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MOLECULAR CHARACTERIZATION OF THYROID CANCER IN PASHTUN ETHNIC FAMILIES OF KHYBER PAKHTUNKHWA, PAKISTAN

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Abstract
Medullary thyroid carcinoma (MTC) inherits as an autosomal dominant trait with almost complete penetrance. The condition arises due to mutations in the *RET* proto-oncogene. Herein the present study, two Pashtun origin families, suffering from MTC, were ascertained for genetic and *in Silico* functional characterization. Both the families were segregating MTC disorder in autosomal dominant fashion. Whole exome analysis in family 1 and 2 found missense mutations c.C1902G: p.Cys634Trp & c.G1901A:p.Cys634Tyr respectively in the 11th exon of *RET* gene. *In silico* analysis, for both mutant RET proteins, determined drastic effects of identified mutation on protein folding pattern as well as its interaction properties. Among all report mutations of *RET* gene, c.C1902G: p.Cys634Trp accounts for more than 50% of all MEN-2A mutations. The current study signifies the importance of onco-genetic testing & counseling for early intervention. This research opens a new avenue for investigating the genetic landscape of MTC and encourages further exploration into the intricate genetic factors that contribute to MTC's hereditary nature.

Key words: *RET*, Thyroid, cancer, Mutation, Pashtun

1. Introduction
Thyroid cancer is the most common type of endocrine cancer and the sixth most common cancer among women [1,2]. The probability of developing thyroid cancer is 5% in patients having thyroid nodules [3]. In Pakistan, the prevalence of thyroid malignancy in nodules varies from 11% [4–6] to 14.35% [7]. Among all globally reported cases, the prevalence of thyroid carcinoma is 1-2%, however, the annual incidence varies in different parts of the world from 0.5-10 cases per 100,000 individuals [2,8] and hence, ranked among the top 20 carcinomas in the world. It is reported as three times more common in females compared to males [9]. This makes thyroid carcinoma the seventh most common female malignancy. The prognosis of thyroid carcinoma is good with an excellent disease-free survival. In Pakistan, it is estimated that 1.2% cases among all malignant tumors are of thyroid cancer [10]. Male to female ratio in Pakistan is between 2.5 to 4.1, which matches with the international data set [7]. Its incidence is still increasing besides early detection and appropriate management of thyroid nodules. Among all sub-types of thyroid cancer, most common histological types are papillary and follicular thyroid carcinomas, which represent about 85% of all thyroid cancer

cases. This type of cancer is unusual during childhood, but its incidence increases with the age [9]. Radiation [11]**,** intake of iodine, variations in the population [10], and irregularities in Thyroid stimulating hormone (TSH) are some of the agents that may lead to thyroid cancer [12]. Additionally, Body weight, diet, lifestyle, contaminated environment and genetic factors also contribute in inducing thyroid carcinoma. Medullary thyroid carcinoma (MTC) is the third sub-type of thyroid cancer, which accounts for 3-10% of all sub-types of thyroid cancers. It develops from the para-follicular cells of the thyroid gland, which produces calcitonin [12,13]. MTC is most common in adults and affect only one lobe of thyroid gland. Most cases of medullary thyroid carcinomas are sporadic, but 20% of cases are due to germline mutations in the RET proto-oncogene [13]. Hereditary forms are transmitted with an autosomal dominant pattern with high penetrance (>90%). It accounts for 5–10% of all thyroid cancers. MTC is hereditary in about 25% of cases [14,15]. Here in the current investigation, we performed molecular characterization of familial MTC cases to find any relationship between clinical and molecular findings. We report Cys634Trp at position 1902 and Cys634Tyr at position 1901 as the only point mutations responsible for MTC in our familial cases characterized during this study.

2. Materials and Methods

Herein the present study, two Pashtun origin families, suffering from MTC, were ascertained for molecular characterization. As an inclusion criterion, only families with clinical diagnosis and family history of MTC were included in the study. The study was approved by ethical review board of Gomal University, D. I. Khan. Information, regarding patient's clinical history, disease onset, exposure to radiation, smoking, and treatment regimen, were recorded on a study-designed performa and informed consent from each patient was obtained prior to the sample collection. Later, blood samples were collected in EDTA tubes and DNA extraction was done using salting out method [16]. Subsequently, pedigrees were drawn wherein, squares were used to indicate males and circles to indicate females.

2.1. Biochemical Testing.

Different biochemical tests including Calcitonine, Carcinoembryonic antigen (CEA), Thyroid Hormone level T3, Thyriod Hormone level (T4) and triglycerides (Tg) were also performed on all patients.

2.2. Exome sequencing

Whole exome sequencing was done commercially (GENEWIZ, Inc. China). DNA samples were fragmented using ultrasonicator and accordingly constructed into high throughput sequencing library through terminal repair, adding A tail, adapter ligation, purification and pre-amplification, quantitative, exon capture and PCR enrichment. The size and concentration of each sample was determined using Qbit flourometer and assessed accordingly.

2.3. Data analysis

Variants in the reported genes of *RET* family were manually screened for the presence of possible pathogenic variant. In the filtration criteria, only those variants were screened which were protein coding, genotypically homozygous and nonsynonymous.

To know about the pathogenicity and their functional consequences, bioinformatics tools like SIFT (Sorting Intolerant From Tolerant) (http://SIFT.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), LinkResearchTools (LRT) and hidden Markov models (HMMs) were used.

2.4. Mutation analysis

After identifying the pathogenic variants, primers were designed using online tool; Primer3, v.0.4.0 in the flanking regions of the candidate pathogenic variant. Subsequently, polymerase chain reaction was performed to amplify the target region containing pathogenic variants. PCR products were purified by PCR Advanced PCR Clean Up System (VIOGENE, Taiwan). Later, the sample was

submitted for Sanger sequencing, which was done commercially (GENEWIZ, Inc. China). For mutation detection, sequence data were matched to the reference sequence available in UCSC Genome Browser. For sequence alignment, the online tool BLAST (Basic Local Alignment Search Tool) (Kent, 2002) was used. Alongside, BioEdit (v7.0.5) was also used to analyze the genetic variation and capturing the sequence chromatogram.

2.5. *In silico* Analysis

In silico analysis involved variety of investigation i.e. from protein stability analysis to protein modeling and docking studies.

2.5.A. Protein Stability Analysis

Protein stability was evaluated using I-Mutant (http: //gpcr2.biocomp.unibo.it/cgi/predictors/ Mutant3.0 / Mutant3.0.cgi,) and MUpro (http://mupro.proteomics.ics.uci.edu).

2.5.B. Conservation Analysis

Conservation analysis was performed using Clustal omega (https://www.ebi.ac.uk/ Tools / msa / clustalo) and ConSurf tools (http://consurf.tau.ac .il,). Multiple sequence alignment was performed to determine the amino acid conservation among different animal species.

2.5.C. Protein Structure Prediction

The secondary folding of normal and mutant RET protein was analyzed by using online tool PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). While, I-TASSER tool (https://zhanglab.ccmb.med. umich.edu/TASSER/) was used to predict the 3D conformation of the RET protein. Missense3D (http://missense3d.bc.ic.ac.uk/missense3d/) was used to predict structural changes in proteins by addition of amino acids. Later, UCSF Chimera (candidate version 1.15) was used to visualize the 3D structures of proteins downloaded from I-TASSER.

2.5.D. Protein–Protein Interactions

The interaction of RET protein with other proteins was investigated using the STRING online tool (https://string-db.org), which predicts the close functional interactor. ClusPro 2.0 tool was used to estimate the positioning of one molecule with respect to the other, when they are bound to each other to make a stable complex [17,18].

3. Results

Inherited Medullary thyroid carcinoma (MTC), associated with MEN 2 syndrome, is caused due to mutations in the RET proto-oncogene, situated on chromosome 10. It inherits in an autosomal dominant manner with high penetrance. In the present genetic study, two consanguineous families from Khyber Pakhtunkhwa province were mapped, which revealed mutations in the *RET* gene. These mutations affect the cysteine-rich extracellular domain, leading to ligand-independent dimerization and tyrosine kinase receptor activation. The most common mutation, occurring in over 80% of cases, involves codon 634 (within exon 11) in the *RET* gene. This mutation replaces cysteine with tryptophan and tyrosine (c.C1902G: p.Cys634Trp and c.G1901A:p.Cys634Tyr)

3.1. Family 1

Family 1 belonged to Pashtun ethnicity (Figure 1a), documented a three-generation pedigree presenting autosomal dominant inheritance for MTC. The family included were four MTC-affected individuals i.e., two males and two females. Clinical finding in the patients of current family are shown in table 1. Results of biochemical tests shown that the Calcitonin level was greater than 100 pg/mL (pictogram per milliliter) in all the patients. Carcinoembryonic Antigen (CEA) was greater than 5 ng/mL (nanograms per milliliter) in the female patients. Serum chemistry tests of affected individuals indicated normal levels of LDH, ALT/SGPT, ALP, Serum creatinine and urea, while the

levels of T3 and T4 was observed higher in all the three patient's biochemistry profile is illustrated in Table 2.

Whole exome analysis in the affected individuals revealed a missense mutation c.C1902G (p.Cys634Trp) in the 11th exon of *RET* gene. At protein level, this alteration results in the substitution of cysteine with tryptophan amino acid at position 634. Varsome classified this variant as pathogenic, indicating its disease causing potential. Further to this, one unaffected male family member was also carrying the same *RET* gene mutation c.C1902G. This highlights the need for comprehensive genetic counseling and diagnostic assessments for all family members.

The identified *RET* gene mutation [c.C1902G:(p.Cys634Trp)] showed perfect segregation with the disease phenotype across the family lineage. The affected individuals were heterozygous for the said mutation while normal individual was homozygous wild-type of the identified variant (Figure 1-c).

3.2. Family 2

The documented three generation family pedigree indicated one affected individual each in the first, 2nd and 3rd generation. Among three patients, there were two males and one female. Clinical details of all the patients are summarized in table 1. Analysis of patient's various biochemical tests identified higher level of Calcitonin level than 100 pg/mL in all the patients. Carcinoembryonic Antigen (CEA) was greater than 5 ng/mL in the female patients. Serum chemistry tests of affected individuals indicated normal levels of LDH, ALT/SGPT, ALP, Serum creatinine and urea, while the levels of T3 and T4 was observed higher in all the three patients' biochemistry profile is illustrated in Table 2.

The pedigree analysis showed autosomal dominant mode of inheritance (Figure 1b). Sanger sequencing analysis determined a missense mutation [c.G1901A:(p.Cys634Tyr)] (Figure 1d) in the *RET* gene, resulting in substitution of Cysteine with Tyrosine at position 634. The prediction tool and mutation analysis revealed a remarkable alignment between the mutation and the disease phenotype across the entire family, it was determined as pathogenic variant.

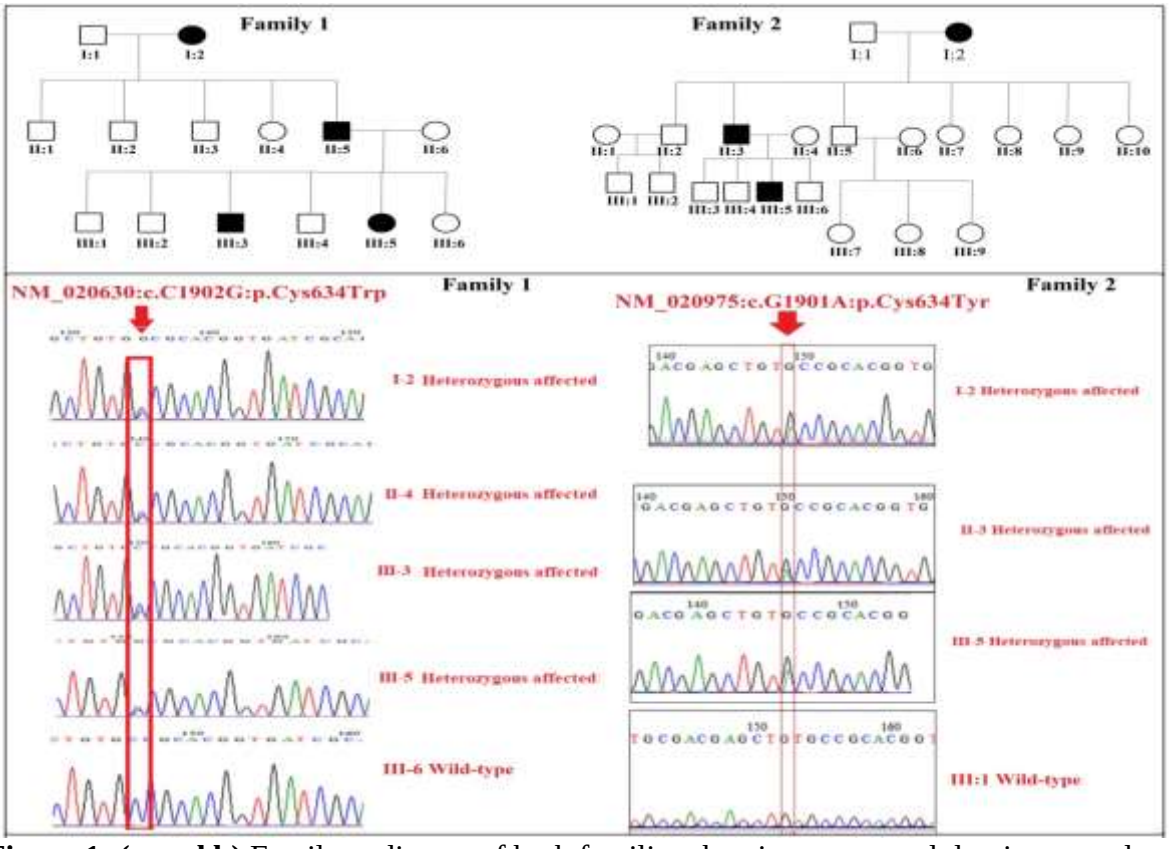

Figure 1: (a and b) Family pedigrees of both families showing autosomal dominant mode of disease inheritance, **(c,d)** Sanger sequencing chromatograms showing segregation of identified variant in the family with the disease phenotypes.

Table 1: Phenotypic features of patients in both families

Phenotypes	**Family 1**				**Family 2**		
	I-2	**II-4**	**III-3**	**III-5**	**I-2**	**II-3**	**III-5**
Gender	Female	Male	Male	Female	Female	Male	Male
Age (years)	75	50	17	20	68	42	21
General physique	Normal	Normal	Normal	Normal	Normal	Normal	Normal
IQ level	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Voice level	Difficulty	Difficulty	Normal	Difficulty	Difficulty	Difficulty	Normal
Smoking	No	Yes	No	No	No	Yes	No
Neck swelling	No	No	No	No	No	Yes	No
Throat pain	Yes	Yes	No	No	Yes	Yes	No
Fatigue	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Diarrhea	No	Yes	No	No	No	Yes	No
Facial flushing	Mild	Mild	Normal	Normal	Mild	Mild	Normal
Breathing	Difficulty	Difficulty	Normal	Normal	Difficulty	Difficulty	Normal
Cough	Yes	Yes	No	Yes	Yes	Yes	No
Muscular pain	Yes	Yes	No	No	Yes	Yes	No
Loss of appetite	Yes	Yes	No	No	Yes	Yes	No

Table 2: Biochemical findings in patients from current study

S.No	**Test**	**Normal value**	**Family 1**				**Family 2**		
			I-2	**II-4**	**III-5**	**III-3**	**I-2**	**II-3**	**III-5**
1	Calcitonine	<10 pg/ml	~480 pg/ml	~410 pg/ml	~380 pg/ml	~100 pg/ml	~450 pg/ml	~480 pg/ml	~390 pg/ml
2	Carcinoembryonic antigen (CEA)	<5 ng/ml	~25 ng/ml	~30 ng/ml	~27 ng/ml	~4.5 ng/ml	~37 ng/ml	~35 ng/ml	~30 ng/ml
3	Thyroid Hormone level T3	100-200 ng/dL	~150 ng/Dl	~170 ng/dL	~165 ng/dL	~140 ng/dL	~400 ng/Dl	~430 ng/dL	~600 ng/dL
4	Thyriod Hormone level T4	4.5-12.5 ug/dL	~8 µg/Dl	~10 µg/dL	~10 µg/dL	~5.5 µg/dL	~18 µg/Dl	~35 µg/dL	~26 µg/dL
5	Tg	Upto 55 ng/ml	~140 ng/ml	~180 ng/ml	~160 ng/ml	~40 ng/ml	~130 ng/ml	~260 ng/ml	~190 ng/ml

3.3. Protein structural findings

On examining and comparing 3D structure of wild-type and mutant (p.Cys634Trp) RET protein, similarity index of 33.6% was found, showing notable alterations in the protein's tertiary structure. The mutation gave rise to helices, loops, and strands at specific points. However, Similarity index of only 29% were observed in wild-type and mutant (p.Cys634Tyr) RET protein. The N-terminal and C-terminal segments of the wild type and mutant *RET* proteins exhibited a lack of superimposition,

gave rise to helices, loops, and strands at specific points while being lost at other locations (Figure 2).

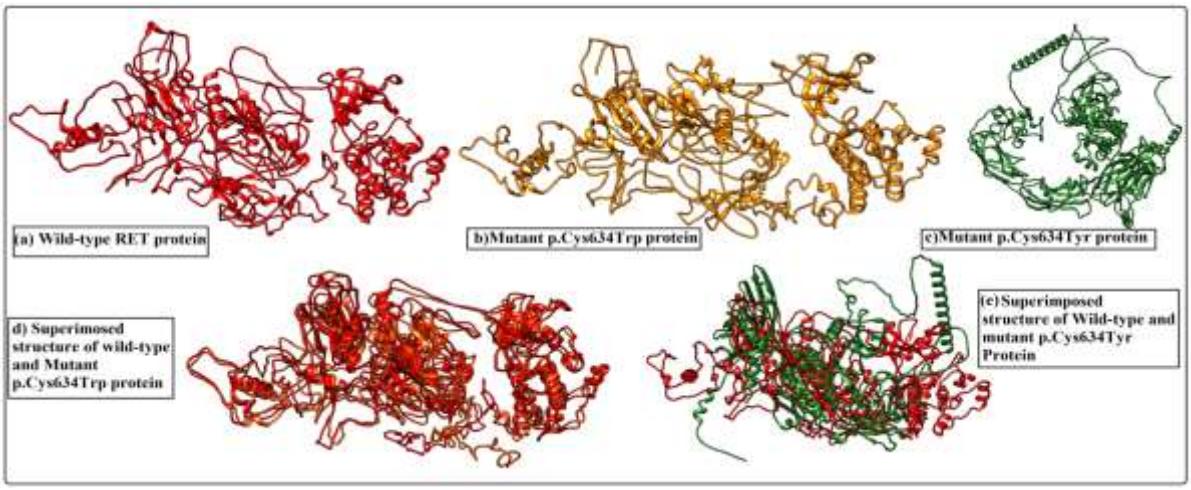

Figure 2: (a) 3D model of wild-type RET protein, **(b)** Mutant p.Cys634Trp protein **(c)** Mutant p.Cys634Tyr protein, **(d)** Superimposed structure of wild-type and mutant p.Cys634Trp protein RET protein **(e)** Superimposed structure of wild-type and mutant p.Cys634Tyr protein RET protein

3.4. Protein docking outcomes

The wild-type *RET* protein interacted with close interactor GFRA3 through 26 H-bonds via 16 amino acid residues (Val383, Ser388, Cys389, Arg94, Asn372, Arg124, Asn95, Leu393, Leu391, Gln380, Arg378, Leu397, Leu396, Try400, Leu399 and Ser398) while, the mutant (p.Cys634Trp) protein *RET* protein showed docking with GFRA3 through 15 H-bonds and 1 salt bridge involving only 11 amino acid residues (Ile394, Leu395, Try400, Leu393, Ser388, Phe378, Leu386, Trp382 and Arg378). While, in case of mutant (p.Cys634Tyr) protein only 13 amino acid residues (Ser398, Leu397, Leu395, Ile394, Cys389, His273, Glu324, Arg94, Asn374, Glu88, Arg39, Asn42 and Asp85) of the mutant *RET* protein docked with GFRA3 through 17 H-bonds and one salt bridge. With regard to residue number, amino acid type, and position, the interaction patterns of wild-type and mutant *RET* proteins with GFRA3 were different showing significant alterations (Figure 3).

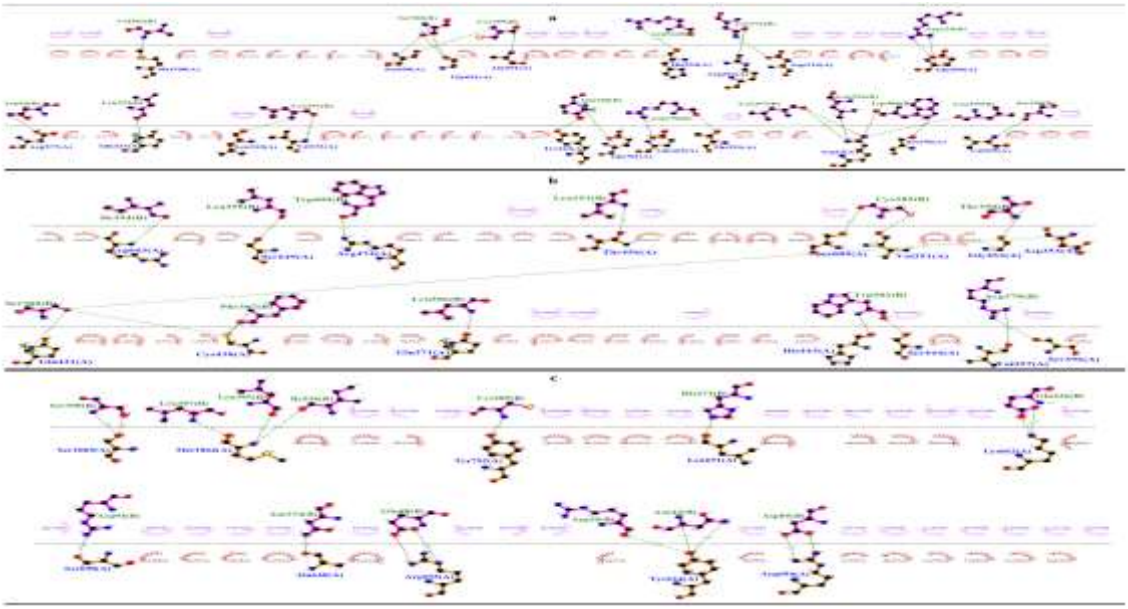

Figure 3: (a) Protein-Protein interaction wild-type and Close interactor GFRA3 protein. **(b)** Protein-Protein interaction Mutant p.Cys634Trp protein and Close interactor GFRA3 protein **(c)** Protein-Protein interaction Mutant p.Cys634Tyr protein and Close interactor GFRA3 protein

3.4. Multiple Sequence Alignment:

Multiple sequence alignment of the RET protein confirms the conservation of substituted amino acid as shown in figure 4.

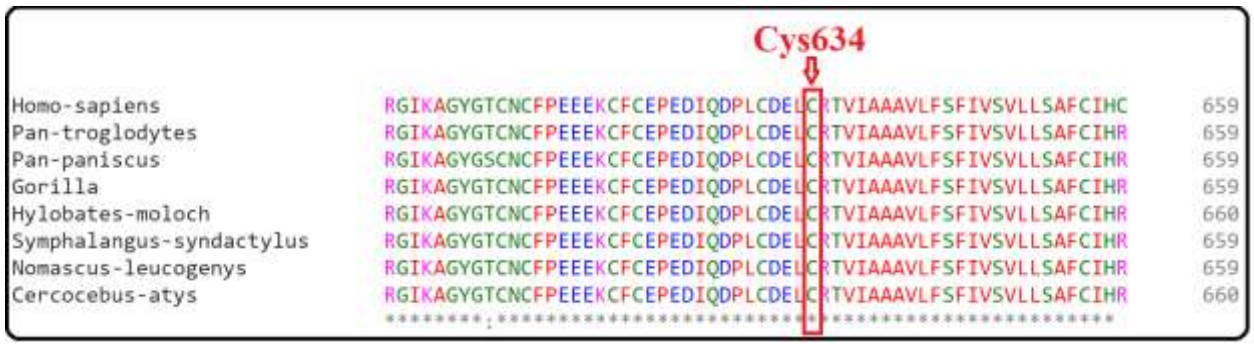

Figure 4: Multiple sequence alignment of RET protein showing the conservation of substituted amino acid Cys at position 634 throughout multiple species.

4. Discussion

Thyroid cancer is regarded as one of most prevalent cancer globally, and its prevalence is increasing over the past decade [19,20]. In Pakistan, its incidence varies from accounting for 1.2% of all malignant tumor cases [21–31]. In an intriguing genetic scenario, all the members of three families were characterized who share a common RET mutation, the Cys634Trp and Cys634Tyr mutation. Medullary Thyroid Carcinoma is a recognized as a genetic disorder that is associated with mutations in the *RET* gene, and this particular mutation is known for its significant role in driving the development of MTC. In this family, the three affected individuals display clinical manifestations consistent with MTC, indicating the potential hereditary nature of the condition within the family lineage.

In this study, we identified that Cys634Trp and Cys634Tyr mutations are the most prevalent mutations in individuals with family history of the MTC disease, however, individuals without a family history are more likely to experience mutations that damage other structural components. This is due to the fact that cysteine mutations, particularly the Cys634Tyr, are typically more damaging and have a stronger association with the disease onset. The severity and timing of the disease's symptoms are also impacted by these mutations. The clinical practice of RET genetic screening in patients with hereditary and sporadic MTC has been introduced shortly after the discovery of the driving role of RET mutations in the pathogenesis of MTC. This is done to identify RET positive subjects and their relatives who are at high risk of developing MTC during their lifetime. In a study, the entire RET coding sequence was examined in patients with familial MTC who tested negative on the initial screen. In keeping with previously published data, we have reported that RET germline mutations cause roughly 98.5% of hereditary MTC, however a small number of cases are still RET negative even though the screening included all RET exons. The occurrence of a mutation in a gene other than RET or the existence of an RET intronic variant with the ability to alter RET expression levels could be suggested to explain the hereditary nature of RET cases [32]. In line with previous data [33–47], we showed that the patients with MTC had germline RET mutation, indicating the critical significance of RET genetic screening for the discovery of unknown MEN2 families. Once an apparently sporadic MTC patient is reclassified as hereditary, RET genetic screening should be carried out in all first-degree family members. This will help identify gene carriers who can then benefit from appropriate screening, an early diagnosis, and prompt or preventative treatment measures. Earlier findings [20] showed that 94% of patients had RET germline mutations affecting one of the following codons 609, 611, 618, 620, 634, 768, 804, or 918. The inclusion of a larger section of the *RET* gene and possibly advancements in sequencing capabilities may help to explain this finding.

The cysteine-rich domain mutations of the *RET* gene, which are known to account for 95% of MEN2B cases [48], were present in all of the MEN2B patients in our series. The RET mutations in MEN2B were all de novo in our individuals as well, as has been described previously as well [20].
A significant genotype-phenotype connection has been confirmed in our Pakistani families, correlating with earlier series [21]. Particularly, the finding that MTC is highly related with RET mutations supports the notion that changes in *RET* gene alone are sufficient to cause tumoral transformation, as shown by *in vitro* investigations [15]. The 3D protein structure and its interaction with other proteins have been dramatically affected by the discovered mutation. The discovered mutation alters the protein's three-dimensional structure significantly, changing its helices, loops, and strands. These modifications may affect the protein's general stability, folding, and functional areas, which may jeopardize its capacity to interact with other molecules and to carry out biological functions. Based on the information on these families characterized, it is recommended that oncologists in Pakistan should genetically characterize the patients because early diagnosis and the beginning of treatment can significantly slow the growth of tumor and prevention further segregation. What adds an element of complexity to this familial case is the presence of a seemingly unaffected male with no MTC record but still carrying the same p.Cys634Tyr mutation in *RET* gene. The unaffected status of this individual is intriguing, as this mutation is often associated with MTC. Genetic and clinical investigations are warranted to understand the reason behind the phenotypic variability observed within the family. Factors such as modifier genes, genetic background, and environmental influences might contribute to the varying expressivity of the mutation. This scenario underscores the importance of thorough genetic counseling and diagnostic assessments for all family members. Understanding the underlying genetic factors and potential risks associated with carrying the p.Cys634Tyr mutation is critical not only for the affected individuals but also for the apparently unaffected male. Further research and investigation into the genetic and molecular mechanisms involved in this family's unique case could potentially provide insights into the variable penetrance of the RET mutation and aid in refining personalized medical management strategies.

5. Conclusion

Current genetic study was done on two families diagnosed with MTCs. The majority of sporadic MTCs cases also contain somatic mutations in *RET* gene. Analysis of *RET* gene identified two previously reported mutation i.e. p.Cys634Tyr and p.Cys634Trp. During this study, it was found that the most prevalent *RET* mutation in the Pakistani population was p.Cys634Tyr and also p.Cys634Trp. All the patients of thyroid cancer must be molecularly characterized to determine the course of disease and proper streamlining the patient follow-up.

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Authors Contribution: I.U.R: Research and Family sampling, J.K: supervision, Biochemical testing, S.S: Biochemical testing, S.S Sanger sequencing, M.M. Drafting manuscript and Software, M.M: Software, I.U.R Software, and Sampling.

Statements and Declarations

Data Availability Statement: The data (sequence, photographs, and pedigrees) is stored in a password protected computer at the Institute of Biological Sciences at Gomal University, D.I.Khan, Pakistan and are available upon request. Furthermore, the reference sequences were obtained through freely available genome databases.

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Conflict of Interest Disclosure: None declared by all authors

Ethics Approval Statement: The institutional ethical review board of Gomal University D.I.Khan, KPK, Pakistan, gave its approval to the current medical genetic study. All study participants, as well as their parents or legal guardians, gave their informed consent.

Patient Consent Statement: The patient's guardians provided their consent to publish their clinical information and photographs.

Permission to Reproduce Material from Other Sources: Not applicable

Clinical Trial Registration: Not applicable

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