains structure

1.7.1.4.4.3 Pathway

Initially each receptor consists of a single polypeptide chain. The encoded pre protein is reported to processed proteolytically within the trans-Golgi network. Furin like Convertase cleave these polypeptide chain at NOTCH S1 site to create two polypeptide chains that undergoes heterodimerization to produce the mature cell-surface receptor.

Expression of notch ligand on the surface of one cell and NOTCH1 receptor on the surface of another cell stimulate notch signaling. Binding of ligand to its receptor leads to activation of NOTCH1 protein receptor. In ligand binding, there are two types of interactions:

- Trans interaction
- Cis interaction

Trans interaction occurs between the notch ligand and its receptor by cell to cell interaction. This interaction causes the activation of NOTCH signaling. While cis interaction is believed to occur by binding of notch ligand to its receptor within the same cell. It causes the inhibition of NOTCH signaling (Kopan *et al.,* 2009). These cis and trans interactions are facilitated by EGF Notch domain (de Celis *et al.,* 2000).

Before ligand binding to NOTCH1 receptor, the receptor express resistance toward the activated proteolysis. An extracellular domain containing EGF like repeats and 3 LNR Notch repeats provide security against cleavage site NOTCH1 S2 in the ectodomain. S2 site are located almost 12 amino acid sequence before the transmembrane domain. A cascade of proteolytic cleavage is initiated after the binding of ligand to its NOTCH1 receptor, which regulate the first cleavage at S2 and thus allows the ADAM/TACE metalloproteinase to have access on NOTCH1 S2 site for cleavage, fallow by the loss of ectodomain (Kopan *et al.,* 2009). The second cleavage is reported at S3 site. metalloprotease-cleaved form of NOTCH1 protein form upon the removal of ectodomain. This cleaved intermediate act as a substrate for S3 cleavage by gamma secretase complex. Gamma secretase is an intramembrane protease (Kopan *et al.,*2009) which undergoes

NOTCH cleavage within the transmembrane domain at S3. Thus this NOTCH cleavage causes the release of NOTCH intracellular domain from membrane in to cytoplasm. Where NOTCH intracellular domain (NICD) translocate in to the nucleus. In nucleus, NICD interact with the transcriptional machinery through the mastermind transcriptional coactivator (MAML 1-3), NOTCH ankyrin repeats and NOTCH RAM 23 domain motif (Kovall., 2008) to develop the transcriptional activation. The transcriptional complexes machinery includes the DNA binding transcription factor CSL which is CBF1 in homosapians. Interaction of NICD with these transcriptional complexes mediate the transcriptional activation of various target genes such as HEY and HRT gene family. When the NOTCH1 signaling is inhibited, CSL transcription factor attract with the promoters of these target genes as a result bind to the promotors and leading toward the activation of corepressors which causes transcriptional inhibition of these targeted genes. The corepressor molecules include SMRT/NcoR. Upon activation of NICD, it interacts with CSL. It alters CSL from a transcriptional repressor and convert it into a transcriptional activator which is CBF1 for human. This mechanism involves the displacement of corepressor complex and activation of coactivators MAML-1 (Cayo *et al.,* 2008). After the transcriptional activation of targeted genes by NOTCH1 signaling expression. Its degradation is caused by ubiquitination mechanism on the completion of its function. NOTCH1 expression is reported to alter by various known proteins such as numb and numb like, these are phosphotyrosine binding domain (PTB) containing proteins and E3 ubiquitin ligase. These proteins cause the positive or negative regulation of NOTCH1 signal.

PEST domain is located near the carboxyl terminal of NICD. FBXW7 targeted PEST domain as a degradation signal and ubiquitinized it, which causes the destruction of NICD. As the PEST domain function to keep NOTCH1 activation by maintaining its signal strength and duration. After the recruitment of RNA polymerase II holoenzyme to the transcriptionally active complex, it causes the phosphorylation of PEST domain. Which make it susceptible to be target by FBXW7 and thus cause its degradation (Bhanushali., 2010).

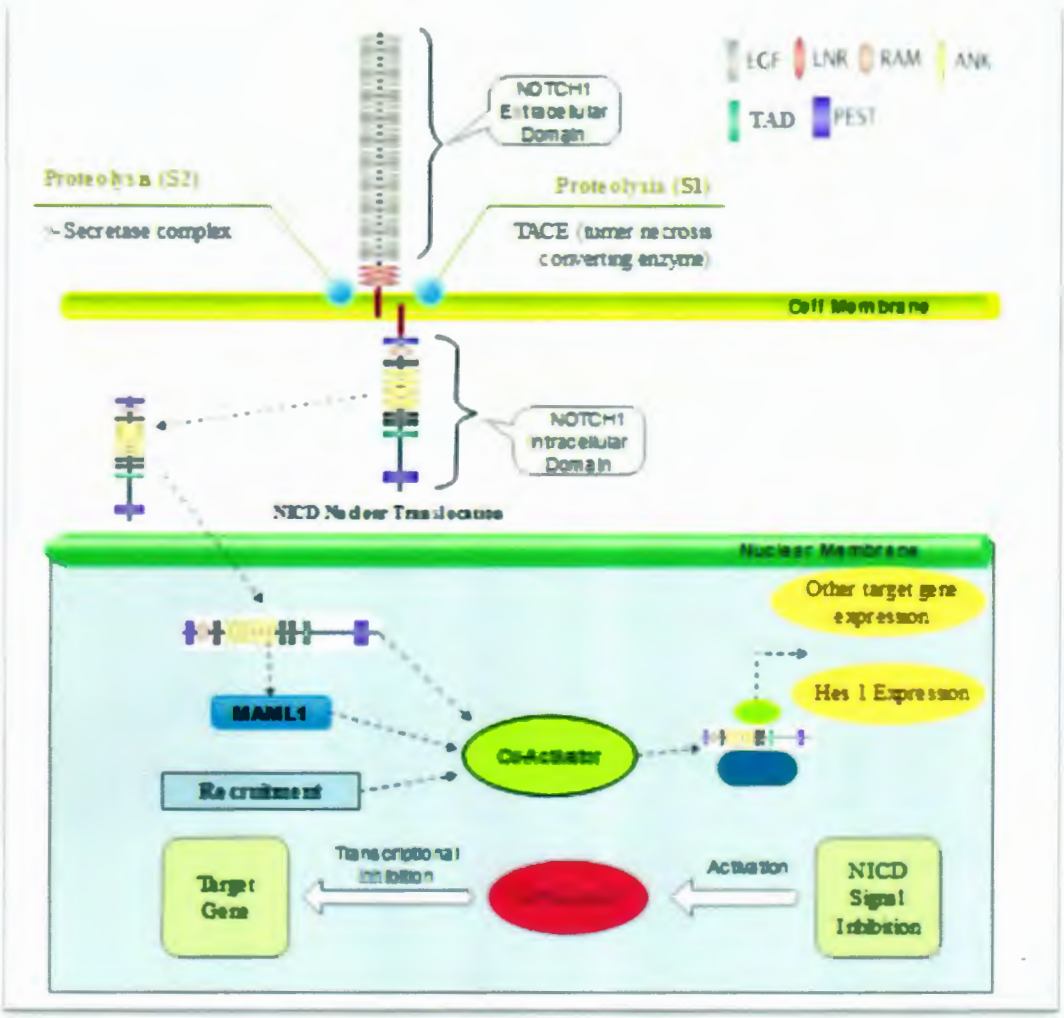

Figure 2 Schematic Representation of NOTCH1 Receptor Signaling Pathway in transactivation of target genes

1.7.1.4.4.4 Mutations

The research has revealed that NOTCH1 observed as performing tumor suppressor function. The reports have demonstrated that the occurrence of frame shift, missense and nonsense mutation is observed in 18.6 cases (Cerami *et al.*, 2012) these mutations have been detected in EGF like domain repeats, which play an important role in ligand binding. It suggests the function of NOTCH1 as a tumor suppressor gene. Moreover, it also has been observed that Hyperplasia might been result due to the loss of NOTCH function in a murine model inducing skin carcinogen, thus supporting NOTCH as a tumor suppressor gene (Nicolas *et al.*, 2003). Mediation of NOTCH oncogenic function is cell cycle progression and apoptosis inhibition is illustrated (Ranganathan *et al.*, 2011).

NOTCH1 ligand mutations truncate the growth and development of several organs and tissues. These mutations have been identified in skeleton, cardiac and pulmonary. Rib fusion is caused by the DLL3 NOTCH1 ligand mutation. The experimentation has been reported in which the mutant NOTCH1 cell when got infection with a retrovirus, it transduces the ras oncogene transformation, as a result an active form of squamous cell carcinoma arises when it is inserted in to nude mice. The same experiment is conducted on wild type cells, interestingly did not developed the disease. Keratinocyte tumor arises due to the loss of NOTCH1 activity.

In the development of leukemia and lymphomas, aberrant NOTCH1 signaling have been demonstrated as the causative agent. Currently it have been illustrated that the NOTCH1 heterodimerization domain (HD) and PEST domain is associated with T-cell acute lymphoblastic leukemia and lymphoma (T-ALL) and involve the translocation of t(7;9). In T- ALL patients, 3' part of notch1 gene juxtaposition with locus of T cell receptor beta is characterized by this translocation, leading towards the truncated NOTCH1 transcript expression. Thus causes the production of ligand independent NOTCH1 receptor and ultimately develop T-ALL (Cayo *et al.*, 2008).

Mutation in NOTCH receptors are reported in 24% cases. It also has been that FBXW7, which is a ubiquitin ligase, it can target NOTCH for degradation that is reported to be mutated in 4.7% of cases of Head and neck carcinoma (Welcker *et al.,* 2008). Aberrant NOTCH protein and ligand signaling is associated with the occurrence of skin, mammary, cervical, prostate carcinomas. The absence of NOTCH1 expression causes the development of neuroendocrine tumors which suggest the role of NOTCH1 as a tumor suppressor gene in neuroendocrine region (Cayo *et al.,* 2008). The role of NOTCH1 have been well illustrated in the maintenance of epithelial integrity and reliability (Sakamoto *et al.,* 2012) as terminal differentiation of squamous epithelium cells produced by the truncated expression of NOTCH1 protein causes the formation of immature and undeveloped epithelia. In Alzheimer's disorder, the inhibitors of gamma secretase are reported to downstream NOTCH1 signaling pathway. An elevated association is demonstrated with the risk of the skin carcinoma development (Teodorczyk *et al.,* 2014). NOTCH1 has been considered as one of the key innovative finding in causing head and neck carcinoma (Toustrup *et al.,* 2012).

1.7.1.4.4.5 Single nucleotide polymorphism

Several single nucleotide polymorphism (SNP) of NOTCH1 gene has been reported. A high frequency of NOTCH1 somatic mutations causing head and neck cancer were reported to be missense mutation. These missense mutations were demonstrated to occur near or within extracellular domain of NOTCH1 protein and causes the production of NOTCH1 protein which lack its extracellular domain. Normally, extracellular region comprises of 36 EGF like repeat domains which contain Ca^{2+} binding conserved sequence. EGF repeats create interaction between NOTCH1 receptor and its ligand, causing its activation. NOTCH1 receptor missing extracellular domain were identified to be inactivated by somatic mutations. These mutations cause the development of head and neck cancer (Sakamoto *et al.,* 2012). Several frameshift and nonsense somatic mutations were also reported. These mutations truncate and reduce the expression of NOTCH1 protein. The truncated NOTCH1 protein lack intracellular domain (NICD). In a normal pathway, upon

activation of NOTCH1 receptor. NICD translocate into the nucleus and cause transcriptional activation of targeted gene. However, the truncated NOTCH1 protein missing NICD, inhibit the transactivation of the targeted genes. This suggest the role of NOTCH1 protein as a tumor suppressor gene in occurrence of head and neck cancer (Cancer Genome Atlas, 2015).

The missense somatic mutations 1363G →A, 1070T→C, 1055A→T and 2898C→G have been identified in the Ca^{2+} binding EGF repeats domain. These missense mutations were illustrated to effect the efficiency of Ca^{2+} binding EGF repeats function.1396A→G and 1363G→A were two somatic mutations identified in the NOTCH1 receptor ligand binding region. The somatic mutation 466T→A were observed to enhance the affinity of NOTCH1 receptor for DLL116 and Jagged 1 ligands. Its location has been identified in the domain within O-fucosylation site conserved sequence. Two novel missense mutations 1127G→T,4070G→A were reported in Taiwan population. It effects the functionality of EGF repeats by mutating its structure. In EGF repeats, these mutations have been observed between disulfide bonds of double stranded beta sheets within cysteine loops (Liu *et al.,* 2016). In EGF like repeats domain, five somatic mutations with amino acid change 455E→K, 966S→R, 466T→A,1108Q→X and 385S→Y were identified. The SNP rs139994842 were identified to be associated with these NOTCH1 mutations in Taiwan population (Liu *et al.,* 2016) Which indicate that it might be a functional variant. Based on this speculation, variant at site (rs139994842) identified in the 28th EGF like repeat of NOTCH1 was selected in foremost study.

Objectives of the Study

The current study was designed for

- Assessment of most relevant risk factor leading to Head and Neck Cancer in selected Pakistani Population.
- Analyzing NOTCH1 gene (rs139994842) association in patients of Head and Neck Cancer.

Chapter 2

2.1 Sample Collection

For the analysis of association of NOTCH1 gene with Head and Neck cancer, blood samples were collected from the selected Pakistani population. Total one hundred blood samples were collected from Nuclear Medicine Oncology and Radiotherapy Institute (NORI) Hospital and Institute of Nuclear Medicine and Oncology Lahore (INMOL) Hospital. For comparison with the samples, fifty controls were collected randomly for this study. Consent forms were filled for the blood sample collection. Risk factors include alcohol consumption, smoking, age, nutritional practices and physical inactivity. Sample tubes were labeled with the name of patients. Blood samples were takes in total quantity of 1.5ml. Gently inverted the tubes after blood collection for proper mixing of additives and blood. Vacutainer containing blood samples were kept in refrigerator at 20°C. Specific ID's were given to the blood samples starting from HNC-001 to HNC-100 and were stored in refrigerator.

2.2 DNA Extraction using Organic Method

DNA of samples was extracted by using organic method.

2.2.1 Day-1

Vacutainers containing blood samples were kept in racks for one hour at room temperature. 750µl of blood was taken in Eppendorf's and then added 750µl of Solution A. Eppendorf's were inverted for 4-6 times for proper mixing and were kept for 10mins at room temperature. Then centrifuged the Eppendorf's at 13000rpm for 1min. Supernatant was discarded. Nuclear pellet was re-suspended in 400µl of Solution A. Mixed well to dissolve the pellet. Centrifuged again for 1min at 13000rpm. Discarded the supernatant. Pellet was re-suspended in 400µl of Solution B, 12µl of 20% SDS (25µl of 10% SDS) and 5-8µl of proteinase K (20µl/mL stored at 20°C). Pellet was dissolved by tapping and incubated overnight at 37°C.

2.2.2 Day-2

Added 500µl of fresh mixture of equal volume of Solution C and Solution D. Mixed and centrifuged at 13000rpm for 10mins. Upper layer (aqueous phase) was collected into new tubes. Add equal quantity (500µl) of Solution D in aqueous layer. Centrifuged for 10mins at 13000rpm. Upper aqueous layer was again transferred into different tubes. DNA was precipitated by adding 55µl of sodium acetate and 500µl of chilled iso-propanol (-20°C). Tubes were inverted several times to precipitate the DNA. Tubes were centrifuged at 13000rpm for 10mins and then supernatant was discarded (carefully without disturbing the DNA pellet). 70% ethanol (-20°C) about 200µl was added and centrifuged for 7mins at 13000rpm. Ethanol was discarded. Pellet was dried in an incubator at 37°C for 20min. Then precipitated DNA was dissolved in 80-100µl of TE/ Deionized water and incubated over-night at 37°C.

2.3 Agarose Gel Electrophoresis

2% gel was prepared by adding 0.5g of agarose in 45ml of distilled water and 5ml of 10X TBE in a graduated flask. Solution was heated for 2-3min to dissolve the agarose completely and allowed to get cool for some time. 5µl of Ethidium Bromide was added into the solution and mixed. Solution was poured into the gel caster and combs were inserted. For 30min, solution was allowed to get solidify. After solidification of the gel, combs were removed carefully. Gel caster was placed into the gel tank and 1X TBE solution was added. Before loading into the wells, samples were centrifuged for 1min. 2.5 µl DNA and 2.5 µl loading dye were mixed and loaded into the wells carefully. Voltage of 115V was supplied to the tank by attaching electrodes for 30min. After the gel electrophoresis, bands were analyzed on Gel Documentation System by turning on the UV-Trans-illuminator. Gel picture was taken and saved.

2.3.1 Compositions and Functions of Solutions

All stock solutions were prepared in the laboratory and were autoclaved before using. Compositions and concentrations of all the solutions are shown in table 2.1

Table 2.1:

Compositions of Solutions

	Components	Concentrations	Functions
Solution A	**Sucrose**	0.32 M	Makes the sample heavier
	Tris-HCl	10mM	Maintains the pH
	MgCl2	5mM	It acts as a detergent and helps in bursting cell membranes
Solution B	**Tris-HCl**	10mM	Maintains the pH
	NaCl	400mM	Increases buffer capacity and causes buffer precipitation
	EDTA	2mM	Captures Mg2+ to protect the DNA from the action of nucleases (Chelating agent / Enzyme inhibitor)
Solution C	**Phenol**	100ml	Protein denaturation
	Hydroxyquiniline	10 mg	Chelating agent
	Tris-Cl	0.1M	Lysis buffer
	βmercaptoethanol	200µl	Irreversibly denature RNase
Solution D	**Chloroform**	24ml	Protein denaturation
	Isoamylalcohal	1ml	Increases efficiency of chloroform to denature

2.4 Quantification of Extracted DNA

DNA quantification was done by using spectrophotometer. Quantification of all the samples (Patients and Controls) was performed to determine either DNA is contaminated or not. The standard value of quantification ranges from 1.7-1.8, below or above this range, DNA was re extracted. Optical density (OD) of the samples was taken at 260nm and 280nm. Lid factor 10 was used.

2.5 Primer Sequence

Sequence of all four primers is given in table 2.2.

2.6 Tetra primer Amplification Refractory Mutation System (ARMS) PCR

Tetra primer ARMS PCR was performed for all 100 samples along with the controls. PCR conditions were altered for the optimization of samples. Extracted DNA was amplified in a total reaction volume of 25ul. Reactions for wild-type allele and mutant allele were prepared in same tube. According to the Tetra primer ARMS-PCR conditions for NOTCH1 variant C1083C, 1 cycle of denaturation was carried out at 96°C for 3 minutes followed by the 35 cycles of annealing carried out at 96°C for 40sec, at 70°C for 40sec and at 72°C for 50sec. The final cycle of extension was carried out at 72°C for 15 minutes as shown in table 2.3.

Table 2.2

Thermal cycling PCR conditions for amplification

Primer	Sequence
Common Forward	5'-AGCCAGGGCCTGGTGTGTGGC-3'
Common Reverse	5'-GCCCCTGGACTCCCCACCAC-3'
Allele Specific Forward	5'-TCCAGCCGCTGGGGCACGCA-3'
Allele Specific Reverse	5'-GCCGCGGTACTGGGTGTGGGT-3'

Table 2.3

Thermal cycling PCR conditions for amplification

Steps	Time	Temperature	No. of Cycles
Denaturation	3min	96°C	1 cycles
Annealing	40sec	96°C	35 cycles
	40sec	70°C	
	50sec	72°C	
Extension	15min	72°C	1 cycles

2.7 Gel Electrophoresis

Products obtained from tetra primer ARMS-PCR were analyzed on 3% agarose gel. Gel was prepared by inserting two combs. Products for wild-type allele were loaded in upper row and products for mutant allele were loaded in lower row to differentiate the both alleles easily. In all the wells, 6X loading dye (Thermo Scientific) was poured. To analyze the band size 100bp DNA ladder (O'GeneRuler) was used. After loading the samples, voltage was supplied and then gel was analyzed on Gel Documentation System.

2.8 Statistical Analysis

For the interpretation and analysis of data, IBM SPSS software was used. Chi-square and p-value for age, gender and histopathology were calculated. Genotypic and allele frequency were also calculated.

Chapter 3

A complex disease, Head and Neck cancer have been reported as serious cause of mortality. Occurrence of Head and Neck cancer might involve different factors including genetic and environmental factors. Genetic variation in genes that regulate the mechanism in an individual may develop the risk of Head and neck cancer. NOTCH1 gene have been reported as tumor suppressor gene which participates in a wide range of squamous cell differentiation. Its mutational inactivation has been involved in the development of Head and Neck cancer. Different SNPs have been analyzed to find the association with HNC showing either null association or significant association. In this study, NOTCH1 gene has been selected to determine the association with Head and neck cancer in specific Pakistani population, as this type of association has not been yet reported previously in Pakistani population.

3.1 SNP Genotyping of NOTCH1 C1083 (rs139994842)

To determine the association of NOTCH1 gene with Head and Neck cancer, SNP (rs139994842) of NOTCH1 gene was genotyped using Tetra primer ARMS-PCR technique.

3.2 Tetra primer ARMS-PCR analysis of NOTCH1 (rs139994842)

The product obtained after Tetra primer ARMS-PCR amplification was analyzed on 2% agarose gel. Gel picture showed the band size of 645bp and 440bp of wild type allele G and 245bp of mutant allele A (fig. 3). Genotype were analyzed by visualizing the gel image that was obtained through gel documentation system (BioRad).

3.3 Genotypic Analysis of NOTCH1 (rs139994842)

Among three genotypes, homozygous major GG have been identified in patients and controls. Genotype heterozygous GA have been found in patients and not a single homozygous minor AA genotype have been seen in both patients and controls. Chi square test was applied to predict the significance of the study and p-value was calculated. Genotypic and allelic frequencies of the patients were also calculated.

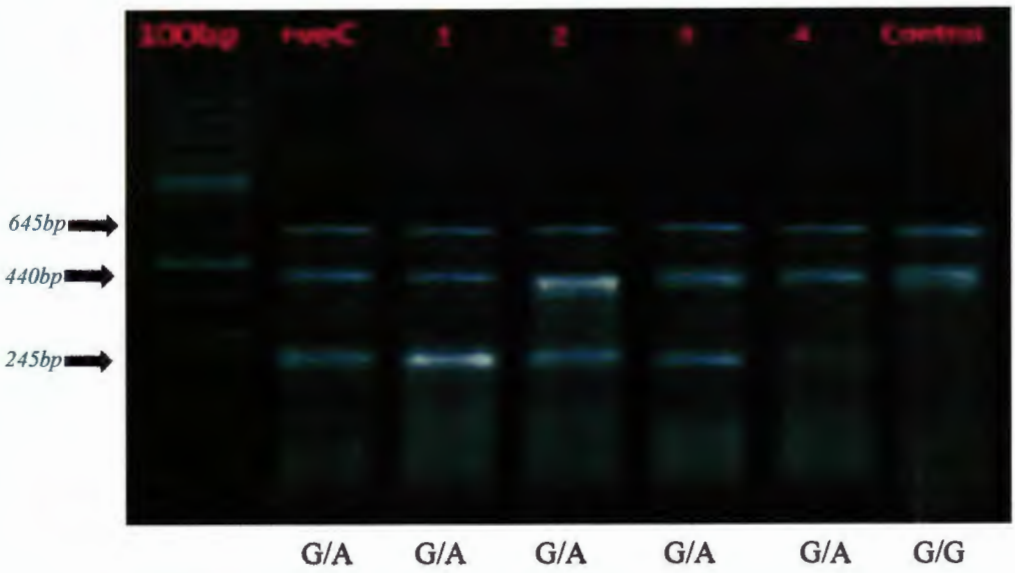

Figure 3 Analysis of NOTCH1 C1083 amplified products by using Tetra primer ARMS-PCR

First and second row showing band size for wild-type allele at 645bp and 440bp respectively. Third row showing band size for specific allele at 245bp. First lane is for positive control and lane 1, 2, 3 and 4 are showing results for HNC patients while last lane is showing result for control. All three rows are showing results for same individuals. 100bp ladder was used (O' Gene Ruler).

3.4 Distribution of Genotypes

Distribution of genotypes GG, GA and AA have been analyzed in both patients of HNC and controls (Table 3.1). Analysis showed that genotypic frequency of GG was 42% and 100% in HNC patients and controls respectively. GA genotype showed frequency of 58% in HNC patients and showed 0% frequency in controls. Genotype AA showed 0% frequency in both HNC patients and controls. To determine the significant association, p-value was calculated. The p-value was significant (0.0000) (Table 3.2). the resultant value showed the association of NOTCH1 C1083 (rs139994842) with HNC.

3.4.1 Allelic Frequency

Frequencies of both A and G allele were calculated for HNC patients and controls. Allele A showed frequency of 0.58% in HNC patients and 0% in controls while G allele showed 0.71% frequencies in HNC patients and 2% frequency in controls. Allelic frequencies have been showed in (Table 3.3). This analysis revealed significant statistical difference.

Table 3.1

Genotypic distribution in Controls and Patients

Genotype	Control	Patient
GG	50	42
GA	0	58
AA	0	0
Total	**50**	**100**

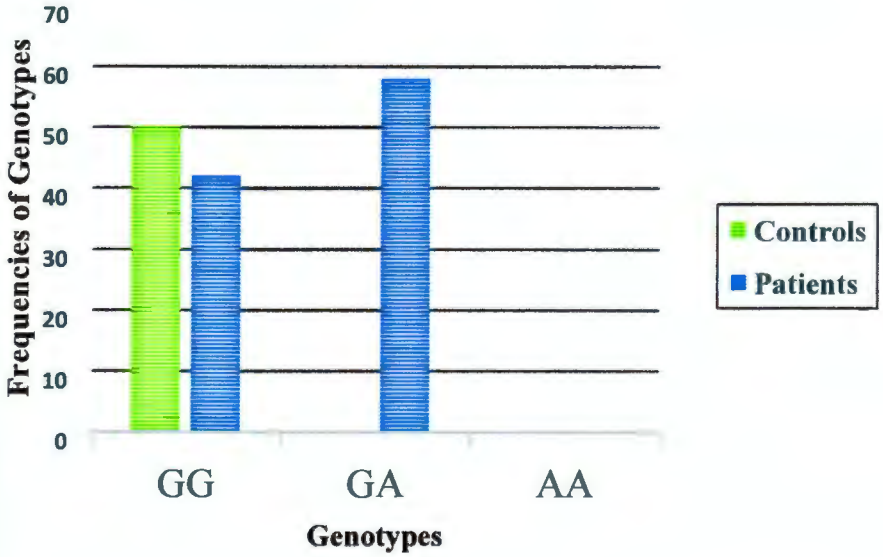

Figure 4 Bar Chart representing frequency of all three Genotypes

Table 3.2

P-value for Genotypes

Genotypes	Value of x	rs139994842	
		Degree of Freedom	P-value
GG	0.363	1	0.546
GA	42.42	1	0.0000

Table 3.3

Distribution of Allelic Frequency in Patients

Alleles	Allelic frequency in Controls	Allelic frequency in Patients
G	100 2%	142 0.71%
A	0 0%	58 0.58%

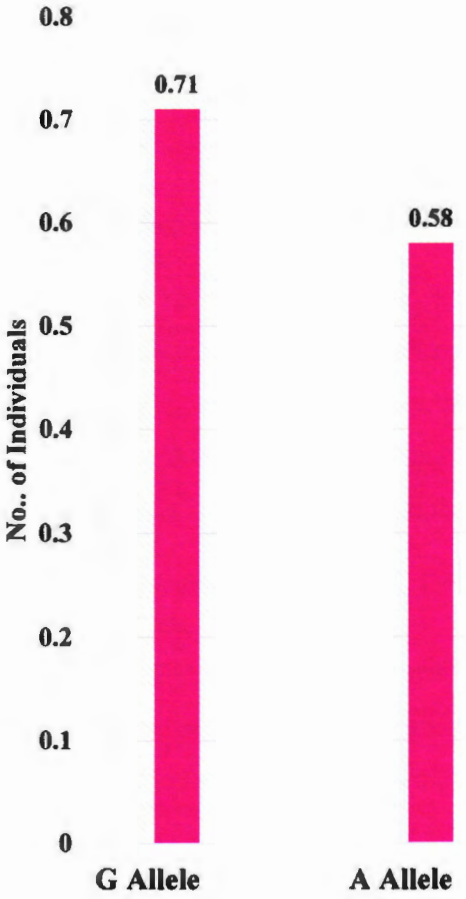

Figure 5 Bar chart representing individual Allelic frequencies

3.5 Genotypic Distribution between Males and Females

All the three genotypes were also analyzed in male and female HNC patients and controls. Genotype GG was found to be 12 and 30 in HNC males and females respectively while GA genotype was found to be 50 and 8 in HNC males and females respectively (Table 3.4). GA genotype was not found in control groups. The value of odd ratio for gender was, OR 15.625 with 95% CI (5.733-42.583). Chi-square test was applied to gender to find out association between gender and HNC (Table 3.5). The value of Chi-square was 34.346 with p-value 0.000. it was found that p-value was lesser than the standard significant p-value (<0.05).

3.6 Distribution of HNC subtypes among Patients

Among all the 100 HNC individuals, frequency of HNC sub types was also calculated. Number of patients with Nasopharynx (0.20%) and Buccal Mucosa (0.20) were found to be higher than the patients carrying other sub types cancer. The frequency of other HNC subtypes was larynx (0.16%), hypopharynx (0.15%) Tongue (0.11%) Max Sinus (0.10%) oropharynx (0.05%) lips (0.03%). The result shows that Nasopharynx and Buccal cancer were more prevalent.

3.7 Age Distribution among HNC Patients

Distribution of HNC patients according to different age groups have been shown in table 3.7. Analysis of data showed that Head and neck cancer is more common between the age group of over 30 and below 65. This age group had both genders. The calculated mean and standard deviation for age was 47.27±13.46.

3.8 Ethnic study among HNC patients

Among all the 100 HNC individuals, Ethnic frequency was also calculated. In current study, number of Punjabi patients carrying Head and Neck cancer (0.75%) was found to be higher. Ratio of Pathan patients were 0.11%, Kashmiri patients were 0.10%, Sindhi 0.03 %and Balochi patient was found to be 0.01%.

Table 3.4

Genotypic Distribution in Males and Females

				OR (95%CI)
		GG	GA	
Gender	**Female**	30	8	**15.625 (5.733-42.58)**
	Male	12	50	
Total		42	58	

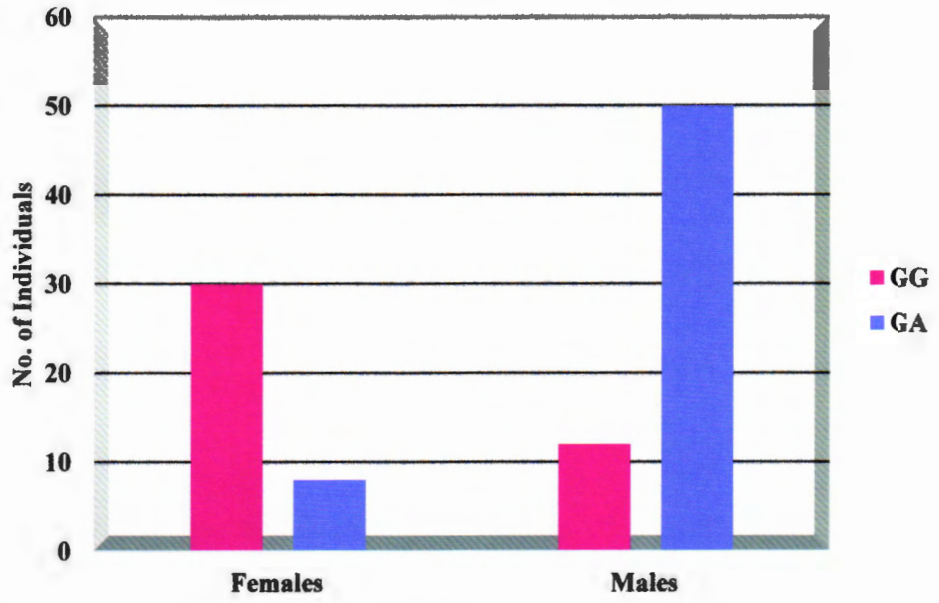

Figure 6 Bar chart representing Genotypic distribution between females and males

Table 3.5

Distribution of HNC subtypes in Patients

Types of HNC	No. of Individuals N=100	Percentage
Nasopharynx	20	0.20%
Buccal Mucosa	20	0.20%
Larynx	16	0.16%
Hypopharynx	15	0.15%
Tongue	11	0.11%
Maxilla Sinus	10	0.10%
Oropharynx	5	0.05%
Lip	3	0.03%

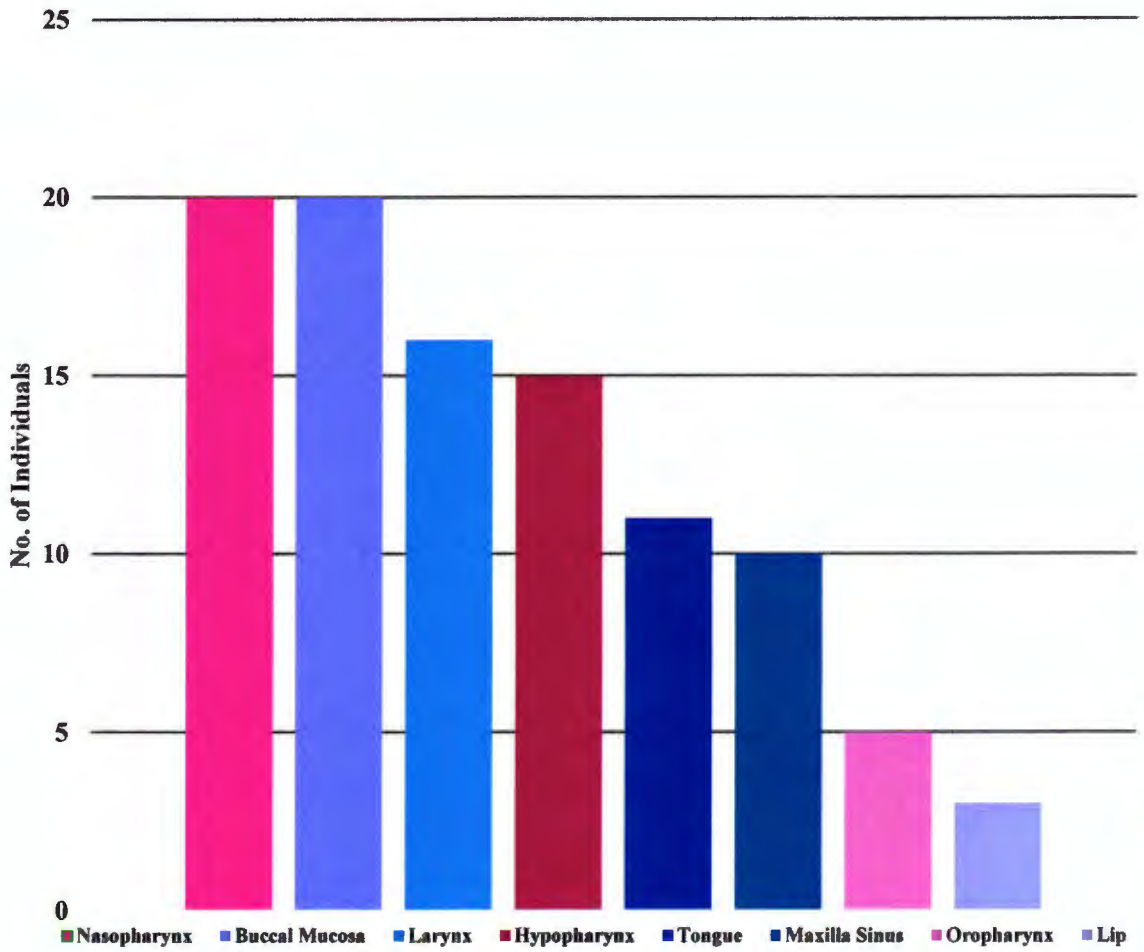

Figure 7 Bar chart representing Head and Neck cancer subtypes in Patients

Table 3.6

Distribution of HNC in Different Age Groups

		Results		Total
		GG	GA	
Age Groups	15 years or below	1	1	2
	16-25 years	4	1	5
	26-35 years	3	8	11
	36-45 years	11	14	25
	46-55 years	12	22	34
	56-65 years	8	8	16
	65years or above	3	4	7
Total		42	58	100

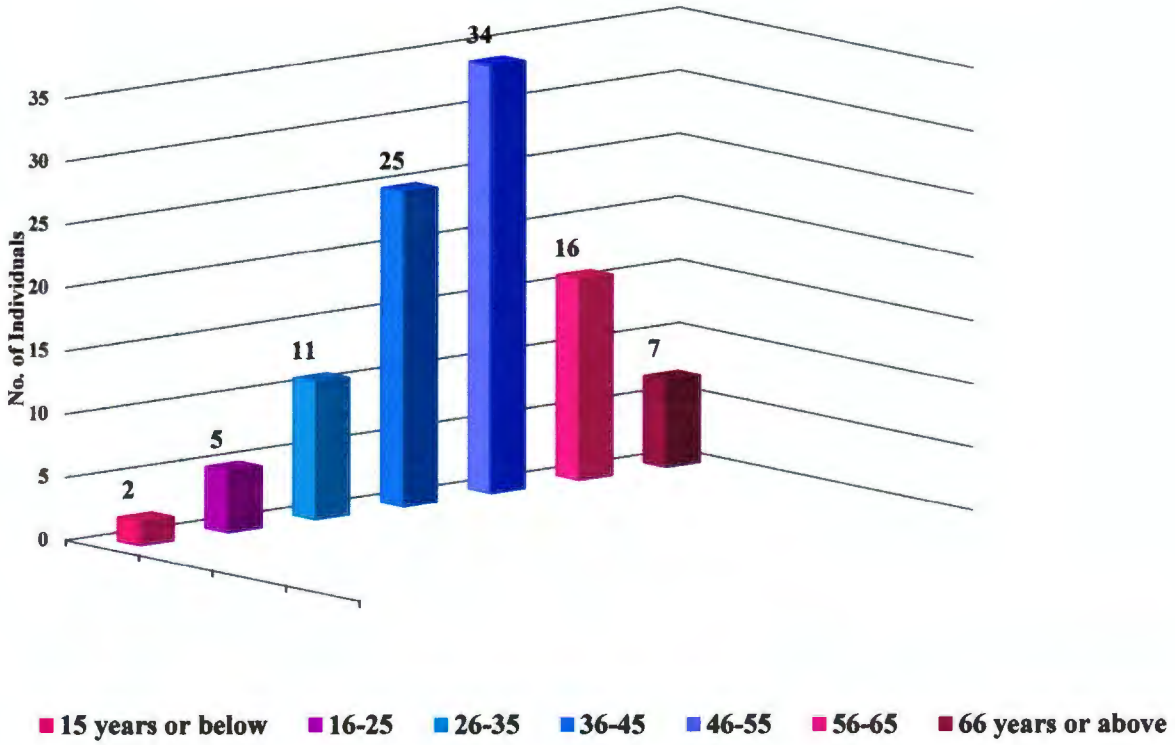

Figure 8 Bar chart representing distribution of HNC in different Age groups

Table 3.7

Ethnic study among patients

		Results		Total
		GG	GA	
Ethnic Groups	Punjabi	31	44	75
	Pathan	4	7	11
	Sindhi	1	2	3
	Balochi	1	0	1
	Kashmiri	5	5	10
Total		42	58	100

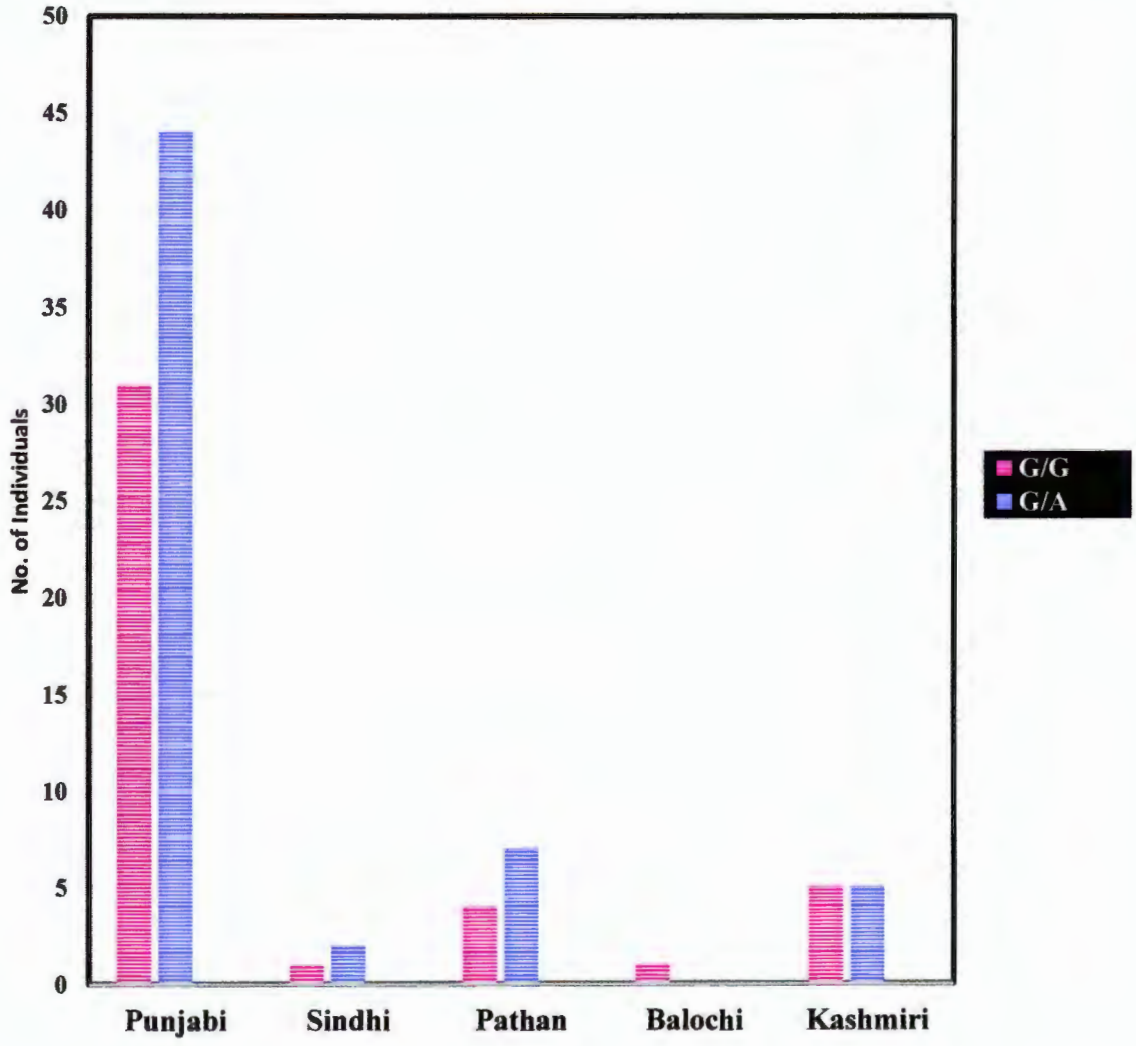

Figure 9 Bar chart representing distribution of HNC in different Ethnic groups

3.9 Smoking history of HNC patients

In this study, smoking reports of HNC patients were also collected. Analysis of data showed that Head and Neck cancer is more prevalent among smokers (0.58%) as compared to nonsmokers (0.42%). Chi-square value for smoking was calculated as 30.131 and p-value was 0.000. As p value was significant, it can be concluded that smoking might be the relevant risk factor for developing HNC.

Table 3.8

Distribution of HNC in Different Smoking Groups

		Results		Total
		GG	GA	
Smoking	Heroine	1	0	1
	Cigarette	2	27	29
	Hookah	0	1	1
	Betel Quid	3	10	13
	Paan	2	1	3
	Non Smokers	34	19	53
Total		**42**	**58**	**100**

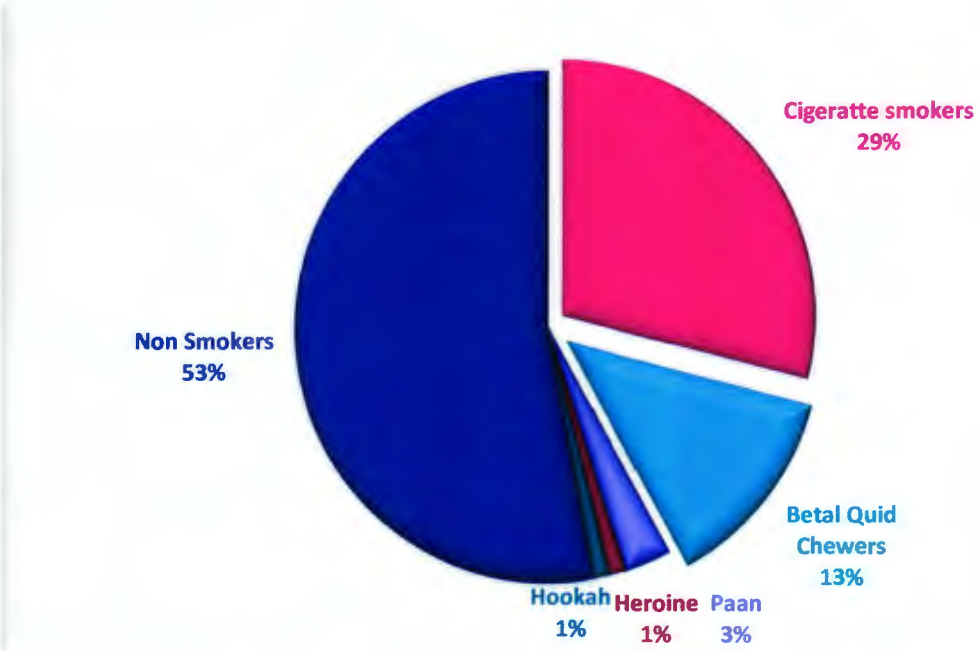

Figure 10 Pi-chart representing frequency of Smokers

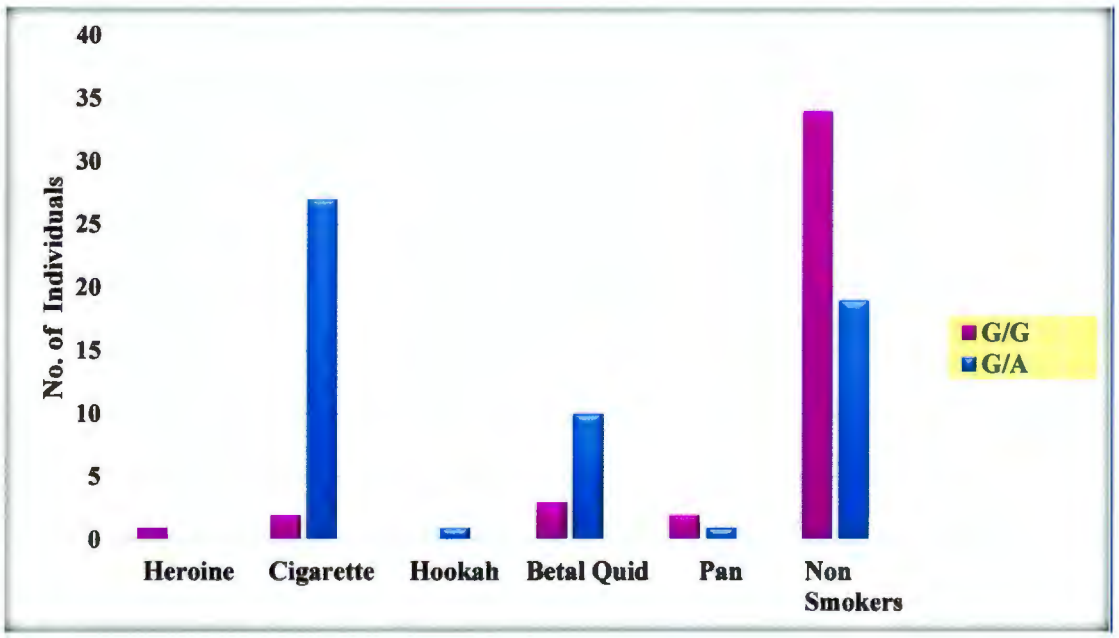

Figure 11 Bar chart representing distribution of HNC in different Smoking groups

3.10 Discussion

Head and neck cancer have been considered as seventh most common carcinoma in the world. In 2012, 599,000 new cases and 325,000 death cases were reported globally (Ferlay *et al.,* 2010). Head and neck cancer involves the carcinomas of oral cavity, larynx, pharynx, lip, buccal and tongue. Few single nucleotide polymorphisms have been investigated to show mutations among specific populations as in different studies, association between genetic variants and Head and neck cancer have been concluded (Sharan *et al.,* 2012). After the development of high-throughput techniques such as exome sequencing that particularly detect the association of related genes link-with the development and growth of the disease, the attentions have turned around (Cancer Genome Atlas, 2015). The role of NOTCH1 gene have been investigated by Whole exome sequencing technique in the development of Head and neck cancer about 15-19% (Agarwal *et al.,* 2011).

The functional inactivation of NOTCH1 have been researched to link with the squamous cell differentiation and Head and neck cancer progression (Sakamoto *et al.,* 2012). Another studies conducted by utilizing cultured esophageal keratinocytes, which also shows the role of NOTCH1 gene in squamous cell differentiation (Feigelson *et al.,* 2014). The role of NOTCH1 gene has been illustrated as tumor suppressor gene in Head and neck cancer occurrence (Cancer Genome Atlas, 2015).

The aim of this study was to determine the role of NOTCH1 C1083 rs139994842 with Head and neck Cancer in selected Pakistani population. Total 100 samples were collected to conduct the study along with the 50 controls. According to our knowledge, this study is the first study being conducted among Pakistani population.

Current study showed association of NOTCH1 C1083 rs139994842 with HNC by giving P-value 0.000 that was less than the standard P-value 0.05. A study on population of Taiwan also show significant association of NOTCH1 C1083 rs139994842 with HNC. In this study, NOTCH1 germline variant rs 139994842 shows significant association with increased risk of Head and neck Carcinoma. The observed OR value 3.46 and 95% CI, 1.11–10.84 and p-value 0.03 (Liu *et al.,* 2016). In current study, the observed OR value was 15.625 with 95% CI (5.733-42.583) and p-

value was 0.000. In present study, allelic frequencies were calculated for patient and controls. Allele G showed frequency of 0.71% in HNC patients and 2% in controls while A allele showed 0.58% frequency in HNC patients and 0% frequency in controls. According to Taiwan study, A allele of NOTCH1 C1083 rs139994842 was found higher in Patients (0.17%) than controls (0.08%) (Liu *et al.*, 2016).

Two genotypes, GG and GA were identified in present study. These genotypes were analyzed in patients and controls along with the comparison in gender. In females, GG genotype was higher (0.30%) than males (0.12%) among HNC patients. GA genotype was also found higher in males (0.50%) than females (0.08%) in HNC patients and this genotype was absent in control cases (male and female). Risk of occurrence of Head and neck cancer is higher in men (86.2%) than women (13.7%). The fact and figures from 1995 to 2006 have demonstrated that the ratio of developing head and neck carcinoma among males have increased from 7.6% to 17.7% (Chen et al., 2008). In Taiwan study, the proportions of males were reported 94.7% among patients (Liu *et al.*, 2016).

In current study, Nasopharynx cancer (0.20%) and Buccal Mucosa Cancer (0.20%) was found to be more frequent than Larynx (0.16%) Hypopharynx (0.15%) Tongue (0.11%) Maxilla (0.10%) Sinus (0.05%) Lip (0.03%). It has been found that Head and Neck cancer occurs in different ratios with the advancing age. Previously it has been reported in Taiwan study among 128 samples that 0.55% patients had Buccal cancer, 0.42% patients have Tongue cancer, 0.15% have Gum cancer, 0.07% have Palate cancer, 0.03% have Oropharynx,0.02 % have Hypopharynx and Larynx cancer (Liu *et al.*, 2016). In present study, age was one of the parameter. This study showed that most of the patients with the age group above 30 and below 65 were found to have more mutations than the age group below 30. The calculated mean and standard deviation for age was 47.27±13.46. The mean age of Head and neck cancer patient reported in Taiwan study were 53.8 years. In this particular study, no significant association were detected for the cancer stages, subtypes and cancer therapies with age.

On the other hand, Head and neck carcinoma reoccurrence and death rate show significant association. P-value calculated for tumor reoccurrence were (P=0.02) and (P=0.06) calculated for death rate (Liu *et al.*, 2016). In 2005 the particular study was conducted about Australian

population, in which the approximate age of HPV associated HNC male patients were 59.8 years and female patients were observed 63.6 years (Hocking *et al.*, 2011).

In current study, one of the important parameter was smoking. The analysis of data shown that Head and Neck cancer is more prevalent among smokers (0.58%) as compared to nonsmokers (0.42%). Chi-square value for smoking was calculated as 30.131 and p-value was 0.000. As p value was significant, it can be concluded that smoking might be the relevant risk factor for developing HNC. In current study, 0.29% were cigarette smokers,0.13% were betel quid chewers, 0.03% were Pan chewers and 0.01% were heroine and hookah addicts among 100 samples.

In Taiwan study, the ratio of betel quid chewers was 82.3% however 67.4% cases were observed as alcohol users and the percentage of cigarette smokers were 86.2% recorded among the sample size of 282 cases (Chiang *et al.*, 2008). In this study, the OR value for cigarette smoking were 1.34 calculated with (95% CI, 0.77-2.34) and P-value (0.24). The OR value for alcohol users were 1.46 (95% CI, 0.88-2.42) and P-value (0.21). The OR value for Betel chewing were 22.45 observed with (95% CI, 13.39–37.64 and P-value (<.0001) calculated. Due to significance of p value, betel quid chewers show significant association with Head and neck Cancer (Liu *et al.*, 2016). In this study, head and neck cancer associated NOTCH1 rs 139994842 show elevation with the chewing of betel quid (Chiang *et al.*, 2008) observed in incidence of 18% larynx and 79% oral carcinoma (Lee *et al.*, 2012). In this study, Betel quid were classified as human carcinogens and chewing of betel quid shows significant association with NOTCH1 gene linked Head and Neck cancer (Liu *et al.*, 2016). In this study, the chewing of betel quid had shown stronger effect which may observed to masked the cigarette smoking properties. The chewing of betel quid shown strong associations with the occurrence of Head and neck cancer by causing somatic mutations. These mutations cause abnormal alterations in the structure of NOTCH1 gene and increases the risk of Head and neck cancer, reoccurrence and death rate as well. This investigated NOTCH1 as a tumor suppressor gene in Head and neck cancer development (Liu *et al.*, 2016).

The SNP rs139994842 were identified to be associated with these NOTCH1 mutations in Taiwan population, which indicate that it might be a functional variant. Based on this speculation, germline variant at site (rs139994842) identified in the 28th EGF like repeat of NOTCH1 (Liu *et al.*, 2016).

Exon 20 of NOTCH1 gene is the large exon and contains 75% of gene coding sequence. Mutations found on this exon are either frameshift synonymous or nonsense mutations. NOTCH1 C1083 rs139994842 is a synonymous mutation that lies on this exon. The NOTCH1 C1083 rs139994842 polymorphism had been investigated as genetic variant link with the risk of Head and neck cancer in Taiwan population. It is evident from studies that loss in functionality of NOTCH1 gene leads to the development of Head and neck cancer. Current study was designed to determine the association of NOTCH1 gene with Head and neck cancer in selected Pakistani population that has not been performed earlier. By viewing the statistical results and discussion mentioned above, current study showed a significant association of NOTCH1 C1083 rs139994842 with Head and neck cancer in selected Pakistani population. Calculated value of p-value was 0.000 showing the significance of the study. It can also be seen that most of the mutation of NOTCH1 rs139994842 was found in smoker patients that gave significant p-value (0.000). Most of the patients carrying NOTCH1 rs139994842 mutation were above the age group of 30 and below 65. The calculated p-value for gender were significant (0.000) revealed that this factor is relevant with head and neck cancer in current study.

In this study not a single homozygous recessive AA genotype was found. There might be a low penetrance of NOTCH1 C1083 rs139994842 allele in patients showing GG genotype. NOTCH1 germline variant rs139994842 can statistically help as an early prognostic biomarker for Head and neck cancer occurrence and development and this data can be used effectively in prevention, observation of patients at risk, and early detection for reducing disease and death ratio in Head and neck cancer patients (Liu *et al.*, 2016).

Although in current study, selected population showed a significant association but there is a possibility that by conducting current study on large sample size of this population, more strong association could be seen. This association has not been reported previously in Pakistani population therefore we might say that NOTCH1 rs139994842 polymorphism is novel finding in this population. The findings of current case-control study are consistent with the proposal that Pakistani subjects differ from other subjects with respect to phenotypic expression of Head and neck cancer and NOTCH1 risk. The results of current study are based on small sample size, but in future more fact and figures can investigate by increasing the population sample size of study.

Regardless of some limitations, the results of the study provide a complete and systematic image of NOTCH1 polymorphisms role in the risk of developing Head and neck cancer and may provide genetic insight into possible strategies for the prevention of Head and neck cancer. On clinical ground, more effort is required to investigate the Head and neck cancer risk with NOTCH1 variant and to illustrate the best mode for the determination of NOTCH1 C1083 rs139994842 mutations.

In current study, only single germline variant rs 139994842 was examined for evaluating the association with Head and neck Cancer. However other somatic mutations can also determine for association with the development of Head and neck cancer. In future study, other NOTCH1 exons can also be selected for investigating Head and neck cancer genetic variants. The effort of current study can investigate on other populations as well. Future study is required to determine that whether other populations carry the same NOTCH1 association with Head and neck cancer or not. And whether the risk factors calculated in current study would show the similar association with Head and neck cancer. A more appropriate study design is required to determine the role of NOTCH1 polymorphism in the development of Head and neck cancer with sufficient data for investigating how genotypic and polymorphic frequency along with environmental factors could elevate the risk of NOTCH1 C1083 rs139994842 carriers in Head and neck Cancer patients.

References

References

Amelia AS, Maria TR, Carlos R., Jose IA, Marian MP, Joseph AC, (2009) P53 mutation is rare in oral mucosa brushings from patients previously treated for a head and neck squamous cell carcinoma, *Oral Oncol*; 45: p. 661-664

Agrawal N, Frederick J, Pickering R, Bettegowda C, Chang K, Li J, (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1, *Science;* 333: p. 1154–1157

Ariyawardana A, Johnson W, (2013) Trends of lip, oral cavity and oropharyngeal cancers in Australia 1982-2008: overall good news but with rising rates in the oropharynx, *BMC Cancer;* 13: p. 333

Ang K, Harris J, Wheeler R, (2010) Human papillomavirus and survival of patients with oropharyngeal cancer, N Engl J Med; 363: p. 24–35

Angadi PV, Rao SS, (2011) Areca nut in pathogenesis of oral sub mucous fibrosis: revisited. *Oral Maxillofac Surg*; 15: p. 1–9

Allocati N, DiIlio C, De Laurenzi V, (2012) p63/p73 in the control of cell cycle and cell death, *Exp Cell Res;* 318: p. 1285–1290

Agarwal AK, Sethi A, Sareen D, Dhingra S, (2011) Treatment delay in oral and oropharyngeal cancer in our population: the role of socio-economic factors and health-seeking behavior, *Indian J Otolaryngol Head Neck Surg*; 63: p. 145–150

Bhurgri Y, Bhurgri A, Usman A, Pervez S, Kayani N, Bashir I, (2006) Epidemiological review of head and neck cancers in Karachi, *Asian Pac J Cancer Prev*; 7: p. 195-200

Brennan J.A, Boyle J.O, Koch. WM, Goodman. S.N, Hruban. R.H, (1995) Association between cigarette smoking and mutation of the p53 gene in squamous-cell carcinoma of the head and neck, *N Engl J Med*; 332: p. 712–717

Brooks P.J, Theruvathu. J.A, (2005) DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis, *Alcohol*; 35: p. 187–193

Blot WJ, McLaughlin JK, Winn DM, Austin DF, Greenberg RS, Preston-Martin S, Bernstein L, Schoenberg JB, Stemhagen A, Fraumeni JF, (1988) Smoking and drinking in relation to oral and pharyngeal cancer, *Cancer Res*; 48(11): p. 3282–3287

Basu R, Mandal S, Ghosh A, Poddar TK, (2008) Role of tobacco in the development of head and neck squamous cell carcinoma in an eastern Indian population, *Asian Pac J Cancer Prev*; 9(3): p. 381–386

Bhanushali, Aparna A, Suresh B, Veera R, Renjith P, Pratiksha C, and Bibhu R, (2010) Mutations in the HD and PEST Domain of Notch-1 Receptor in T-Cell Acute Lymphoblastic Leukemia: Report of Novel Mutations from Indian Population, *Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics;* 19.2: p. 99-104

Bisht M, Bist SS, (2011) Human papilloma virus: a new risk factor in a subset of head and neck cancers, *J Cancer Res Ther*; 7(3): p. 251-255

Buchkovich K, Duffy LA, Harlow E, (1989) The retinoblastoma protein is phosphorylated during specific phases of the cell cycle, *Cell*; 58: p. 1097-1105

Boren J, Brindle M, (2012) Apoptosis-induced mitochondrial dysfunction causes cytoplasmiclipid droplet formation, *Cell Death Differ;* 19: p. 1561–1570

Bray F, Ren JS, Masuyer E, Ferlay J, (2013) Global estimates of cancer prevalence for 27 sites in the adult population in 2008, *Int J Cancer*; 132: p.1133

Bray F, Sankila R, Ferlay J, Parkin DM, (2002) Estimates of cancer incidence and mortality in Europe in 1995, *Eur J Cancer*; 38: p. 99–166

Carvalho AL, Singh B, Spiro R.H, Kowalski L.P, Shah J.P, (2004) Cancer of the oral cavity: a comparison between institutions in a developing and a developed nation, *Head Neck*; 26: p. 31-38

Cerami E, Gao J, Dogrusoz U, (2012) The Bio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data, *Cancer Discov*; 2(5): p. 401-404

Cho H, Kim J, Kim J, Kim R, (2012) POZ/BTB and AT-hook-containing zinc finger protein 1 (PATZ1) inhibits endothelial cell senescence through a p53 dependent pathway, *Cell Death Differ;* 19: p. 703–712

Chiang L, (2008) Up-regulation of inflammatory signaling by areca nut extract and role of cyclooxygenase-2 -1195G>A polymorphism reveal risk of oral cancer, *Cancer research;* 68: p. 8489–8498

Cunningham J, Rumbold AR, Zhang X, JR C, (2008) Incidence, aetiology, and outcomes of cancer in Indigenous peoples in Australia, *Lancet Oncol*; 9: p. 585–595

Chen Y, Chang J, Liao C, (2008) Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis, *Cancer Sci;* 99(8): p. 1507–1514

Canova C, Hashibe M, Simonato L, Nelis M, Metspalu A, Lagiou P, Trichopoulos D, Ahrens W, Pigeot I, Merletti F, Richiardi L, Talamini R, Barzan L, Macfarlane J, Macfarlane V, Holcátová I, Bencko V, Benhamou S, Bouchardy C, Kjaerheim K, Lowry R, Agudo A, Castellsagué X, Conway DI, McKinney A, Znaor A, McCartan E, Healy M, Marron M, Brennan P, (2009) Genetic associations of 115 polymorphisms with cancers of the upper aerodigestive tract across 10 European countries: the ARCAGE project, *Cancer Res;* 69: p. 2956–2965

Cruz B, Snijders J, Steenbergen D, (1996) Age dependence of human papillomavirus DNA presence in oral squamous cell carcinomas, *Eur J Cancer B Oral Oncol;* 32: p. 55–62

Chaudhry S, (2008) Estimating the burden of head and neck cancers in the public health sector of Pakistan, *Asian Pac J Cancer Prev;* 9: p. 529-532

Cancer Genome Atlas, (2015) Comprehensive genomic characterization of head and neck squamous cell carcinomas, *Nature;* 517: p. 576–582

Cayo M, Greenblatt D, Kunnimalaiyaan M, Chen H, (2008) NOTCH1 (Notch homolog 1, translocation-associated Drosophila), *Atlas Genet Cytogenet Oncol Haematol;* 12(2): p. 111-119

Celis F, Bray J, (2000) The Abruptex domain of Notch regulates negative interactions between Notch its ligands and Fringe, *Development;* 127: p. 1291–1302

Dayal K, Mani J, Bhargava K, (1978) Prevalence of oral cancer and precancerous lesions in 'pan'/'supari' chewers, *Indian J Public Health*; 22: p. 234–245

Dayyani F, Etzel J, Liu M, Ho C, Lippman M, Tsao S, (2010) Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC), *Head Neck Oncol;* 2(1): p. 15

D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, (2007) Case-control study of human papillomavirus and oropharyngeal cancer, N *Eng J Med*; 356(19): p. 1944-1956

D'Souza G, Fakhry C, Sugar E.A, Seaberg EC, Weber K, (2007) Six-month natural history of oral versus cervical human papillomavirus infection, *Int J Cancer*; 121: p. 143–150

DeSantis C, Naishadham D, Jemal A, (2013) Cancer statistics for African Americans, *CA Cancer J Clin*; 63: p. 151

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM, (2010) Estimates of worldwide burden of cancer in 2008, *Int J Cancer*; 127(12): p. 2893–2917

Feigelson H, (2014) Approaches to integrating germline and tumor genomic data in cancer research, *Carcinogenesis,* 35: p. 2157–2163

Gupta PC, Warnakulasuriya S, (2002) Global epidemiology of areca nut usage. *Addict Biol*; 7: p. 77–83

Graham S, (1977) Dentition, diet, tobacco and alcohol in the epidemiology of oral cancer, *J Natl Cancer Inst*; 59: p. 1611–1618

Gupta PC, Ray CS, (2004) Epidemiology of betel quid usage. *Ann Acad Med Singapore*; 33: p. 31–36

Gupta PC, Sinor PN, Bhonsle RB, Pawar VS, Mehta HC, (1998) Oral sub-mucous fibrosis in India: a newepidemic, *Natl Med J India*; 11: p. 13–16

Goon P, Stanley M, Ebmeyer J, Steinsträsser L, Upile T, Jerjes W, (2009) HPV & head and neck cancer: a descriptive update, *Head Neck Oncol;* 1: p. 36

Gatta G, Botta L, Sanchez MJ, (2015) Prognoses and improvement for head and neck cancers diagnosed in Europe in early 2000s: The EUROCARE-5 population-based study, *Eur J Cancer*; 51: p. 21-30

Hocking S, Stein A, Conway L, Regan D, Grulich A, Law M, Brotherton M, (2011) Head and neck cancer in Australia between 1982 and 2005 show increasing incidence ofpotentially HPV-associated oropharyngeal cancers, *Br J Cancer;* 104(5): p. 886–891

Haddad R.I, Shin D.M, (2008) Recent advances in head and neck cancer, *N Eng J Med*; 359(11): p. 1143-1154

Ho T, Wei Q, Sturgis E.M, (2007) Epidemiology of carcinogen metabolism genes and risk of squamous cell carcinoma of the head and neck, *Head Neck*; 29: p. 682–699

Haines G.K, (2013). Pathology of Head and Neck Cancers I: Epithelial and Related Tumors, *Springer Science & Business Media;* 3: p. 257–287

Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun M.J, (2007) Cancer statistics, *CA Cancer J Clin*; 57: p. 43–66

John R, Bonnie S, Glisson, Miriam N, Lango, Feigenberg S, (2014) Head and neck tumors, *Maryland Cancer management*

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun M.J, (2009) Cancer statistics, *CA Cancer J Clin*; 59(4): p. 225-249

Jemal A, (2011) Global cancer statistics, *CA: a cancer journal for clinicians;* 61(2): p. 69-90

Kreimer R, Clifford M, Boyle P, Franceschi S, (2005) Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review, *Cancer Epidemiol Biomarkers Prev;* 14: p. 467–475

Kopan R, Ilagan X, (2009) The canonical Notch signaling pathway: unfolding the activation mechanism, *Cell;* 137: p. 216–233

Krishnatreya M, Rahman T, Kataki A, Das A, Das K, Lahkar K, (2013) Synchronous primary cancers of the head and neck region and upper aero digestive tract: defining high-risk patients, *Indian journal of cancer;* 50 (4): p. 322–326

Kovall A, (2008) More complicated than it looks: assembly of Notch pathway transcription complexes, *Oncogene;* 27: p. 5099–5109

Kandoth C, McLellan D, Vandin F, Ye K, Niu B, Lu C, (2013) Mutational landscape and significance across 12 major cancer types, *Nature;* 502: p. 333–339

Kreimer A, Clifford G, Boyle P, Franceschi S, (2005) Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review, *Cancer Epidemiol Biomarkers Prev;* 14(2): p. 467–475

Lee H, (2012) The neoplastic impact of tobacco-free betel-quid on the histological type and the anatomical site of aerodigestive tract cancers, *International journal of cancer;* 131: p. 733–743

Laimer K, Spizzo G, Gastl G, Obrist P, Brunhuber T, Fong D, (2007) High EGFR expression predicts poor prognosis in patients with squamous cell carcinoma of the oral cavity and oropharynx: a TMA-based immunohistochemical analysis, *Oral Oncol*; 43: p. 193-198

Levine A.J, (1993) The tumor suppressor genes, Annu Rev *Biochem*; 62: p. 623–651

Lane D.P, (1992) Cancer. p53, guardian of the genome, *Nature*; 358: p. 15-16

Liu Y, Chiang S, Lin C, Chang J, Chung C, Ko A, Lin Y, Lee C, Lee K, Chen M, Hua C, Tsai M, Chen Y, Ko Y, (2016) Somatic Mutations and Genetic Variants of NOTCH1 in Head and Neck Squamous Cell Carcinoma Occurrence and Development, *Nature;* 6: p. 24014

Leonova I, Brodsky L, Lipchick B, Pal M, Novototskaya L, Chenchik A, (2013) p53 cooperates with DNA methylation and a suicidal interferon response to maintain epigenetic silencing of repeats and noncoding RNAs, *Proc Natl Acad Sci USA;* 110: p. 89–98

Lee KS, Conigrave KM, Patton GC, Clough AR, (2009) Cannabis use in remote Indigenous communities in Australia: endemic yet neglected, *Med J Aust*; 190(5): p. 228–239

Lambert R, Sauvaget C, de Camargo Cancela M, Sankaranarayanan R, (2011) Epidemiology of cancer from the oral cavity and oropharynx, Eur J Gastroenterol Hepatol; 23: p. 633

Leemans CR, Braakhuis BJ, Brakenhoff RH, (2011) The molecular biology of head and neck cancer, Nature reviews. Cancer; 11: p. 9–22

Mehanna H, Paleri V, West C, Nutting C, (2010) Head and neck cancer—Part 1: epidemiology, presentation, and prevention, *BMJ;* 341: p. 46–84

Marcu G, Yeoh E, (2009) A review of risk factors and genetic alterations in head and neck carcinogenesis and implications for current and future approaches to treatment, *J Cancer Res Clin Oncol;* 135(10): p. 1303–1314

Muñoz N, Bosch F, de Sanjosé S, (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer, *N Engl J Med;* 348: p. 518–527

Morton P, Timpson P, Karim A, Ridgway A, Athineos D, Doyle B, (2010) Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer, *Proc Natl Acad Sci USA;* 107: p. 246–251

Murray CJL, Lopez AD, (1996) The Global Burden of Disease: A Comprehensive Assessment of Mortality and Disability from Diseases, Injuries, and Risk Factors in 1990 and Projected to 2020, *Boston: Harvard University Press*

Nicolas M, Wolfer A, Raj K, (2003) Notch1 functions as a tumor suppressor in mouse skin, *Nat Genet*; 33(3): p. 416-421

Panzarella V, Falaschini S, Russo A, Matranga D, Lo Muzio L, (2008) HPV in oral squamous cell carcinoma vs head and neck squamous cell carcinoma biopsies: a metaanalysis (1988–2007), *Ann Oncol;* 19(10): p. 1681–1690

Posner M, Lorch J, Goloubeva O, (2011) Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial, *Ann Oncol;* 22: p. 1071–1077

Proia N, Paszkiewicz G, Nasca M, Franke G, Pauly J, (2006) Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer-a review. *Cancer Epidemiol Biomarkers Prev*; 15: p. 1061–1077

Poeta M.L, Manola J, Goldwasser M.A, Forastiere A, Benoit N, Califano JA, (2007) Tp53 mutations and survival in squamous cell carcinoma of the head and neck, *N Engl J Med*; 357: p. 2552-2561

Polager S, Ginsberg D, (2009) p53 and E2f: partners in life and death, *Nat Rev Cancer;* 9: p. 738–748

Perea-Milla López E, Miñarro-Del Moral M, Martínez-García C, Zanetti R, Rosso S, Serrano S, Aneiros F, Jimenez-Puente A, Redondo M, (2003) Lifestyles, environmental and phenotypic factors associated with lip cancer: a case–control study in southern Spain, *Br J Cancer;* 88: p. 1702–1707

Park JH, Slack-Smith L, Smith A, Frydrych AM, O'Ferrall I, Bulsara M, (2011) Knowledge and perceptions regarding oral and pharyngeal carcinoma among adult dental patients, Aust Dent J; 56(3): p. 284–289

Poeta L, Manola J, Goldwasser A, Forastiere A, Benoit N, Califano A, (2007) TP53 mutations and survival in squamous-cell carcinoma of the head and neck, *N Engl J Med;* 357: p. 2552–2561

Parkin DM, Bray F, Ferlay J, Pisani P, (2002) Global cancer statistics, *CA Cancer J Clin*; 55(2): p. 74–108

Ranganathan P, Weaver KL, Capobianco AJ, (2011) Notch signalling in solid tumours: a little bit of everything but not all the time, *Nat Rev Cancer*; 11(5): p. 338-351

Raybaud-Diogene H, Tetu B, Morency R, Fortin A, Monteil RA, (1996) P53 overexpression in head and neck squamous cell carcinoma: review of the literature, *Oral Oncol Eur J Cancer*; 32B: p. 143-149

Rasmussen UB, Wolf C, Mattei M. G, (1993) Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma, *Cancer Res*; 53: p. 4096-4101

Stricker TP, Kumar V, (2007) Neoplasia Robin's basic pathology 8th ed. Philadelphia, *W.B. Saunders*; p. 173-223

Schlecht N.F, Franco E.L, Pintos J, Kowalski L.P, (1999) Effect of smoking cessation and tobacco type on the risk of cancers of the upper aero-digestive tract in Brazil, *Epidemiology*; 10: p. 412–418

Sturgis E.M, Wei Q, Spitz MR, (2004) Descriptive epidemiology and risk factors for head and neck cancer, *Semin Oncol*; 31: p. 726–733

Sana M, Irshad S, (2012) P53 as a biomarker of breast cancer, *Res Cancer Tumor*; 1: p. 5-8

Sharan N, Mehrotra R, Choudhury Y, Asotra K, (2012) Association of betel nut with carcinogenesis: revisit with a clinical perspective, *PLos One;* 7: p. 427-459

Sugerman P, Savage N, (2002) Oral cancer in Australia: 1983– 1996, *Aust Dent J;* 47(1): p. 45–56

Sherr J, McCormick F, (2002) The RB and p53 pathways in cancer, *Cancer Cell;* 2: p. 103–112

Sakamoto K, (2012) Reduction of NOTCH1 expression pertains to maturation abnormalities of keratinocytes in squamous neoplasms. Laboratory investigation, *a journal of technical methods and pathology;* 92: p. 688–702

Silveira A, Goncalves J, Sequeira T, Ribeiro C, Lopes C, Monteiro E, Pimentel FL, (2012) Head and neck cancer: health related quality of life assessment considering clinical and epidemiological perspectives, *Rev Bras Epidemiol*; 15(1): p. 38–48

Siegel RL, Miller KD, Jemal A, (2016) Cancer statistics, *CA Cancer J Clin*; 66: p. 7

Turati F, Edefonti V, Bosetti C, Ferraroni M, Malvezzi M, Franceschi S, Talamini R, Montella M, Levi F, Dal Maso L, Serraino D, Polesel J, Negri E, Decarli A, La Vecchia C, (2013) Family history of cancer and the risk of cancer: a network of case-control studies, *Ann Oncol;* 24(10): p. 2651–2656

Teodorczyk M, & Schmidt H, (2014) Notching on Cancer's Door: Notch Signaling in Brain Tumors, *Front Oncol;* 4: p. 341

Toustrup K, Sorensen BS, Lassen P, (2012) Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck, *Radiother Oncol*; 102(1): p. 122-129

Victoria Department of Health (2008) Preventing alcohol-related harm in Australia: a window of opportunity. *Victoria: Department of Health*

Vogelstein B, Lane D, Levine J, (2000) Surfing the p53 network, *Nature;* 408: p. 307–310 Pelucchi C, Gallus S, Garavello W, (2008) Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver, Eur *J Cancer Prev*; 17: p. 340–344

Wen Q.H, Miwa T; Yoshizaki T, (1996) Prognostic value of EGFR and TGF-alpha in early laryngeal cancer treated with radiotherapy, *Laryngoscope*; 106: p. 884-888

Weinberg R, (1991) Tumor suppressor genes, *Science*; 254: p. 1138–1146

Welcker M, Clurman BE, (2008) FBW7 ubiquitin ligase: a tumour suppressor at the crossroads of cell division, growth and differentiation, *Nat Rev Cancer*; 8(2): p. 83-93

Wu TC, Tashkin DP, Djahed B, Rose JE, (1988) Pulmonary hazards of smoking marijuana as compared with tobacco, *N Engl J Med*; 318(6): p. 347–351

Warnakulasuriya S, (2009) Global epidemiology of oral and oropharyngeal cancer, *Oral Oncol*; 45(4): p. 309–316

Warnakulasuriya S, Trivedy C, Peters TJ, (2002) Areca nut use: an independent risk factor for oral cancer, *BMJ*; 324: p. 799–800

Zhang X, Condon JR, Rumbold AR, Cunningham J, Roder DM, (2011) Estimating cancer incidence in Indigenous Australians, Aust N Z J Public Health; 35(5): p. 477–485

Zheng T, Boyle P, Hu H, (1990) Dentition, oral hygiene, and risk of oral cancer: a case-control study in Beijing, People's Republic of China, *Cancer Causes Control*; 1: p. 235–241