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# Mutation Landscape in Thyroid Cancer: Implications for Tumor Grading and Prognosis

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## ABSTRACT

**Background:**The Aim Of The Study Was To Estimation Of The Association Between The Genetic Alterations In Proto-Oncogenes (Egfr) And Overall Thyroid Cancer.

**Methods**:In A Prospective Study, A Total Of Sixty (N=60) Histologically Confirmed, Previously Untreated Thyroid Cancer Patients Are In This Study. Tumour Tissue Along With Corresponding Normal Tissue Underwent An Egfr Mutation Test. The Association Of Egfr Mutation In Thyroid Cancer And Family History Were Evaluated Using Allele Specific Pcr Was Setup To Detect Mutations, If Any, In Exon 19, 20 And 21 Of Egfr.

**Results:**Total Of 32 Patients 53.3% (32/60) Were Having 15bp Deletion In Exon 19 Of Egfr (P≤0.05). The Total Mutational Rate Of T790m In Egfr Tyrosine Kinase Domain (Exon 20) Among 60 Patients Was Found To Be Only 8.4% (05 Of 60) (P>0.05). The Total Of 43.3% (26 Of 60) Of Thyroid Cancer Patients Were Positive For Egfr L858r (T2573g) Mutation In Exon 21(P≤0.05).

**Conclusion**:A Significant Association Was Found Between Tumour Grading And T858r Mutational Status Of Egfr Gene As 34.2% Of Patients With Well Differentiated Carcinoma Were Positive For The Mutation As Opposed To 63.2% Of Patients With Poorly Differentiated Grade (P≤0.05). Significant Relations Of Multiple Variables Were Seen In Associations With Egfr Domain.

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## 1. . INTRODUCTION

Thyroid cancers are malignant tumours in the thyroid gland, a butterfly-shaped organ draped around the front and sides of the wind pipe (or trachea) in the lower neck (1-8). In our region the incidence of thyroid cancer is not known due to the non-availability of cancer registry, but our observational study has revealed that the cases are increasing manifold particularly in women. The incidence of thyroid cancer in women has increased by 34 percent in the last decade (compared with 17 percent in men). The age-adjusted incidence rate per 100,000 women in California was 9.9 in 2000 compared with 7.4 only a decade earlier [9, 10]. In areas without endemic goitre, over 80 percent of all thyroid cancers are of papillary histology (or its variant, mixed papillary-follicular). The prognosis for this cancer is very good, especially for young women, with a 10-year survival rate of over 99 percent for women diagnosed before the age of 45 years [11]. Other than ionizing radiation and proliferative benign thyroid disease, the causes of thyroid cancer are still largely unknown. Dietary factors may also influence risk. Recent studies have found that greater consumption of soy-based foods and vegetables is associated with a reduction in thyroid cancer risk [12, 13]. Conventional clinicopathological evaluation is currently the basis upon which risk stratification is pursued for patients with thyroid cancer [14, 15]. There are several clinicopathological characteristics that are classical high-risk factors, including old patient age at the time of diagnosis, male gender, large tumour size, extra thyroidal invasion, lymph node metastasis, distant metastasis, and advanced disease stages [16-22]. Each of these clinic-pathological risk factors has been shown to be associated with an increased risk for the progression, recurrence, and even morbidity and mortality of thyroid cancer. Cancers arise owing to the accumulation of mutations in critical genes that alter normal programmes of cell proliferation, differentiation and death. As the first stage of a systematic genome wide screen for these genes, we have prioritized for analysis signalling pathways in which at least one gene is mutated in human cancer. Thyroid cancer harbours several highly prevalent genetic alterations, some of which are seen only in this cancer. The classical oncogenic genetic alterations commonly seen in thyroid cancer include RAS mutations [23-25], RET/PTC rearrangements [26-28], and PAX8-peroxisome proliferator-activated receptor-g (PPARg) fusion oncogene [29,30]. Various activating RAS mutations, widely seen in other cancers as well, occur mainly in FTC and the follicular variant of PTC [31,32]. mutation in BRAF (the gene for the B-type RAF kinase, BRAF) the most common genetic alteration in thyroid cancer. Discovery of this genetic alteration has created the opportunity to develop novel clinical strategies for the management of thyroid cancer, as EGFR mutations are now increasingly seen as a new

research topic with thyroid cancers. We will define Epidermal growth factor receptor (EGFR) as a transmembrane glycoprotein belonging to the [erbB] family of receptor tyrosine kinases [33]. A large number of somatic mutations have been reported in EGFR in several cases of non-small cell lung cancers.[35] These are mainly localized to the tyrosine kinase domain of the protein. Activation of EGFR promotes processes responsible for tumour growth and progression, including proliferation, maturation,

angiogenesis, invasion, metastases and inhibition of apoptosis. EGFR has been detected in varying degrees in a wide range of solid tumors [36]. Cetuximab is an approved monoclonal antibody therapy targeting EGFR for colorectal and squamous cell carcinoma of head and neck cancer. Erlotinib is a tyrosine kinase inhibitor (TKI) which is an FDA approved drug for non-small cell lung cancer and pancreatic cancers and is in clinical trial in thyroid cancers in particular. There has been exciting progress in understanding its molecular pathogenesis in recent years with the thyroid cancers molecular biology [37] as best exemplified by the elucidation of the fundamental role of several major signalling pathways and related molecular derangements.

Many of these molecular alterations represent novel diagnostic and prognostic molecular markers and therapeutic targets for thyroid cancer, which provide unprecedented opportunities for further research and clinical development of novel treatment strategies for this cancer. As the focus of our study is EGFR mutation in thyroid cancers we will try to elaborate the concern molecular marker with the latest research on it. Epidermal growth factor receptor gene mutations in papillary thyroid cancers have shown in recent studies,[38] which indicated that somatic mutations in the epidermal growth factor receptor (EGFR) gene have been identified in a subset of patients with non-small cell lung cancer (NSCLC) and are associated with sensitivity to the EGFR-tyrosine-kinase inhibitors. These mutations have been reported to be almost exclusively found in a pulmonary adenocarcinoma subgroup of NSCLC, with a low frequency in other solid tumours. In other studies, it was found that Epidermal Growth Factor Receptor Over expression is a Marker for Adverse Pathologic Features seen in Papillary Thyroid Carcinoma [39]. It is suggested that the EGFR status should be analysed at diagnosis in any patient with a poorly differentiated tumour. The presence of an EGFR mutation may provide an effective therapeutic pathway for these patients.

## 2. MATERIAL AND METHODOLOGY

This was a prospective study and 60 patients of thyroid cancers were included in this study. The aim of this study is

1. Collection of biopsies/samples from operated patients with thyroid cancers for screening of EGFR mutations.

2. Development of gene mutational profile of EGFR in thyroid cancer patients from Kashmiri population.

**Patients and Controls**

A total of sixty (n=60) histologically confirmed, previously untreated Thyroid cancer patients attending Department of General and Minimal Access Surgery at our terttiary care Centre hospital were included in this study. Tumour tissue along with corresponding normal tissue was available for all 60 patients. A written pre informed consent was obtained from all cases and controls. Demographic and clinicopathological characteristics of each patient were recorded in a Questionnaire. This study was approved by the Ethical committee of the institution.

**Sample collection/storage**

The surgically resected tissue samples either by total thyroidectomy/hemi-thyroidectomy or Lobectomy, were collected directly into sterile vials containing chilled PBS (pH=7.2) and frozen at -80°C for molecular investigations. Adjacent normal tissues were resected from outside the margins of resection. Histopathologically confirmed Thyroid cancer tissues and corresponding normal tissues were used for mutational analysis of *EGFR* gene.

**4.3 Extraction and Quantitation of genomic DNA**

Methodology of DNA extraction

High-molecular-weight DNA was isolated by using *proteinase-K* and phenol method

The frozen tissue was allowed to thaw at room temperature.

The tissue was chopped with fresh surgical blades in a sterile petri dish.

The chopped tissue was then transferred into a sterile polypropylene tube (15ml) containing 3ml of 1X TE (see appendix II), 2ml of lysis buffer (see appendix II) and *Proteinase-K* to a final concentration of 100µg/ml was added to it.

The mixture was incubated at 37°C in a water bath for overnight.

Next day, equal volume of TE saturated phenol (see appendix II) was added and the mixture was gently mixed by inversion of tubes on overhead shaker for 15 minutes.

The tubes were then centrifuged at 3000-4000 rpm at 4°C for 15 minutes.

The supernatant aqueous phase was collected in a fresh polypropylene tube without disturbing the interphase with the help of a micropipette fitted with a wide bored tip.

To the supernatant from above step, equal volume of TE saturated phenol chloroform-isoamylalcohol (25:24:1) was added and the mixture was shaken on overhead shaker for 15 minutes and steps 6 and 7 repeated.

To the supernatant thus obtained in fresh tube, equal volume of chloroform isoamylalcohol (24:1) was added and each tube was shaken, and step 6 and 7 repeated.

To the supernatant from the above step, 1/10 volume of chilled 3M sodium acetate solution (pH=5.2) and 2.5 volumes of chilled ethanol or equal volume of isopropanol was added and mixed by gently inverting the tube. If visible precipitate of genomic DNA appeared, it was transferred to 1.5ml microfuge tube and centrifuged at 6000 rpm for 5 minutes. The pellet thus obtained was washed with 500µl of 70% ethanol and re-centrifuged. The washing was repeated.

If the precipitate of genomic DNA was not visible, the tubes were then allowed to stand at either -70°C for 10 minutes or at -20°C for overnight. Next day, the tube was centrifuged at 6000 rpm at 4°C for 45 minutes. The pellet thus obtained was washed twice with 70% ethanol as above.

Air/vacuum dried DNA pellet was dissolved in 200µl of DNA storage buffer and stored at 4°C or at -20°C for longer periods.

**4.4 Polymerase chain reaction**

*Allele-specific PCR (AS-PCR)*

Given the high frequency of *EGFR* mutations and the possible implication of this receptor in the development of thyroid cancer, it was important to develop a simple, fast, and reliable method to identify these mutations in greater detail as a potential tool for the diagnosis and follow-up of these patients. The mutations in exon 19, 20 and 21 of EGFR gene account for more than 95% of total mutations in the gene. These mutations therefore represent an excellent target for assays, such as allele-specific PCR (AS-PCR) that depends on the specific detection of point mutations. The general principle underlying the AS-PCR technique is to design a mutation-specific primer that produces the preferential amplification of a specific mutant allele **(Bakkar et al., 2005)**. The schematic representation of this AS-PCR is shown in *Figure 3.11.*

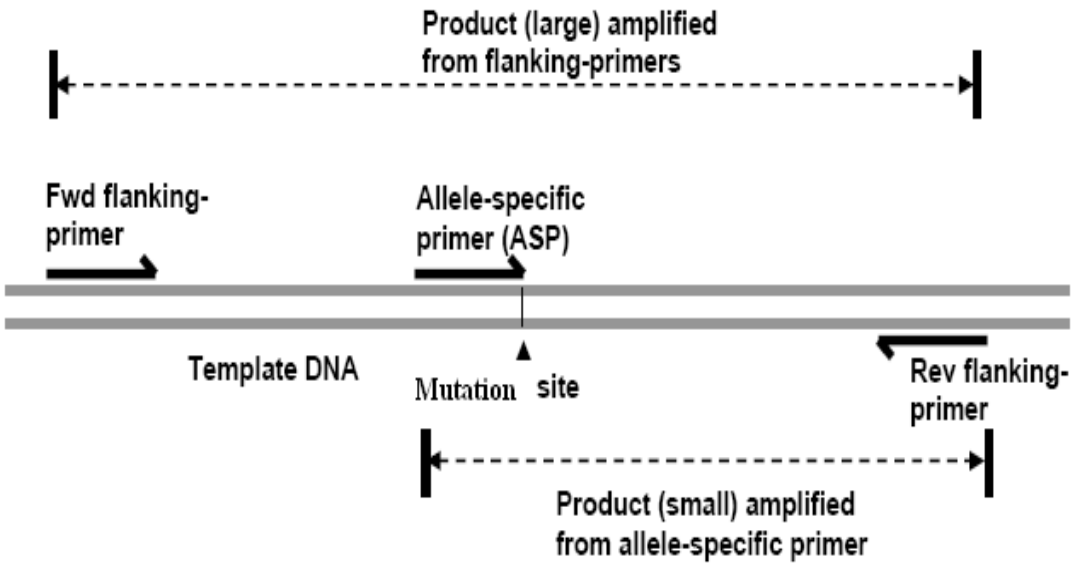


**Figure 4.1: Schematic representation of Allele-specific PCR.**

*Procedure for AS-PCR amplification of various exons of EGFR genes*

All precautions were taken to ensure contamination free amplification of DNA. Prevention of carry-over by separation of pre and post-PCR steps, and use of aerosol-free tips were strictly followed. Results were considered valid only on reproduction of same in two independent experiments.

*Equipment's and Reagents*

Thermal cycler (Eppendorf Thermocycler).

Micropipettes

0.2 ml PCR tubes

Genomic DNA: 250 ng/µl

10X PCR buffer: 100mM Tris-HCL, pH 8.3; 500mM KCL; 15mM $MgCl_2$; 0.1% gelatine and 1% Triton X100.

Deoxyribonucleotide triphosphate: 10mM each dATP, dCTP, dGTP and dTTP.

Primers: 10µM in sterile deionised water.

*Taq* DNA polymerase: 5U/µl.

*Primers for amplification*

Four primers were used in a single tube to setup an ARMS PCR for exon 19 (15 bp deletion; codons 746-750) of EGFR gene (Table 3.1).

Four primers were used in a two-tube reaction for setting up of AS-PCR for the detection of mutation in exon 20 (T790M) (Table 3.1).

Two allele specific primers and a single common primer were used in two tubes to determine the exon 21 mutations (L858R) in EGFR gene of thyroid cancer patients (Table 3.1).

*Protocol*

The amplification reaction was carried out in 25µl reaction volume in a 0.2ml PCR tubes. The reaction contained the following reagent volumes: -

| | |
|---|---|
| 1. 10X PCR buffer | 2.5µl |
| 2. 10mM dNTP mix | 0.5µl |
| 3. Primer no. 01 | 0.5µl |
| 4. Primer no. 02 | 0.5µl |
| 5. Primer no. 03 | 0.5µl |
| 6. Primer no. 04 | 0.5µl |
| 5. *Taq* DNA polymerase (5U/ µl) | 0.2µl |
| 6. Genomic DNA | 1.0µl |
| 7. Distilled water | (25-rest of components) µl |
| *Total volume* | *25.0µl* |

The above-mentioned reagents were pipetted in a 0.2ml thin-walled PCR tube and placed in Thermocycler. The following temperature profile was used for amplification: -

| | |
|---|---|
| 1. Initial denaturation | 95°C for 4 minutes |
| 2. Denaturation | 95°C for 30 seconds |
| 3. Annealing | x°C for 30 seconds* |
| 4. Extension | 72°C for 30 seconds |
| 5. Final Extension | 72°C for 7 minutes |

Temperature profile from step 2-4 was used for 35 cycles before final extension

*x was 3-5°C lower than the melting temperature ($T_m$) of the primers and was calculated by using the following formulae.

1. For primers 14-25 nucleotides in length:

$T_m$ = [2°C x (number of A and T bases)] + [4°C x (number of G and C bases)]

**Table 4.1:** Primers, product size and annealing temperatures used to detect mutations, if any, in various exons of *EGFR* gene by ARMS-PCR and AS-PCR.

**4.5 Statistical Analysis**

| Amplicon | Change | Primer sequence* | Annealing Temp. (°C) | Product size (bp) |
|---|---|---|---|---|
| **Exon 19** | 15 bp deletion; codons 746-750 | P - 5'-GTAACATCCACCCAGATCACTG-3'<br>Q - 5'-GTGTCAAGAAACTAGTGCTGGG-3'<br>A - 5'-CCCGTCGCTATCAAGGAATTAA-3'<br>B - 5'-GTTGGCTTTCGGAGATGTTTTGATAG-3' | 60 | **(Single tube reaction)**<br>**PQ=444bp (control)**<br>**AQ=325bp (deletion absent)**<br>**PB=134bp (deletion present)** |
| **Exon 20** | T790M | E - 5'-GAAGCCACACTGACGTGCCT-3'<br>F - 5'-GCCGAAGGGCATGAGCTGTG-3'<br>G - 5'-ACCATGCGAAGCCACACTGACG-3'<br>H - 5'-GCCGAAGGGCATGAGCTGGA-3' | 56 | **(Two tube reaction)**<br>**EF= 139bp (for wild allele)**<br>**GH=146bp (for variant allele)** |
| **Exon 21** | **L858R (T2573G)** | **P - 5'-GGGTCTTCTCTGTTTCAGGGCAT-3'**<br>**A - 5'-TTCCGCACCCAGCAGTTTGGCTA-3'**<br>**B - 5'-CGCACCCAGCAGTTTGGTTC-3'** | **60** | **(Two tube reaction)**<br>**PA=137 bp (wild allele)**<br>**PB=134 bp (variant allele)** |

Statistical analysis was performed by using SPSS software (V. 20.0). Chi Square test for homogeneity of proportions was used to determine significance of mutation pattern and Odds ratio was used to determine association of presence of mutations with various Clinico-epidemiological characteristics such as age, site of tumour, clinical tumour stage and histo-pathological grade of tumour. Statistical significance was considered when $p \leq 0.05$.

## 3. RESULTS:

**Mutational analysis of EGFR gene**

*5.1.1 Patient Selection and Characteristics of the study subjects*

A total of sixty (n=60) newly diagnosed thyroid cancer patients who underwent total thyroidectomy/hemi-thyroidectomy/lobectomy in the Department of General and Minimal Access Surgery at our tertiary care centre were selected for the study. Those patients who received previous cytotoxic chemotherapy or radiation were excluded from the analysis. There were no restrictions on age, sex, histology or stage, but patients with a prior history of cancer other than thyroid cancer were excluded from the study. Clinical information, including gender, age, tumour stage, tumour grade, smoking history and histopathology were obtained from the review of patients/medical records. There were 16 males and 44 females with an age at diagnosis ranging from 14 years to 44 years. There were 50 smokers and only 10 non-smokers. Based on histopathological examination there were 48 Papillary thyroid carcinomas (PTC) and 12 Follicular thyroid carcinomas (FTC). The present study also categorized patients based on grade of differentiation, into well differentiated (n=41) and poorly differentiated (n=19). Lymph node metastasis was present in 32 patients and absent in 28 patients.

For the mutational analysis of *EGFR* gene, sixty (n=60) thyroid cancer patients were studied. Somatic mutation screening was done on surgically resected and histo-pathologically confirmed tumor and the corresponding normal tissues of thyroid cancer patients Various clinico-pathological features of the patients are summarized in *Table 4.1.*

*5.1.2 DNA Extraction*

High molecular weight DNA was isolated from the samples and confirmed by electrophoresis on 1% agarose gel as shown in *Figure 4.1.* The 260/280 of the isolated DNA ranged from 1.6-1.8.

*4.1.3 Allele Specific Polymerase chain reaction (AS-PCR) for EGFR amplification*

Allele specific PCR was setup to detect mutations, if any, in exon 19, 20 and 21 of *EGFR* gene using primers specific for the mutations.

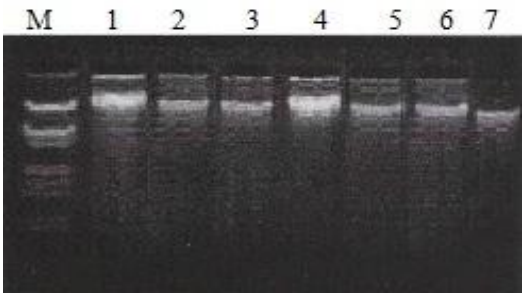


**Figure 5.1: Representative gel picture of genomic DNA isolated from blood, tumor tissue, and adjacent normal tissue of thyroid cancer patients.**

*Lane M: lambda DNA EcoRI and Hind III digest*

*Lanes 1-7: DNA extracted from different tissues/blood samples*

**Table 5.1: Clinico-epidemiological and clinico-pathological variables of thyroid cancer patients used for molecular analysis of EGFR gene.**

| Variable | Parameter | Cases (n = 60) | |
|---|---|---|---|
| | | n | % |
| **Gender** | Female | 44 | 73.0 |
| | Male | 16 | 27.0 |
| **Age in years** | <45 | 45 | 75.0 |
| | ≥45 | 15 | 25.0 |
| **Habitation** | Rural | 51 | 85.0 |
| | Urban | 09 | 15.0 |
| **Marital Status** | Unmarried | 25 | 41.6 |
| | Married | 35 | 53.4 |
| **Use of OCP** | Yes | 05 | 8.4 |
| | No | 55 | 91.6 |
| **Smoking status** | Non-smoker | 50 | 83.3 |
| | Smoker | 10 | 16.7 |
| **TSH levels** | Elevated | 27 | 45.0 |
| | Normal | 33 | 55.0 |
| **Serum Calcium** | Normal | 40 | 66.6 |
| | Decreased | 20 | 33.4 |
| **Initial PPX** | Swelling | 55 | 91.6 |
| | Incidental detection | 05 | 8.4 |
| **BTD** | Yes | 05 | 8.4 |
| | No | 55 | 91.6 |
| **Histological types** | Papillary | 48 | 80.0 |
| | Follicular | 12 | 20.0 |

| | | | |
|---|---|---|---|
| **Grade** | WD<br>PD | 41<br>19 | 68.4<br>31.6 |
| **Stage, < 45 years** | Stage I<br>Stage II | 28<br>17 | 46.6<br>28.4 |
| **Stage, ≥ 45 years** | Stage I&II<br>Stage III/above | 09<br>06 | 15.0<br>10.0 |
| **LN metastasis** | Present<br>Absent | 32<br>28 | 53.4<br>46.6 |
| **V/C Invasion** | Present<br>Absent | 17<br>43 | 28.4<br>71.6 |

*n; Number, OCP; Oral contraceptive pills, TSH; Thyroid stimulating hormone, PPX; Prophylaxis, BTD; Benign Thyroid Disease, WD; Well differentiated, PD; Poorly differentiated, LN; Lymph node, V/C; Vascular/Capsular.*

**5.2 *EGFR mutational spectrum***

*5.2.1 EGFR 15 bp deletion (codons 746 to 760; exon 19)*

The representative PCR gel picture for the detection of 15bp deletion in exon 19 of *EGFR* gene is shown in *Figure 4.2.* Total of 32 patients were having 15bp deletion in exon 19 of *EGFR.* The overall 15bp deletion rate in *EGFR* tyrosine kinase domain (exon 19) among 60 patients was found to be 53.3% (32/60). Among patients with elevated TSH levels, 77.7% (21 of 27) were having 15bp deletion in exon 19 compared to only 22.3% (06 of 21) which did not have deletions (P≤0.05). 60.5% (29/48) deletions were detected in Papillary thyroid carcinomas (PTC) correlated to only 25.0% (03/12) in Follicular thyroid carcinomas (FTC). Association of *EGFR* 15 bp deletion with different clinicopathological variables are shown in *Table 4.2.*

*5.2.2 EGFR T790M mutation in exon 20*

The representative PCR gel picture for the detection of T790M mutation in exon 19 of *EGFR* gene is shown in *Figure 4.3.* The total mutational rate of T790M in *EGFR* tyrosine kinase domain (exon 20) among 60 patients was found to be only 8.4% (05 of 60). Only 8.3% of mutations were detected in PTC (04 of 48) as well as FTC (01 of 12) patients (P>0.05). T790M mutation was not found in any of the patients positive for vasculocapsular invasion compared to 17.8% (05 of 28) of patients who were absent for vasculocapsular invasion (P≤0.05). None of the other clinicopathological parameters were found to be associated with T790M mutation in exon 20 of *EGFR* gene. Association of *EGFR* T790M mutation with different clinicopathological variables are shown in *Table 4.2.*

*5.2.3 EGFR L858R (T2573G) mutation in exon 21*

The representative PCR gel picture for the detection of L858R mutation in exon 21 of *EGFR* gene is shown in *Figure 4.4.* The total of 43.3% (26 of 60) of thyroid cancer patients were positive for L858R mutation in *EGFR* tyrosine kinase domain. Only 28.0% of married individuals (07 of 25) were harboring L858R mutations compared to 54.4% (19 of 35) of married individuals with L858R mutation present in married individuals and the association was statistically significant (P≤0.05). A significant association was found between tumor grading and T858R mutational status of *EGFR* gene as 34.2% of patients with well differentiated carcinoma were positive for the mutation as opposed to 63.2% of patients with poorly differentiated grade (P≤0.05). None of the other clinicopathological parameters were found to be associated with L858R mutation of EGFR gene. Association of *EGFR* L858R mutation with different clinicopathological variables are shown in *Table 4.4*

**Table 5.2: Association of EGFR 15bp deletion in exon 19 with different variables of thyroid cancer patients**

| **Variable** | **Cases n=60** | ***EGFR* *15 bp Deletion in exon 19* (n=60)** | | **OR (95% CI)** | **P value** |
|---|---|---|---|---|---|
| | | **Positive n=32 (53.3%)** | **Negative n=28 (46.7%)** | | |

| | | | | | |
|---|---|---|---|---|---|
| **Gender** | | | | | |
| **Female** | 44(73.0) | 22(50.0) | 22(50.0) | 1.6(0.5-5.3) | 0.2 |
| **Male** | 16(27.0) | 10(62.5) | 06(37.5) | | |
| **Age in years** | | | | | |
| **< 45** | 45(75.0) | 25(55.5) | 20(44.4) | 0.7(0.2-2.2) | 0.5 |
| **≥ 45** | 15(25.0) | 07(46.6) | 08(53.4) | | |
| **Habitation** | | | | | |
| **Rural** | 51(85.0) | 25(78.1) | 26(50.9) | 3.6(0.6-19.2) | 0.1 |
| **Urban** | 09(15.0) | 07(21.8) | 02(22.2) | | |
| **Marital Status** | | | | | |
| **Unmarried** | 25(41.6) | 09(36.0) | 16(64.0) | 1.9(0.6-5.9) | 0.2 |
| **Married** | 35(53.4) | 23(65.7) | 12(34.3) | | |
| **Use of OCP** | | | | | |
| **Yes** | 05(8.4) | 03(60.0) | 02(40.0) | 0.7(0.1-4.8) | 0.5 |
| **No** | 55(91.6) | 29(52.7) | 26(47.3) | | |
| **Smoking status** | | | | | |
| **Non-Smoker** | 50(83.4) | 24(48.0) | 26(52.0) | 4.3(0.8-22.4) | 0.06 |
| **Smoker** | 10(16.6) | 08(80.0) | 02(20.0) | | |
| **TSH levels** | | | | | |
| **Elevated** | 27(45.0) | 21(77.7) | 06(22.3) | 0.1(0.04-0.45) | **0.0006** |
| **Normal** | 33(55.0) | 11(33.3) | 22(66.7) | | |
| **Serum Calcium** | | | | | |
| **Normal** | 40(66.6) | 20(50.0) | 20(50.0) | 1.5(0.5-4.4) | 0.4 |
| **Decreased** | 20(33.4) | 12(60.0) | 08(40.0) | | |
| **Initial PPX** | | | | | |
| **Swelling** | 55(91.6) | 30(54.5) | 25(45.4) | 0.5(0.08-3.5) | 0.4 |
| **Incidental detection** | 05(8.4) | 02(40.0) | 03(60.0) | | |
| **BTD** | | | | | |
| **Yes** | 05(80.0) | 03(60.0) | 02(40.0) | 0.7(0.1-4.8) | 0.5 |
| **No** | 55(20.0) | 29(52.7) | 26(47.3) | | |
| **Histological types** | | | | | |
| **Papillary** | 48(80.0) | 29(60.5) | 19(39.5) | 0.2(0.05-.09) | **0.02** |
| **Follicular** | 12(20.0) | 3(25.0) | 09(75.0) | | |
| **Grade** | | | | | |
| **WD** | 41(68.4) | 19(46.3) | 22(53.7) | 2.5(0.7-7.8) | 0.1 |
| **PD** | 19(31.6) | 13(68.4) | 06(31.6) | | |
| **Stage, < 45 years** | | | | | |
| **Stage I** | 28(46.6) | 14(50.0) | 14(50.0) | 1.8(0.5-6.3) | 0.3 |
| **Stage II** | 17(28.4) | 11(64.7) | 06(35.3) | | |

| | | | | | |
|---|---|---|---|---|---|
| **Stage, ≥ 45 years** | | | | | |
| **Stage I&II** | 09(15.0) | 05(55.5) | 04(44.5) | 0.4(0.04-3.4) | 0.3 |
| **Stage III & above** | 06(10.0) | 02(33.3) | 04(66.7) | | |
| **V/C Invasion** | | | | | |
| **Present** | 32(53.4) | 16(50.0) | 16(50.0) | 1.3(0.5-3.6) | 0.5 |
| **Absent** | 28(46.6) | 16(57.1) | 12(42.9) | | |
| **LN metastasis** | | | | | |
| **Present** | 17(28.4) | 06(35.2) | 11(64.8) | 2.8(0.9-9.0) | 0.07 |
| **Absent** | 43(71.6) | 26(60.4) | 17(39.6) | | |

*n; Number, OCP; Oral contraceptive pills, TSH; Thyroid stimulating hormone, PPX; Prophylaxis, BTD; Benign Thyroid Disease, WD; Well differentiated, PD; Poorly differentiated, LN; Lymph node, V/C; Vascular/Capsular.*

**Table 5.3: Association of EGFR T790M mutation in exon 20 with different variables of thyroid cancer patients**

| **Variable** | **Cases n=60** | ***EGFR* T790M mutation (n=60)** | | **OR (95% CI)** | **P value** |
|---|---|---|---|---|---|
| | | **Mutants n=05 (8.4%)** | **Wild type n=55 (91.6%)** | | |
| **Gender** | | | | | |
| **Female** | 44(73.0) | 05(11.4) | 39(88.6) | 0.4(0.04-3.5) | 0.3 |
| **Male** | 16(27.0) | 00(0.0) | 16(100.0) | | |
| **Age in years** | | | | | |
| **< 45** | 45(75.0) | 03(6.7) | 42(93.3) | 2.12(0.3-14.2) | 0.3 |
| **≥ 45** | 15(25.0) | 02(13.3) | 13(86.6) | | |
| **Habitation** | | | | | |
| **Rural** | 51(85.0) | 05(9.9) | 46(90.1) | 0.8(0.08-7.2) | 0.6 |
| **Urban** | 09(15.0) | 00(0.0) | 09(100.0) | | |
| **Marital Status** | | | | | |
| **Unmarried** | 25(41.6) | 04(16.0) | 21(84.0) | 0.15(0.01-1.4) | 0.09 |
| **Married** | 35(53.4) | 01(1.9) | 34(97.1) | | |
| **Use of OCP** | | | | | |
| **Yes** | 05(8.4) | 00(0.0) | 05(100.0) | 0.7(0.07-6.8) | 0.5 |
| **No** | 55(91.6) | 05(9.0) | 50(91.0) | | |
| **Smoking status** | | | | | |
| **Non-Smoker** | 50(83.4) | 05(10.0) | 45(90.0) | 0.7(0.07-6.3) | 0.6 |
| **Smoker** | 10(16.6) | 00(0.0) | 10(100.0) | | |
| **TSH levels** | 27(45.0) | | | 3.5(0.3-34.1) | 0.2 |
| **Elevated** | 33(55.0) | 01(3.8) | 26(96.2) | | |

| | | | | | |
|---|---|---|---|---|---|
| **Normal** | | 04(12.2) | 29(87.8) | | |
| **Serum Calcium** | | | | | |
| **Normal** | 40(66.6) | 04(10.0) | 36(90.0) | 0.5(0.04-4.5) | 0.4 |
| **Decreased** | 20(33.4) | 01(5.0) | 19(95.0) | | |
| **Initial PPX** | | | | | |
| **Swelling** | 55(91.6) | 05(9.0) | 50(91.0) | 1.4(0.1-13.8) | 0.5 |
| **Incidental detection** | 05(8.4) | 00(0.0) | 05(100.0) | | |
| **BTD** | | | | | |
| **Yes** | 05(80.0) | 00(0.0) | 05(100.0) | 0.7(0.07-6.8) | 0.5 |
| **No** | 55(20.0) | 05(9.0) | 50(91.0) | | |
| **Histological types** | | | | | |
| **Papillary** | 48(80.0) | 04(8.3) | 44(91.7) | 1.0(0.1-9.8) | 0.7 |
| **Follicular** | 12(20.0) | 01(8.3) | 11(91.7) | | |
| **Grade** | | | | | |
| **WD** | 41(68.4) | 04(9.7) | 37(90.3) | 0.5(0.05-4.9) | 0.5 |
| **PD** | 19(31.6) | 01(5.3) | 18(94.7) | | |
| **Stage, < 45 years** | | | | | |
| **Stage I** | 28(46.6) | 03(10.7) | 25(89.3) | 0.4(0.03-3.5) | 0.3 |
| **Stage II** | 17(28.4) | 00(0.0) | 17(100.0) | | |
| **Stage, ≥ 45 years** | | | | | |
| **Stage I&II** | 09(15.0) | 02(22.3) | 07(77.7) | 0.4(0.03-4.5) | 0.4 |
| **Stage III & above** | 06(10.0) | 00(0.0) | 06(100.0) | | |
| **V/C Invasion** | | | | | |
| **Present** | 32(53.4) | 00(0.0) | 32(100.0) | 8.2(0.9-73.0) | **0.03** |
| **Absent** | 28(46.6) | 05(17.8) | 23(82.2) | | |
| **LN metastasis** | | | | | |
| **Present** | 17(28.4) | 01(5.8) | 16(94.2) | 1.6(0.17-15.8) | 0.5 |
| **Absent** | 43(71.6) | 04(9.4) | 39(90.6) | | |

*n; Number, OCP; Oral contraceptive pills, TSH; Thyroid stimulating hormone, PPX; Prophylaxis, BTD; Benign Thyroid Disease, WD; Well differentiated, PD; Poorly differentiated, LN; Lymph node, V/C; Vascular/Capsular.*

**Table 5.4: Association of EGFR L858R mutation in exon 21 with different variables of thyroid cancer patients**

| **Variable** | **Cases n=60** | ***EGFR L858R mutation* (n=60)** | | **OR (95% CI)** | **P value** |
|---|---|---|---|---|---|
| | | **Mutants n=26 (43.3%)** | **Wild type n=34 (56.7%)** | | |

| | | | | | |
|---|---|---|---|---|---|
| **Gender** | | | | | |
| **Female** | 44(73.0) | 18(41.0) | 26(59.0) | 1.4(0.4-4.5) | 0.3 |
| **Male** | 16(27.0) | 08(50.0) | 08(50.0) | | |
| **Age in years** | | | | | |
| **< 45** | 45(75.0) | 21(46.6) | 24(53.4) | 0.5(0.16-1.9) | 0.2 |
| **≥ 45** | 15(25.0) | 05(33.4) | 10(66.6) | | |
| **Habitation** | | | | | |
| **Rural** | 51(85.0) | 24(47.0) | 27(53.0) | 0.3(0.06-1.7) | 0.15 |
| **Urban** | 09(15.0) | 02(22.3) | 07(77.7) | | |
| **Marital Status** | | | | | |
| **Unmarried** | 25(41.6) | 07(28.0) | 18(72.0) | 3.0(1.1-9.1) | **0.03** |
| **Married** | 35(53.4) | 19(54.3) | 16(45.7) | | |
| **Use of OCP** | | | | | |
| **Yes** | 05(8.4) | 03(60.0) | 02(40.0) | 0.5(0.07-3.1) | 0.4 |
| **No** | 55(91.6) | 23(41.8) | 32(58.2) | | |
| **Smoking status** | | | | | |
| **Non-Smoker** | 50(83.4) | 22(44.0) | 28(56.0) | 0.8(0.2-3.3) | 0.5 |
| **Smoker** | 10(16.6) | 04(40.0) | 06(60.0) | | |
| **TSH levels** | | | | | |
| **Elevated** | 27(45.0) | 13(48.1) | 14(51.9) | 0.7(0.25-1.9) | 0.3 |
| **Normal** | 33(55.0) | 13(39.3) | 20(60.7) | | |
| **Serum Calcium** | | | | | |
| **Normal** | 40(66.6) | 16(40.0) | 24(60.0) | 1.5(0.5-4.4) | 0.3 |
| **Decreased** | 20(33.4) | 10(50.0) | 10(50.0) | | |
| **Initial PPX** | | | | | |
| **Swelling** | 55(91.6) | 24(43.6) | 31(56.4) | 0.9(0.1-5.5) | 0.6 |
| **Incidental detection** | 05(8.4) | 02(40.0) | 03(60.0) | | |
| **BTD** | | | | | |
| **Yes** | 05(80.0) | 01(20.0) | 04(80.0) | 3.3(0.3-31.7) | 0.2 |
| **No** | 55(20.0) | 25(45.5) | 30(54.5) | | |
| **Histological types** | | | | | |
| **Papillary** | 48(80.0) | 21(43.7) | 27(56.3) | 0.9(0.25-3.3) | 0.5 |
| **Follicular** | 12(20.0) | 05(41.6) | 07(58.4) | | |
| **Grade** | | | | | |
| **WD** | 41(68.4) | 14(34.2) | 27(65.8) | 3.3(1.0-10.2) | **0.03** |
| **PD** | 19(31.6) | 12(63.2) | 07(36.8) | | |
| **Stage, < 45 years** | | | | | |
| **Stage I** | 28(46.6) | 12(42.8) | 16(57.2) | 2.3(0.7-7.3) | 0.1 |
| **Stage II** | 17(28.4) | 14(82.3) | 08(17.6) | | |

| | | | | | |
|---|---|---|---|---|---|
| **Stage, ≥ 45 years** | | | | | |
| **Stage I&II** | 09(15.0) | 03(33.4) | 06(66.6) | 1.0(0.1-8.9) | 0.7 |
| **Stage III & above** | 06(10.0) | 02(33.4) | 04(66.6) | | |
| **V/C Invasion** | | | | | |
| **Present** | 32(53.4) | 14(43.7) | 18(56.3) | 0.9(0.3-2.6) | 0.5 |
| **Absent** | 28(46.6) | 12(42.8) | 16(57.2) | | |
| **LN metastasis** | | | | | |
| **Present** | 17(28.4) | 05(29.5) | 12(70.5) | 2.3(0.7-7.6) | 0.1 |
| **Absent** | 43(71.6) | 21(48.8) | 22(51.2) | | |

*n; Number, OCP; Oral contraceptive pills, TSH; Thyroid stimulating hormone, PPX; Prophylaxis, BTD; Benign Thyroid Disease, WD; Well differentiated, PD; Poorly differentiated, LN; Lymph node, V/C; Vascular/Capsular.*

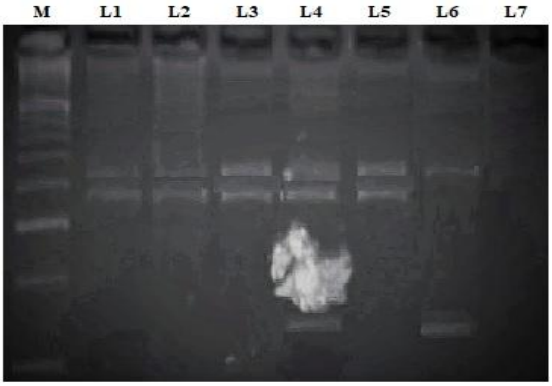


**Figure 5.2: Representative picture of ARMS-PCR products for detection of 15 bp deletion in exon 19 of *EGFR* gene**

*A single tube reaction in which "M" contains molecular marker (100bp); L1, L2, L3 & L4 contain 444bp and 325bp bands representing absence of deletion; L6 contains 444bp and 134bp bands representing the presence of 15bp deletion; L4&L6 contain 444bp, 325bp & 134bp bands representing the heterozygosity; L7 represents negative control.*

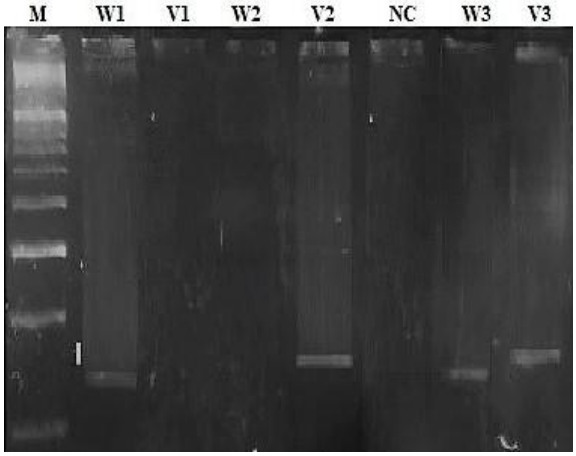


**Figure 5.3: Representative picture of AS-PCR products for detection of T790M mutation in exon 20 of *EGFR* gene**

*Two tube reaction in which lanes marked as "W" contain bands pertaining to wild allele and "V" contains bands pertaining to variant allele of same sample. "M" contains molecular marker (100bp); "W1" contains 139bp band pertaining to wild type allele, "V1" contains 146bp band pertaining to mutant/variant allele; "NC" represents represents negative control.*

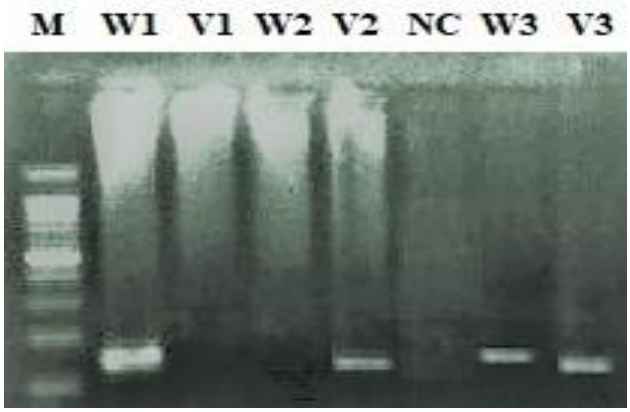


**Figure 5.4: Representative picture of AS-PCR products for detection of L858R mutation in exon 21 of *EGFR* gene**
*Two tube reaction in which lanes marked as "W" contain bands pertaining to wild allele and "V" contains bands pertaining to variant allele of same sample. "M" contains molecular marker (100bp); "W1" contains 137bp band pertaining to wild type allele, "V1" contains 134bp band pertaining to mutant/variant allele; "NC" represents negative control.*

## 4. DISCUSSION

A total of sixty (n=60) newly diagnosed thyroid cancer patients who underwent total thyroidectomy, hemithyroidectomy and lobectomy in the Department of General and Minimal Access Surgery at out tertiary care centre were selected for the study. There were no restrictions on age, sex, histology or stage, but patients with a prior history of cancer other than thyroid cancer were excluded from the study. Clinical information, including gender, age, tumour stage, tumour grade, smoking history and histopathology were obtained from the review of patients/medical records.

There were 16 males (26%) and 44 females (73%) with an age at diagnosis ranging from 26 years to 60 years (mean age 30+/-6 years). Our study was in correlation with study carried out in UK. Where thyroid cancer is the 20th most common cancer (less than 1% of all new male cancer cases). In females in the UK it is the 17th most common cancer (1% of all new female cancer cases).27% of thyroid cancer cases in the UK are in males, and 73% are in females.[49].in our set up as we don't have cancer registry programme and patient general awareness regarding thyroid malignancies, still our study which we have conducted over a period of 2 years found more female with thyroid cancers as compared to males which is in correlation with world incidence of thyroid cancer burden as stated in American society of cancer oncology(ASCO)[50].There were 10 smokers and only 50 non-smokers, as in our study we have 44 females and less than 1% among them do smoke. In real sense we have 62% of smokers among total male thyroid malignant patients. however, in one of the study smoking and association of thyroid malignancy showed a inverse relationship.[51] In one another study cigarette smoking is associated with reduced risks of papillary thyroid cancer and, possibly, follicular thyroid cancer.[52]

Based on histopathological examination there were 48 papillary thyroid carcinomas (PTC) and 12 Follicular thyroid carcinomas (FTC). As we already know the common type of thyroid malignancy is PCT, followed by FTC. [53]

The present study also categorized patients based on grade of differentiation, into well differentiated (n=41) and poorly differentiated (n=19). Differentiation into well grade and poorly differentiated doesn't correlate with the prognosis of thyroid malignancy. Poorly differentiated thyroid carcinoma is a histological type of thyroid cancer with distinctive clincopathological charteristics not yet a synonym for high grade thyroid cancer [54]. Among patients with elevated TSH levels,77.7% (21 of 27) were having 15bp deletion in exon 19 compared to only 22.3% (06 of 21) which did not have deletions ($P \leq 0.05$). studies were done to link between hypothyroid status and thyroid malignancy [55] but a detailed study showing a 15bp deletion in exon19 in relation with hypothyroid status is not in literature yet. But association of thyroid malignancy with benign thyroid diseases is established like thyroid adenoma, multi-nodular goiter,thyroiditis [56-58].

For the mutational analysis of *EGFR* gene, sixty (n=60) thyroid cancer patients were studied. Somatic mutation screening was done on surgically resected and histopathological confirmed tumor and the corresponding normal tissues of thyroid cancer patients.EGFR mutational analysis was seen on exon 19,20,21. [59] In our study Total of 32 patients were having 15bp deletion in exon 19 of *EGFR.* The over all 15bp deletion rate in *EGFR* tyrosine kinase domain exon 19 among 60 patients was found to be 53.3% (32/60). Out of them 60.5% (29/48) deletions were detected in papillary thyroid carcinomas (PTC) correlated to only 25.0% (03/12) in Follicular thyroid carcinomas (FTC).Exon 19 mutational study were also done in relation to lung cancers and were found that the EGFR exon 19 insertions are a newly appreciated family of EGFR-TKI–

sensitizing mutations, and patients with tumors harboring these mutations should be treated with EGFR-TKI. While these mutations may be missed through the use of some mutation-specific assays,the addition of PCR product size analysis to multi-gene assays allows sensitive detection of both exon 19 insertion and deletion mutations.[60] nevertheless detailed studies were not done in case of thyroid malignancy.In future we can predict the use of tyrosine kinase inhibitors [TKI] as a treatment modality for advanced/undifferentiated thyroid malignancy in this part of world.

The T790M mutations seen in *EGFR* tyrosine kinase domain of exon 20 with patients of lung carcinoma have been well documented [61] and the role of TKI against these mutations are already in review as The T790M "gatekeeper" mutation in *EGFR* mediates resistance to low concentrations of an irreversible EGFR inhibitor[62]. In NSCLS most of the patients who receive TKI based targeted chemotherapy respond to early treatment but later on develop to resistance. This resistance is being studied and up to some extent it is been related to T790M mutations seen with EGFR gene domain [62]The total mutational rate of T790M in *EGFR* tyrosine kinase domain (exon 20) among 60 patients was found to be only 8.4% (05 of 60). Only 8.3% of mutations were detected in PTC (04 of 48) as well as FTC (01 of 12) patients (P>0.05). Many studies showed the presence of T790M gene mutations with *EGFR* domain of exon 20, they studies were conducted in lung carcinoma and the effect of targeted based TKI were studied in detail [61-62].the importance of T790M gene mutations with thyroid malignancy needs a detailed study. we have found that these T790M gene mutations with EGFR domain of exon 20 are seen more with papillary thyroid cancers as compared to follicular thyroid cancer. Such interpretations need more detailed study to be conducted on thyroid malignancy in future.

The total of 43.3% (26 of 60) of thyroid cancer patients were positive for L858R mutation in *EGFR* tyrosine kinase domain of Exon-21.Incidence of the L858R and G719S mutations of the epidermal growth factor receptor oncogene in an Ecuadorian population with lung cancer have been studied [63]. The studied individuals with L858R and G719S showed the adenocarcinoma histotype, representing the first study in which all of the individuals with Lung carcinoma had this histotype. Some international research studies, such as the one done by Sharma et al. [64], state that even though most of the affected individuals with EGFR mutations occur in NSCLC, those mutations are also found in SCLC, despite their low incidence [64]. Regarding the L858R mutation, we could observe a higher frequency of the leucine allelein controls than in the affected individuals. In fact, the arginine allele frequency was 0.34 in individuals with Lung carcinoma and 0.09 in healthy ones.

The association of martial status with L858R gene was studied in our setup, and it was a statistically significant association. Only 28.0% of unmarried individuals (07 of 25) were harboring L858R mutations compared to 54.4% (19 of 35) of married individuals. previous studies have revealed that marital status influences the prognosis of patients with various types of cancer. We evaluated the influence of marriage on the survival outcomes in differentiated thyroid cancer (DTC).[65]The Surveillance, Epidemiology and End Results (SEER) database between 2002 and 2012 was used to compare cancer-specific mortality in different marital status, and in each sex, age, and stage stratification by multivariate Cox regression model. In total, 61,077 eligible patients were identified. The widowed group had the highest proportion of women, elderly patients (≥45 years), and advanced stage III/IV tumor ($P = 0.001$), but the total thyroidectomy (TT) performed and radioisotopes therapy rates were lower than those in the married group. Married patients had a better cancer-specific survival (CSS) than the unmarried ($P < 0.05$). These results showed that unmarried status, especially for widowhood, increased the risk of cancer mortality in DTC patients. The presence of L858R mutations with the martial status in thyroid cancers needs more elaborated discussion. However, the influence of marital status in any malignancy is beneficial in terms of good outcome and survival rate.[65]

In our study a significant association was found between tumor grading and T858R mutational status of *EGFR* gene as 34.2% of patients with well differentiated carcinoma were positive for the mutation as opposed to 63.2% of patients with poorly differentiated grade (P≤0.05).in one of the study Prognostic implications of immunohistochemistry markers for EGFR-TKI therapy in Chinese patients with advanced lung adenocarcinoma harbouring EGFR mutations were studied,[66]High scores of mutant EGFR T858R , Napsin-A positivity, TTF-1 positivity, lower Ki67 index, and lepidic pattern were favourable predictors for TKI therapy in patients with advanced lung adenocarcinoma.

## 5. CONCLUSION:

In our study , we extensivley studied the role of EGFR mutations with thyroid cancers, and various mutations on Exon 19,20,21 were studied in detail.Significant relations of multiple variables were seen in associations with above descriebed EGFR domain.this study will provide a nidus for future scope of further elobrating the clinical aspect of EGFR in the management of differentiated as well as aggressively behaving anaplastic thyroid malinanacy. In our part of world and at the same time it will encourge us to take this study further in assuming a vital background for more elobrated work on thyroid malignanacy.

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