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Genetic alteration and prevalence of thyroid cancer.

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Abstract

Cancer is general term that is used for all conditions that may lead towards uncontrolled and undesired cell expansion. When the cells of thyroid gland divides or multiply in uncontrolled manner it leads towards thyroid tumor. Thyroid cancer is the most common endocrine cancer that is classified into four types named as papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), anaplastic thyroid cancer (ATC) and medullary thyroid cancer (MTC). Papillary thyroid cancer is the most common of every single endocrine disease representing 85-90% of every thyroid tumor. The data along with detailed history was collected with the help of clinicians in NORI and entered in the Performa. The current study will determine various types of thyroid cancer with respect to genetic alterations.

Keywords: Thyroid cancer, Oncology, Mutation, Genes.

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Introduction

When the cells of thyroid gland multiply in uncontrolled manner it leads towards thyroid tumor. Thyroid tumor is considered as a standout amongst the most widely recognized endocrine tumors. Histologically it can be isolated into four types, first one follicular epithelial cell determined papillary thyroid cancer which represents more or less 80%, second type is follicular thyroid cancer represents 15%, third type is anaplastic thyroid cancer represents 2% and para-follicular C-cell inferred medullary thyroid cancer.

The papillary thyroid tumor is the most common of every single endocrine disease representing 85-95% of every thyroid tumor. It has a great expectation with normal 10-year survival rate of 93% despite the fact that up to 10% of patients finally die as an after effect of the infection.

Knauf et al. [1] have determined that thyroid cancer harbors a few very common hereditary modifications, some of which are seen just in this disease. They have further described that activation of MAPK pathway initiates starting occasion in thyroid carcinogenesis. This activation of the pathway causes mutation or improvement of the RET and NTRK, or activates mutations in RAS and BRAF which are intermediary molecules of this pathway. In approximately 70% of thyroid carcinomas these occasions occurs in non-overlapping manner that among RAF-kinases group one gene is BRAF which is proto-oncogene situated at 7q24, has played an important role in transduction of signals in MAKP pathway, directing cell growth, differentiation and apoptosis. They have further described that BRAF is the most important and strong activator of RAS/RAF/MEK/ERK pathway among A.

Handkiewicz-Junak et al. [2] have reported that BRAF is much of the time connected with aberrant methylation of a few tumor silencer genes, for example TIMP, DAPK and RARb2. *In vitro* studies have exhibited that expanded articulation of matrix metalloproteinase three, nine and thirteen, vascular endothelial growth factor and stimulation of nuclear transcription factor.

Samuels et al. [3] have determined that V600E BRAF mutation usually occurs in PTC which is typical endocrine malignancy. In current years, this mutation has gotten impressive consideration due to its use in administration of papillary thyroid cancer. In papillary thyroid cancer BRAF mutation is firmly connected with additional thyroidal expansion, LNM, propelled cancer stages, repetition of the disease and with death rate of the patient. BRAF mutation was identified to responsible for various molecular imbalances increase expression of genes that promote tumor and suppression of genes that control iodine in thyroid bringing about hindrance of radioiodine voracity as a result causes disappointment in cure of papillary thyroid cancer by iodine radiation.

Current paper will explore various types of thyroid cancer with respect to genetic alteration. Evaluation of various publications has shown that aberrations in different genes are responsible for thyroid carcinoma in different studied populations. However, no work has found in Pakistan population on genetics of thyroid cancer. This study is designed to evaluate possible contribution of genetic alteration in patients with thyroid cancer in aforementioned population.

The main objective of this study is to determine extent of alterations of genes in patients of thyroid cancer.

Materials and Methods

In the study two genes i.e., BRAF and PIK3CA were selected for mutational analysis. BRAF is the most common genetic alteration in thyroid cancer that activates MAPK pathway leading to oncogenic cell proliferation. PIK3CA gene part of phosphatidylinsitol 3-kinase (PI3K) contributes to a typical cell development and cellular makeover in different types of cancer including thyroid cancer. The purpose of this study was to determine extent of involvement of alterations of these genes in thyroid cancer in Pakistani population. To test the hypothesis that genetic variation in BRAF and PIK3CA plays role in etiology of thyroid cancer. Phenol chloroform method was used for DNA extraction and spectrophotometry. It was done for the

quantification of DNA. A population base case versus control study was conducted in 150 thyroid patients along with 150 aged and gender matched healthy control individuals.

Collections of samples

Patient blood samples were collected at Nuclear Medicine, Oncology and Radiotherapy Institute (NORI), Pakistan. Patients were recognized by oncologist of the (NORI) hospital. Control samples were also collected; they were disease free and healthy individuals having no cancer. Patients were not forced for sampling, on the interest of patients their blood were collected. Blood samples of 2 mL were collected in EDTA-containing tubes. Samples were stored at -2°C until further use.

Setting and study area

The present study was carried out in Nuclear Medicine, Oncology and Radiotherapy Institute (NORI).

General solutions

In this study some solutions are used on daily basis.

1. 5X Tris, boric acid, EDTA (TBE)

539 g of Tris, 274 g boric acid and 45.9 g of disodium EDTA were dissolved in dH_2O and volume 10 L was made.1 L was diluted to 1x concentration before use in agarose gel electrophoresis and keeps the solution at room temperature.

2. Ethidium bromide (EtBr)

0.2 g of ethidium bromide residue was dissolved in 20 mL of dH_2O for the safety of the solution from sun rays cover the holder with aluminium balk and keep in 4ºC securely for further utilization.

3. 70% Ethanol

Take 30 mL dH_2O and dissolve it in 70 mL of unpolluted ethanol.

4. Ammonium per sulfate

2 g of ammonium per sulfate mixed in 20 mL of dH_2O

5. Solution A

Mix 0.31 M sucrose, 10 mM tris and 5 mM $MgCl_2$ in dH_2O autoclave it and then mix 1% vol/vol triton X100 for making solution A.

6. Solution B

Liquefy 10 mM tris, 100 mM NaCl and 2 mM EDTA in dH_2O

7. Solution C

1 volume of Isoamylalcohol was mixed with 24 volume of chloroform to make 1:24 ratio of the solution.

8. Stacking dye

0.0125 g bromophenol blue were mixed with 2 g sucrose in 5 mL water for making stacking dye.

9. 20% sodium dodecyl sulphate

1 g of sodium dodoecyl sulphate was dissolved in 50 mL of water.

Extraction of DNA from blood

Phenol chloroform method was used for DNA extraction.

Quantification

Spectrophotometery and gel electrophoresis was used for extracted DNA from samples.

Spectrophotometry

Taking after steps were utilized for measuring convergence of DNA in the samples tubes.

Step: (1) DNA stock solution was thinned with water in proportion of 1:100.

Step: (2) As kind of perspective 400 µL autoclaved dH_2O was apportioned into the cuvette.

Step: (3) Pour DNA sample for spectrophotometry and evaluation in cuvette. UV radiation was used for scanning of DNA.

Step: (4) Values of UV permeability at 260 nm and 280 nm of radiation were noted.

DNA quantity (ng/µL)=immersion at 260 × 50 × DF

Dilution Factor (DF)=Total vol. of dilution/vol. of stock DNA in the dilution.

Step: (5) 100 µL volume 5 ng/µL dilutions were made and reserved at low temperature for further use. Formula was used to make dilutions which is given below.

V1=(C2 × V2)/C1.

C1=quantity of DNA in stock solution.

V1=solution DNA volume which is to be diluted.

C2=quantity of DNA in dilution to be prepared (5 ng/µL).

V2=volume of DNA dilution to be prepared (100 mL).

Agarose gel electrophoresis

1.5 g of agarose was heated in 100 mL of trisborate EDTA for making the present agarose gel (1%). Cool down the gel at 50°C and then add 5 µL Ethidium bromide to the gel. Poured the gel in caster and hardened at room temperature. In agarose gel wells 2 µL of DNA sample and 2 µL of bromophenol blue were stacked. Time required for agarose gel electrophoresis to complete was 60 min at 150 V. Gel documentation system was used to test enhanced gel products.

Polymerase chain reaction (PCR)

Primers designing for BRAF and PIK3CA: Primer 3 input software versions were used for exon 16 of BRAF and exon 9 of PIK3CA gene (Table 1). Primers were also checked by national center for biotechnology information.

Optimization of primers

It was necessary to optimize BRAF and PIK3CA genes primers for their precise annealing temperature. The primers were run on polyacrylamide gel electrophoresis to find any non-precise bands.

Table 1. Designing of Primers for BRAF gene.

BRAF primers	Sequence	Product mass	Temperature
Forward	TCATAATGCTTGCTCTGATAGGA	224	58
Reverse	GGCCAAAAATTTAATCAGTGGA	224	58
PIK3CA primers	Sequence	Product mass	Temperature
Forward	ATCATCTGTGAATCCAGA	205	58
Reverse	TTAGCACTTACCTGTGAC	205	58

Thyroid tumor samples and controls amplification

10 µL PCR mixture comprising 5 µL master mix, 1 µL primer, 2 µL PCR water and 2 µL PCR water and 2 µL of DNA sample were used to perform polymerase chain reaction. PCR mixtures for all reactions were prepared in PCR workstation to minimize any cross contamination. Reaction mixture was placed in gene AMP PCR system 9700 and verity 96 well thermal cycler were utilized.

Amplification was done for all diseased and normal DNA samples for definite genes with specific genes with specific exon and for precise primers. Negative controls were also used in order to rule out any contamination or non-specificity.

SSCP

Making of sample for SSCP: For determination of genetic changes SSCP is one of the cheapest and specific techniques. Little measure of enhanced items were moved into little tubes. Same quantity of denaturant (NaOH) was also put in these tubes and short was given to samples for blending. A solution of 5 µL enhanced product of PCR and 5 µL of NaOH was used in single strand chain polymorphism.

Samples denaturation: At temperature 95ºC single strand chain polymorphism samples were denatured for 10 minutes than quickly moved on ice. This warmth stun was given for breakage of hydrogen bonds between DNA helix and converted double stranded DNA into single stranded [4] Single stranded DNA was held by keeping it on ice for 5 min after this extraordinary warming at high temperature.

***Making of PAGE*:** For making PAGE gel plates of different chemicals were used and these chemicals were used in different quantity to make 50 mL volume. The volume of chemical are shown as below.

- Acrylamide solution 14 mL
- 10X Tris base EDTA 4.5 mL
- Ammonium persulphate 355 µL
- Tetra methyl ethylene diamine 25 µL.

Add finally water was added for making 50 mL.

Circumstances for electrophoresis

The plates were balanced on vertical gel device as gel was polymerized. The running buffer used in electrophoresis was 1Xtris, borate EDTA. Electrophoresis was run at 130V for one and half hour. The device was placed in cool environment to overwhelmed a change in temperature that may affect the movement of the samples fresh buffer was used for each turn to increase sensitivity.

Imaging and staining gel

Gel documentation system was used for imagining gel was moved to this system for capturing pictures and save those pictures for future analysis.

Variants recognition and their sequencing

Single strand chain polymorphism amplified products which showed unusual pattern were choosing for sequencing in forward direction. Samples were transported to MC lab (California) for sequencing.

Statistical analysis

To test significant association univariate and multivariate analysis were performed (Tables 2 and 3). Those p values were thought to be significant which were equal or less than 0.05.

Results and Discussion

In present study no thyroid cancer patient was found in younger age i.e., less than 20, minimum number of patients were found in 20-30 age group, while number of patients are maximum in age group i.e., 30-40 and 40-50 years. It showed that as the increased the risk for thyroid cancer also increase. The current study was

Table 2. Clinicopathalogical features of thyroid cancer.

Characteristics	Number (%)
Tumor size (cm)	2.5
Histology	
Papillary thyroid cancer	110 (73)
Follicular thyroid cancer	30 (20)
Follicular variant of papillary thyroid cancer	10 (7)
Tumor differentiation	
Well	125 (83)
Moderate	10 (7)
Poor	6 (4)
Unknown	9 (6)
Multicentric	55 (36)
Extra thyroidal invasion	41 (27)
Lymph node metastasis	59 (39)
Distant metastasis	3 (2)
Tumor node Metastasis	
1	116 (77)
2	8 (5)
3	23 (15)
4	4 (2)
Recurrent and persistent disease	100 (66)
Surgery	120 (80)

Table 3. Factors that increase risk for repeat and long lasting PTC.

Factors	Univariate, P value	Multivariate, P value	Odds ratio, (95%) CI
Age	0.6106		
Tumor size	0.0001	0.18727	
Tumor differentiation	0.0516		
Multicentric	0.5117		
Extra thyroidal	0.0021	0.3421	
Lymph node	<0.00001	0.0011	6.664 (2.2-27.9)
Distant metastasis	0.0062	0.8933	
Tumor node metastasis	0.0001	0.1005	
BRAF V 600E	0.008	0.1334	4.1 (1.3-13.9)

accordance with findings of Trovisco et al. [5] reported that in papillary thyroid cancer and FVTC BRAF V600E mutation was linked with advanced age. Some scientists have reported high BRAF V600E mutation in papillary thyroid cancer from aged patients, Fugazzola et al. [6] also have reported the occurrence of thyroid cancer that increases with age.

Percentage difference in gender

In current study it was observed that female percentage (67%) was grater as compared to male percentage (33%). It showed that in Pakistani population the prevalence of thyroid cancer is higher in females than in males. This result was in consistency with other researchers, Glettre and Kravdal [7] analyzed annually that thyroid cancer is 2.9-times less common in men than women. Rahbari et al. reported that the predominance of thyroid malignancy has expanded upto fifty percent since 1973, it is the 2nd common cancer in men and it is the most quickly expending disease among ladies [8]. Jemal et al. have reported that thyroid cancer has been observed more common in females, and males to females ratio was 1:2 [9].

Ahmed et al. [10] publicised a comparatively low male to female ratio of 1.01:1.081. Edward has concluded that different parts of world also showed that thyroid cancer was more prevalent (62%-81%) in females as compared to males [11]. Research from Saudi Arabia revealed that after breast carcinomea, thyroid cancer was the most common malignant tumor in females and it was third most common in a series from UAE [12]. In Filipino women, thyroid tumors were the 4th most common malignant neoplasms thyroid carcinoma incidence is the highest amongst women during reproductive period [13]. The prevalence of thyroid cancer is less in males than females. The female's prevalence recommends that hormonal elements may be included. A few studies propose that hormonal imbalance that happen at the time of pregnancy may expand the danger of thyroid tumor [14,15].

Smoking status

Current study revealed that the numbers of non-smoker (73%) are maximum as compared to smokers (26%). It can be due to the reason that in this study female's ratio was high as compared to males and in the Pakistani population females are usually nonsmokers. Majority of thyroid cancer patient was non-smoker. The result was in accordance with other findings, Davies and Welch [16] reported that as the consumption. Of cigarette will increases the risk for thyroid cancer will be decreases. Sajid and Ringel revealed the association of reduced risk thyroid cancer with cigarette smoking [17]. Smoking reduces TSH secretion as a result body weight of the smokers reduces as compared to nonsmokers, threat for thyroid cancer is linked with obesity and lower body weight reduces the risk of thyroid tumor. Remarkably, in all topographical areas risk for thyroid tumor decrease in males and females smokers equally.

Histopathological characteristics

In current study it was reported that mean tumor size is 2.5 cm. Among different histologic types of thyroid cancer in present study 3 histologic types of thyroid cancer was found i.e., papillary thyroid cancer (110 out of 150 samples i.e., 73%) which was the most abundant one, follicular thyroid cancer (30 out of 150 samples i.e., 20%) and follicular variant of papillary thyroid cancer (10 of 150 samples i.e., 7%). The current results showed consistency with several other studies, Fukushima et al. showed in their results that PTC was eighty percent and FTC was fifteen percent thyroid cancers which is approximately ninety five percent all thyroid carcinomas cases [18]. Rosenbaum et al. reported that among all thyroid cancers PTC is the frequently occurring endocrine cancer, varies from eighty five percent to ninety percent [19]. Lee et al. have analyzed that papillary carcinoma (69%) was the most common thyroid tumor followed by follicular carcinoma (11.6%) [20].

On the basis of tumor differentiation the samples were divided in four groups, the number of well differentiated tumor, moderate differentiated tumor, poor differentiated and unknown are: 125 (83%), 10 (75%), 6 (4%), 9 (6%), respectively. Extrathyroidal invasion, LNM and DM were found in 41(27%). 59(39%) and 3(2%) samples, respectively. Tumor node metastasis is further divided in four stages i.e., stage 1, stage 2, stage 3, and stage 4. In present study the number of patients which laid in stage 1, stage 2, stage 3 and stage 4 are 116 (77%), 8 (5%), 23 (15%), 4 (2%), this study showed that maximum number of samples laid in stage 1 of tumor node metastasis. The current study reported that 66% samples had recurrent and persistent disease. Rodolico et al. [21] reported that advanced age, tumor size, additional thyroidal augmentation, cast, NM and DM have been shown to be associated with diagnosis of thyroid cancer. One percent to two DM cases was in papillary thyroid cancer cases and in current study the distant metastasis was found 2% reported fifty eight percent additional thyroid augmentation in one ninety two patients and LNM forty five percent in one forty seven patients discovered lymph node metastasis in 321 patients (59.1%). Among all PTC patients, 170 patients (31.3%) harbored BRAFV600E, for tumor stage,434 patients (79.9%) were at stage I, 6 patients (1.1%) were at stage II, 70 patients (12.9%) were at stage III, and 33 patients (6.1%) were at stage IV, the current study also showed maximum number of patient in stage 1 of LNM (Table 4).

Thyroid cancer and occurrence of BRAF V 600E Mutation

DNA extraction: DNA was isolated from leukocytes as described before in section DNA of both cancer as well as normal patients. Electrophoresis was carried out on 1% agarose gel. After staining with ethidium bromide in UV transilluminator DNA bands were visualized. T remains for thyroid malignancy patients while C remains for typical controls.

Table 4*. Clinicopathological characteristics of PTC and their association with BRAF Mutation.*

Characteristics	Papillary thyroid cancer, p-Value
Age	0.0271
Tumor size	0.3847
Tumor differentiation	0.3115
Multicentric	0.861
Extra thyroidal	0.6341
Lymph node metastasis	0.0311
Tumor node metastasis	0.0331
Recurrent and persistent disease	0.008
Distant metastasis	0.044

Optimizations of primers: The optimization of BRAF and PICK3 was done according to specified size of product on specified temperature. To obtain a solitary band of specified product size a scope of temperature and reagents fixations were utilized. On 2% agarose gel amplified products were stacked. Electrophoresis delineates the accomplishments of polymerization as well as portrays any possibilities of plausible non specificity. Samples were to be utilized for mutation detection later it was essential to evacuate the shorts of any non-particular binding of primers. Ladder (100 bp) was utilized as ruler for the estimation of product size.

In present study BRAF v600e mutation, was noticed in 52% (58 of 110) of papillary thyroid cancer 30% (3 out of 10) of FVPTCs, and 3% (1 of 30) of FTC. This study presented that papillary thyroid cancer showed maximum number of BRAF mutation (52%) as compare to FVTC (30%) and FTC and (3%). These results are in consistency with other findings of who concluded that in papillary thyroid cancer the occurrence of BRAF mutation varies from 32% to 73.3% revealed that the occurrence of BRAF mutation in PTC varies within the range from 29% to 69% of PTC cases. The occurrence of BRAF mutation in PTC in different population is different, depending on which molecular method was applied for identification real-time PCR reaction detected the presence of BRAF mutation in 5/13 (38.5%) cases of PTC, on the other hand SSCP analysis showed BRAF mutation in 13/33 (39%) cases of PTC. BRAF is a gene present in chromosome 7, and is the strong activator of MAPK pathway. Point mutation is the genetic mutation that is more common in BRAF gene in thyroid cancer. Change of thymine to adenine at 1799 (T1799A) nucleotide, leading a change in valine to glutamine acid. Genetic change like RET readjustment, changes in RAS and BRAF these all are associated with mitogen-activated protein kinase pathway and were found in seventy percent patients of papillary thyroid cancer it showed that the important mitagon-activated protein kinase signaling pathway in papillary thyroid cancer. The frequency of BRAF (v600e) mutation enhanced from 33% in 1996 to 2000, 47,8% in 2001 to 2005, 61.5% in 2007 to 2010.

Linkage of BRAF V600E mutation with histopathological characteristic of papillary thyroid cancer

The current study showed association of BRAF V600E mutation advantage age (p0.0271), LNM (p 0.0323), DM (p 0.044), higher recurrent and persistent disease (p 0.008) and TNM stage (p 0.0331), these results are in accordance with findings that reported greater percentage of extra thyroidal intrusion that was fifty tow percent and thirty tow percent was reported LNM in papillary thyroid cancer revealed that BRAFV600E-PTC showed linkage with additional thyroidal augmentation and progressive disease stage. These finding are consistent with report from [22] progressive disease stage, additional thyroidal augmentation, lymph node metastasis, distant metastasis and tumor node metastasis increase the risk for BRAF v600e mutation in papillary thyroid have revealed that BRAFV600E mutation displayed associated with LNM, more metastatic area, and greater number of lymph node present in papillary in thyroid cancer (Table 3).

Risk factor for repetition and long-lasting PTC

In present study univariate analysis, showed significance association between BRAF V600E mutation, sex, tumor mass, LNM, extra thyroidal instruction, stage of tumor node metastasis, with reappearance and consistent disease, while multivariate analysis showed independent association of BRAF V600E mutation with reappearance and consistent disease current study was in consistency with other result concluded that BRAF V600E mutation bearing tumor were closely linked with reappearance [23] disease reported independent association of BRAF mutation and LNM with reappearance and consistent disease. Risk for reappearance and consistent disease increased by additional no matter what was the size of the tumor. BRAF V600E mutation is linked, with LNM, extra thyroidal intrusion and tumor node metastasis, and all these increase risk for reappearance and consistent papillary thyroid carcinoma.

Conclusion

Present study has concluded that BRAF mutation was most common in papillary thyroid cancer and linked with histopathological characteristics of papillary thyroid cancer. It was found to be a comparatively older age disease as no patient was found in younger age i.e., less than twenty years, while the number of patients was higher in the age groups of 30-40 and 40-50 years.

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