In-silico analysis of highly pathogenic variants of RET gene associated with thyroid cancer

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Abstract

The proto-oncogene *RET* is composed of 21 exons located on chromosome 10 (10q11.2) and encodes for a transmembrane receptor tyrosine kinase for members of the glial cell line–derived neurotrophic factor family (GDNF) and associated ligands, and mutations in this gene can cause Thyroid cancer in humans. Therefore, we anticipated studying the consequences of *RET* non-synonymous variations using advanced bioinformatics tools. Analysis of the genetic mutations in the gene, accomplished using computational methods. The functional and pathogenic effect of missense variations and the deleterious tendency of amino acid substitutions of a gene was investigated using the gnomAD database, SIFT, MutPred, MAESTRO, UCSF Chimera, and cBioportal and survival analysis of a gene was investigated by GEPHA database. This study revealed that “C609Y”,"C618R", “C634R”,"C634F","C634W","L790F, "V804L" and "R813W" variants were predicted to be the most harmful/damaging mutations in *RET* gene and the survival of patients with thyroid cancer was strongly impacted by the downregulated *RET* (low expression). This research renders the next generation of precision medicine approaches possible and provides simple but precious tools for use in clinical settings.

Keywords: Bioinformatics Tools, Thyroid Cancer, RET gene, Precision Medicine, Pathogenic Variants

INTRODUCTION

Thyroid cancer is the fifth most common cancer in women in the USA, and an estimated over 62000 new cases occurred in men and women in 2015[1]. Cancers arise and progress as a result of accumulated genetic damage. The development of methods to clone and characterize genes has dramatically increased our understanding of the molecular basis of cancer[2]. Only 3–4% of all human tumours are thyroid carcinomas. Nevertheless, it is the most frequently reported endocrine neoplasia and is the human tumour with the highest increase in incidence within the past two decades. This increased incidence is probably the result of over diagnosis of thyroid disorders due to the widespread use of neck ultrasonography, which can bring to light many small early-stage tumours that will probably never develop to clinical disease[3].

RET is a single-pass transmembrane receptor tyrosine kinase (RTK) that is required for normal development, maturation and maintenance of several tissues and cell types. The *RET* receptortyrosine kinase is required for the development of neural and genitourinary tissues, but deregulation of *RET* activity is an important contributor to several human cancers[4]

Since the discovery of the *RET* in 1985, alterations of this protein have been found in diverse thyroid cancer subtypes. *RET* gene rearrangements are observed in papillary thyroid carcinoma, which result in *RET* fusion products. By contrast, single amino acid substitutions and small insertions and/or deletions are typical of hereditary and sporadic medullary thyroid carcinoma. *RET* rearrangements and mutations of extracellular cysteines facilitate dimerization and kinase activation, whereas mutations in the RET kinase coding domain drive dimerization-independent kinase activation (Domenico Salvatore et al., 2021).*RET* gene is responsible for three different inherited cancer syndromes namely multiple endocrine neoplasia type 2A (MEN 2A), type 2B (MEN 2B) and familial medullary thyroid carcinoma (FMTC) as well as for Hirschsprung disease (HSCR), a congenital disorder affecting the intestinal motility[5]

The proto-oncogene *RET* is composed of 21 exons located on chromosome 10 (10q11.2) and encodes for a transmembrane receptor tyrosine kinase for members of the glial cell line–derived neurotrophic factor family (GDNF) and associated ligands[6]. The *RET* extracellular segment contains four cadherinlike domains, followed by a domain containing cysteine residues involved in the formation of intermolecular disulfide bonds. *RET* protein is highly glycosylated and N-glycosylation is necessary for its transport to the cell surface[7].Glial cell line-derived neurotrophic factor family ligands (GDNF, NRTN, ARTN, and PSPN) activate *RET* via binding to GDNF family receptor α1–4 (GFRα1–4). Interaction of these ligands with GFRα1–4 induces *RET* dimerization, resulting in intrinsic tyrosine kinase activation[8].

Heritable *RET* mutations that are identified in patients with multiple endocrine neoplasia type 2 (MEN2) are generally point mutations that lie at specific sites in the RET protein and lead to constitutive activation, either by promoting dimerization or by altering conformation and favouring kinase activity. *RET* sequence variants at G691 or Y791are less clearly oncogenic in vivo but can increase oncogenic or other *RET*-associated phenotypes.Mutations of RET that abrogate or reduce RET function are also detected throughout the RET sequence, and they have been reviewed elsewhere[9].

Synonymous SNPs do not lead to a change in the amino acid sequence. Studies conducted over the past few years revealed the effect of synonymous mutations on predisposition to develop cancer. To date, several types of SNPs have been identified in the *RET* gene: G691S/exon 11, GTT/AGT; L769L/exon 13, CTT/CTG; S836S/exon 14, AGC/AGT; S904S/exon 15, TCC/TCG [10]. These changes were present both in affected individuals (endocrine tumors, MTC, PTC, HSCR) as well as in the non-affected population[11]. Among the several known SNPs, only one change, in exon 11 G691S, results in the substitution of another amino acid in the protein chain[12].

Functionally significant mutations in *RET* were screened with the aid of various sequence and structure based *in silico* prediction methods. The deleterious mutants, modelled mutant proteins and deciphered the impact of mutations

on drug binding mechanisms in the *RET* crystal structure of PDB with the potential inhibitor by docking analysis. Furthermore, molecular dynamics simulations were undertaken to understand the mechanistic action of cancer associated mutations in altering the protein kinase structure, dynamics, and stability[13]. The normal RET structure and structure with mutations that leads to thyroid cancer is shown in [Figure 01].

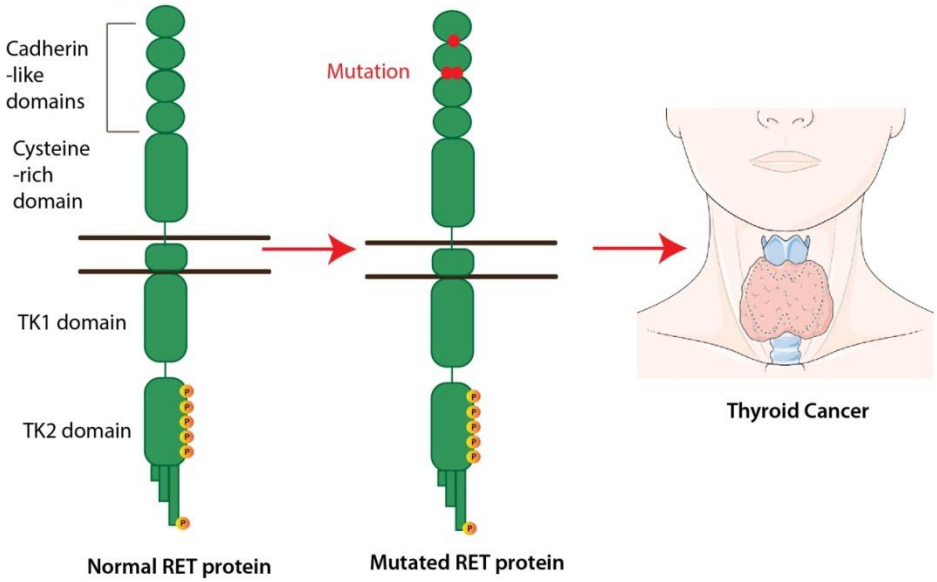


Figure 01: The normal and mutated RET protein is shown

Methodology

General Information

The UniprotKB (https://www.uniprot.org/uniprotkb/P07949/entry), OMIM (https://www.omim.org), and National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov) databases were used to find general information on the *RET* gene by using gene symbol in entry.

Retrieval of *RET* Variants

RET gene variants were acquired from https://gnomad.broadinstitute.org/ on August 11, 2022, and is now accessible at gnomAD 2.1. A global alliance of researchers created the GnomAD, also known as the Genome Aggregation Database Consortium, to compile and unify exome and genome sequencing data from a variety of large-scale sequencing initiatives and provide data summaries to the science establishment.GRCh37/hg19 was the reference

genome adopted for sequence alignment. Due to the subsequent alterations in amino acids in the protein sequence, only missense variants were chosen from the resulting SNPs for further screening.

Functional Analysis of SNPs on *RET*

All missense mutations from these data sets were subjected to pathogenicity predictions using two different predictors. First, the effects of missense SNPs on the functionality of proteins were predicted by SIFT using sequence homology and the physical characteristics of amino acids. When SIFT calculates a score, it assigns a value between 0 and 1; a score between 0 and 0.05 is projected to have a negative impact. (https://sift.bii.a-star.edu.sg/)[14].Second, MutPred, the pathogenicity predictions were performed using an existing random forest-based approach that relies on sequence, conservation, projected structural, and functional factors[15].

Stability Analysis of *RET* Variants

MAESTRO, a flexible method was used for predicting stability changes to RET mutations. For free, non-commercial users can get Windows and Linux executables from http://biwww.che.sbg.ac.at/MAESTRO[16]. A stabilising mutation is indicated if Gpred is less than 0.0, which represents the overall expected change in stability (kcal/mol). A number between 0.0 (unreliable) and 1.0 is provided for the cpred confidence estimation (highly reliable).

Clashes/Contacts Analysis

Prediction of clashes/contacts of highly pathogenic variants was done using UCSF-Chimera. To investigate the impact of mutations, a study was conducted to determine how the altered residue interacted with its adjacent amino acid residues. On the basis of the VDW radii, predictions were generated. The interactions are probably not favorable for the protein's normal operating conditions, suggesting that unfavourable interactions might severely affect the structure.

Lollipop Plot of RET Variants

MutationMapper of cBioPortal (https://github.com/cBioPortal/mutation-mapper) was used to report RET mutations that are extremely pathogenic, alter coding areas, and are strongly linked to thyroid cancer by creating the “Lollipop Plot”.

Survival Analysis of Thyroid Cancer

The prognosis of thyroid cancer, including overall survival time and disease free survival time, was investigated using GEPIA based on TCGA data.

Results

We have estimated the performance of *RET* by mutation analysis using the 06 sequence- and structure-based tools. A total number of 1652 SNPs were reported in the Human *RET* gene using gnomAD database [Figure 02]. On further selection,23 (1.39%) were found to be 3'prime UTRs, 17(1.03%) as 5'prime UTRs,550 (33.29%) as intron variants, 07 (0.43%) in-frame deletion, 07(0.43%) inframeinsertions ,69 (4.18%) as splice regions,329 (19.92%) as synonymous variants and 651 (39.46%) were nsSNPs[Figure 02]. After filtering the large data, 651nsSNPs were further analyzed for the investigation of most pathogenic variants.12 variants predicted to be most deleterious/damaging out of 651 variants by using SIFT algorithm with the threshold score of "less than 0.5" namely: C609Y, C618R, D631Y, C634R, C634F, C634W, K666N, L790F, V804L, R813W, S891A and M918T[Table 01].MutPred algorithm with the threshold score "greater than 0.5" predicted 11 variants except K666N with "0.366" predicted score to be most deleterious out of 12[Table 02]. MAESTERO ($\Delta\Delta G$) defined $\Delta\Delta G \geq 0$ as stabilizing of protein and $\Delta\Delta G \leq 0$ as destabilizing of protein. This algorithm detected 04 stabilizing mutations such as D631Y with predicted score of -0.129, C634R with predicted score of -0.07, K666N with predicted score of -0.1299 and R813W with predicted score of -0.084, and rest of them were predicted as destabilizing mutations with the threshold score of "greater than 0", that affected the function of RET protein[Table 03].

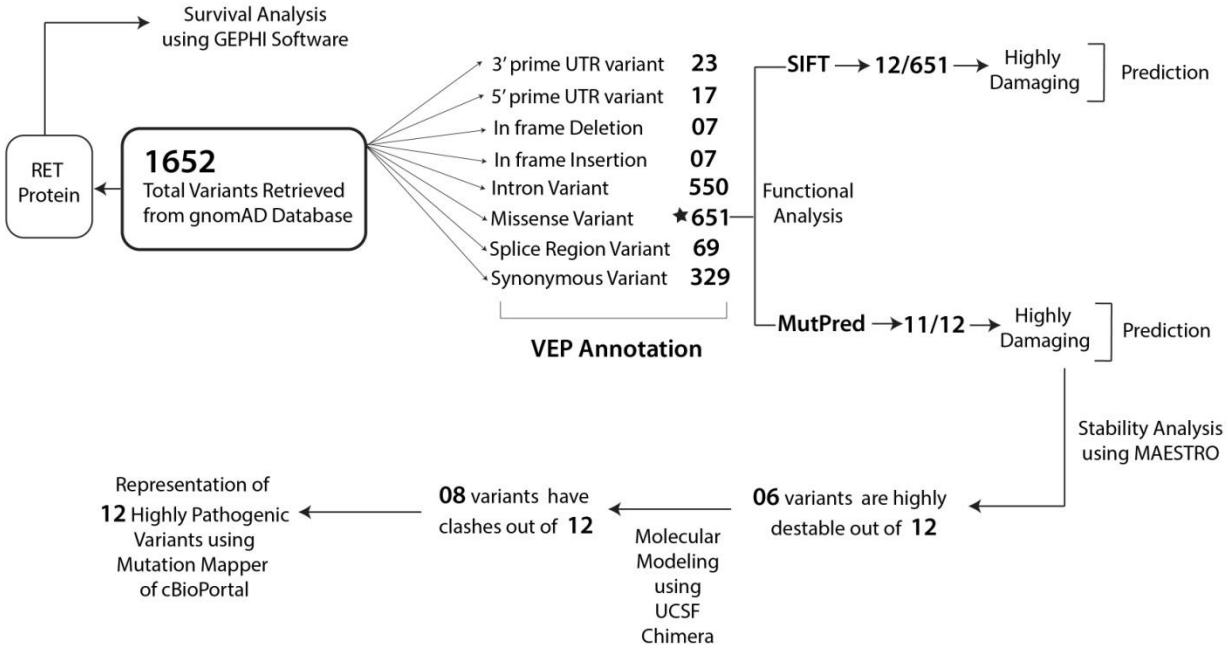


Figure 02: Schematic representation *RET* variants retrieved from insilico tools.

Coordinates	Codons	Substitution	Region	dbSNP ID	SNP Type	Prediction	Score
10,43609070,1,G/A	TGC-TaC	C609Y	EXON CDS	rs77939446:A	Nonsynonymous	DAMAGING	0
10,43609096,1,T/C	TGC-cGC	C618R	EXON CDS	rs76262710:C	Nonsynonymous	DAMAGING	0
10,43609939,1,G/T	GAC-tAC	D631Y	EXON CDS	novel	Nonsynonymous	DAMAGING	0.05
10,43609948,1,T/C	TGC-cGC	C634R	EXON CDS	rs75076352:C	Nonsynonymous	DAMAGING	0
10,43609949,1,G/T	TGC-TtC	C634F	EXON CDS	rs75996173:T	Nonsynonymous	DAMAGING	0
10,43609950,1,C/G	TGC-TGg	C634W	EXON CDS	rs77709286:G	Nonsynonymous	DAMAGING	0
10,43610046,1,G/T	AAG-AAt	K666N	EXON CDS	rs146646971:T	Nonsynonymous	DAMAGING	0.02
10,43613906,1,G/C	TTG-TTc	L790F	EXON CDS	rs75030001:C	Nonsynonymous	DAMAGING	0.01
10,43614996,1,G/T	GTG-tTG	V804L	EXON CDS	rs79658334:T	Nonsynonymous	DAMAGING	0.03
10,43615023,1,C/T	CGG-tGG	R813W	EXON CDS	novel	Nonsynonymous	DAMAGING	0
10,43615592,1,T/G	TCG-gCG	S891A	EXON CDS	rs75234356:G	Nonsynonymous	DAMAGING	0
10,43617416,1,T/C	ATG-AcG	M918T	EXON CDS	rs74799832:C	Nonsynonymous	DAMAGING	0

Table 01: Functional analysis of highly pathogenic variants using SIFT algorithm. SIFT; Sorting Tolerant from Intolerant.

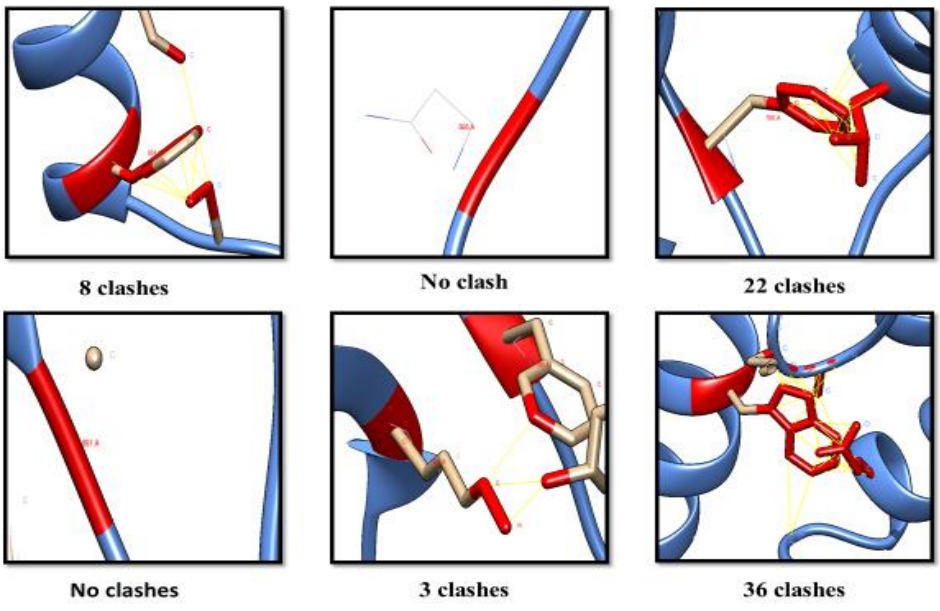
Gene	Variant	Prediction Score of MutPred
sp|P07949|RET_HUMAN	C609Y	0.915
sp|P07949|RET_HUMAN	C618R	0.822
sp|P07949|RET_HUMAN	D631Y	0.596
sp|P07949|RET_HUMAN	C634R	0.778
sp|P07949|RET_HUMAN	C634F	0.694
sp|P07949|RET_HUMAN	C634W	0.753
sp|P07949|RET_HUMAN	K666N	0.366
sp|P07949|RET_HUMAN	L790F	0.745
sp|P07949|RET_HUMAN	V804L	0.781
sp|P07949|RET_HUMAN	R813W	0.832
sp|P07949|RET_HUMAN	S891A	0.777
sp|P07949|RET_HUMAN	M918T	0.925

Table 02:Functional analysis of highly pathogenic variants using MutPredalgorithm. MutPred predicted 11 variants except K666N with “0.366” predicted score to be most deleterious out of 12.

Input	Output	
Mutations	**ddG_pred**	**c_pred**
C609.A{Y}	0.138	0.827
C618.A{R}	0.279	0.8193
D631.A{Y}	-0.1299	0.837
C634.A{R}	-0.076	0.8566
C634.A{F}	0.014	0.8567
C634.A{W}	0.065	0.8368
K666.A{N}	-0.1299	0.8104
L790.A{F}	0.1468	0.884
V804.A{L}	0.1293	0.8495
R813.A{W}	-0.084	0.843
S891.A{A}	0.237	0.875
M918.A{T}	0.4592	0.841

Table 03:The prediction performance of protein unfolding energy ($\Delta\Delta G$) on *RET* variants using MAESTRO bioinformatics tool.The high-risk variants in this performance showed score($\Delta\Delta G \leq 0$) by MAESTERO.

08 amino acid substitutions out of 12 nsSNPs showed the potential to interact negatively with nearby amino acid residues, indicating that they could cause structural disruption[Figure 02].A substitution of the amino acid "C609Y" revealed the formation of 20 clashes, "C618R" revealed the formation of 17 clashes, "C634R" revealed the formation of 3 clashes, "C634F" revealed the formation of 8 clashes, "C634W" revealed the formation of 1 clash, "L790F" revealed the formation of 22 clashes, "V804L" revealed the formation of 3 contacts, and "R813W" revealed the formation of 36 contacts with the surrounding residues[Figure 03]. The substitutions of amino acids “D631Y”, “K666N”, “S891A” and “M918A” showed no clashes with the surrounding residues. Unfavorable encounters could result from these contacts.The molecular simulation with clashes of all the 12 highly predicted variants is shown is figure 03.



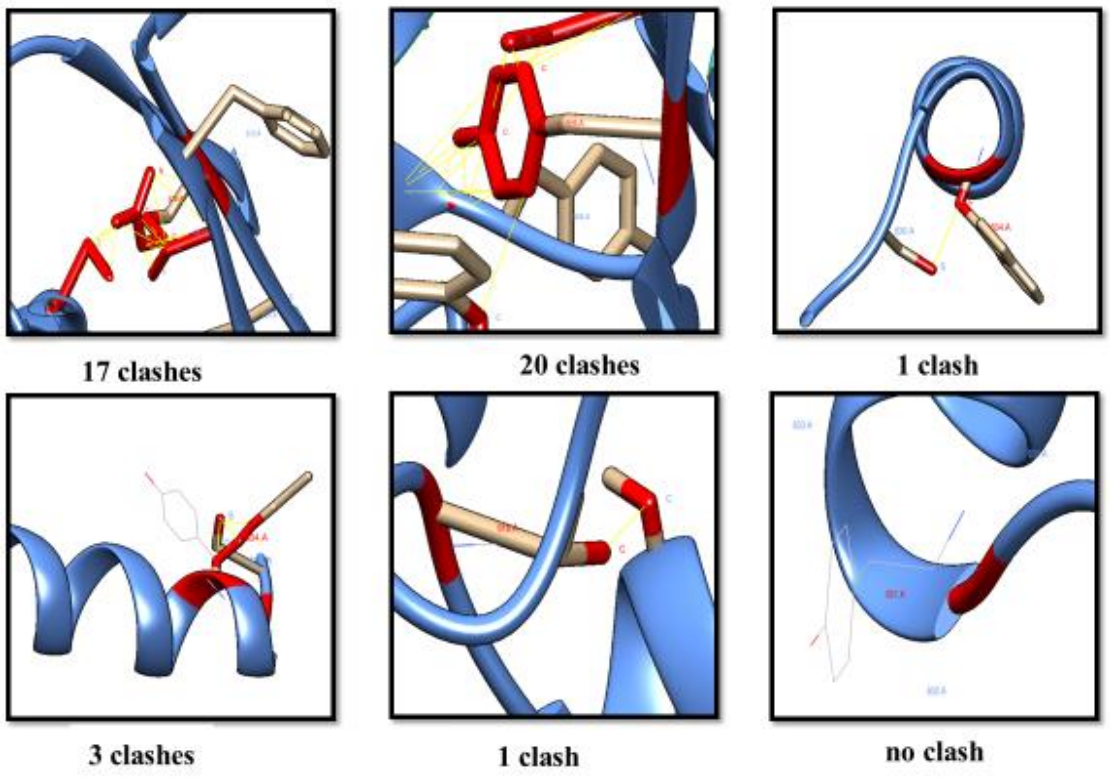


Figure 03: Molecular modeling using UCSF Chimera. All the clashes are shown in yellow colour.

MutationMapper tool of cBioPortal runs without the need for any additional software and is simple to integrate into brand-new pipelines or outputs. The preponderance of the mutations, including L790F, V804L, R813W, S891A, and M918T, were identified in the Pkinase-Tyr domain. C634R/F/W substitutions for amino acids were the most prevalent. This tool reported the high prevalence of the C634R/F/W mutation in the thyroid cancer population [Figure 04]. Low complexity regions are depicted in cyan, signal peptides are depicted in orange, and putatively disordered regions are depicted in dark grey. The next generation of precision medicine applications are made possible by this interpretation, which provides clinical settings with simple and extremely useful resources.

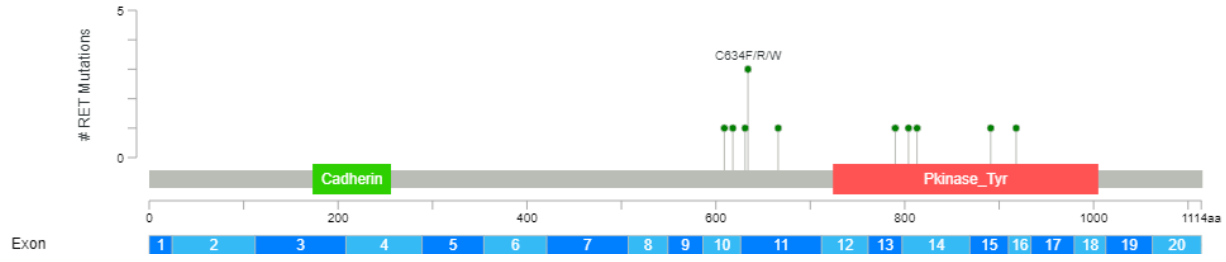


Figure 04: The open box of the *RET* gene as well as the frequency and location of RET mutations in Thyroid cancer in our study, are represented in the "lollipop" figure created by the MutationMapper tool of cBioPortal.

The survival of patients with thyroid cancer was strongly impacted by the downregulated *RET* (low expression). The overall survival time of patients with downregulated *RET* was [log-rank(Mantel-Cox) test P=0.29; hazard ratio (HR) was high with 0.58 value[Figure 5b],and disease free survival [log-rank (Mantel-Cox) testP=0.17; hazard ratio (HR) was high with 0.67 value[Figure 5a]. The lowest *RET* expression was associated with a poor outcome for thyroid cancer patients.

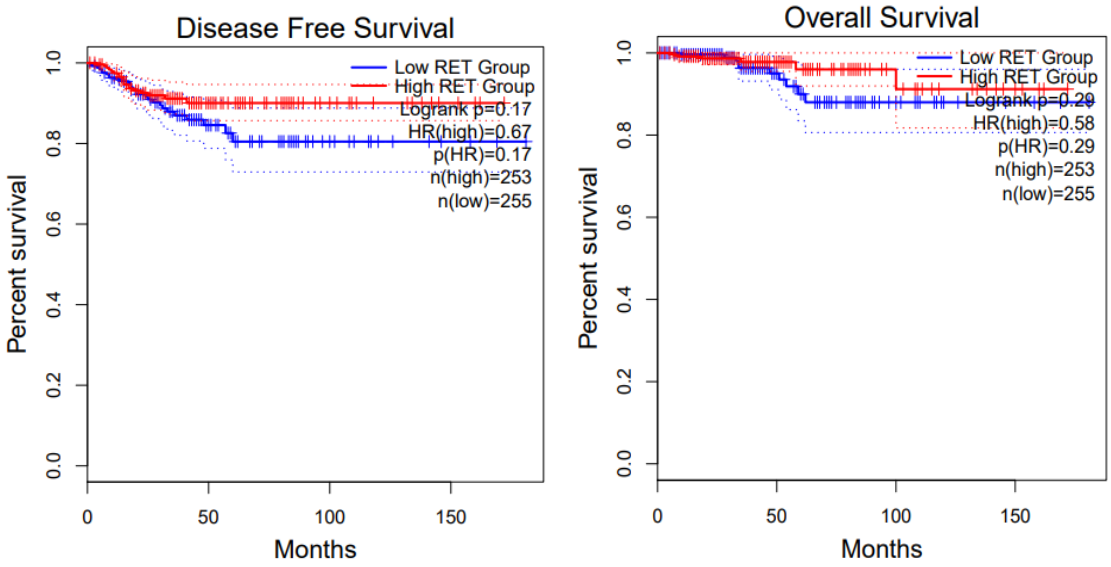


Figure 06 (a,b): Analysis of the *RET* gene with differential expression for survival. Red lines have high expression, whereas blue lines have low expression.P>0.05; Mantel-Cox test on log-rank.

Discussion

Thyroid cancer is one of the most prevalent endocrine tumors, that has been increasing in frequency in recent years. This is probably due to the existing diagnostic techniques' lack of specificity and accuracy, which causes an overdiagnosis of thyroid nodules[17].The incidence of thyroid cancer is one of the few cancers that has risen in recent years.According to the previous findings, important exons of *RET* contained 15 potentially novel genetic variants.Thyroid cancer patients have *RET* proto-oncogene genetic variants distributed at different frequencies and new variants[18].

A progress report was given on the frequency of *RET* indels in medullary thyroid carcinoma (MTC) and examined the effectiveness of selpercatinib in treating patients with advanced MTC who have *RET* indels.With the aid of an Ion S5 targeted sequencing, the MTC tissues of 287 patients were examined. MutationTaster has assessed the reported indels' functional significance. We gathered and examined the clinical and pathological information of MTC patients who had *RET* indels. Selpercatinib was administered to two patients who had *RET* indel mutations.That findings demonstrate how *RET* indels are common and are associated with virulent diseases. When treated with a highly selective *RET* inhibitor, two *RET* indel-positive patients demonstrated a partial response; as a result, these *RET* indels

can be regarded as actionable mutations[19].Another study was focused to identify the underlying mechanism in a male patient who was diagnosed with MTC at age 51. The discovery in our patient with MTC was a p.C630 deletion, a 3-base-pair deletion, in exon 11 of the *RET* gene. The harmful aspect of the mutation is demonstrated by the fact that the p.C630del *RET* promotes cell proliferation by enhancing ligand-independent phosphorylation and activation of the MAPK/ERK pathway. Therefore, it was urged that MTC patients with signs of a genetic aetiology to have a screening panel sequence of *RET[20]*.

In this study, gnomAD 2.1 was used for the variants retrieval of *RET* gene [Figure 02]. The Genome Aggregation Database Consortium (GnomAD), a global alliance of researchers, was established to gather and harmonise exome and genome sequencing data from numerous large-scale sequencing initiatives and to provide data summaries to the scientific community. The reference genome chosen for sequence alignment was GRCh37/hg19. Only missense variants were chosen from the resulting SNPs for further screening because the subsequent changes in the protein sequence's amino acids affected the amino acid sequence.After filtering the large data, 651 nsSNPs were further analysed to identify the most harmful variants. Twelve variants, identified by the SIFT algorithm with a threshold score of "less than 0.5," were predicted to be the most harmful/damaging out of the 651 variants.However, earlier research found that *RET* gene mutations in thyroid cancer are more evenly spread throughout the gene, including mutations at codons 532 and 533 (exon 8), 609, 611, 618, 620 (exon 10), 630 and 634 (exon 11), 768 and 790 and 791 (exon 13), V804M, 844 and 891 (exon 14), and 912 (exon 15)[21]. Only families with thyroid cancer have mutations at codons 532, 533, 768, 844, and 912 been found[22]. Although, it was initially considered that mutations at codon 804 were associated to thyroid cancer[23].Presently, the study revealed that C609Y, C618R, D631Y, C634R, C634F, C634W, K666N, L790F, V804L, R813W, S891A and M918T are highly associated with thyroid cancer.

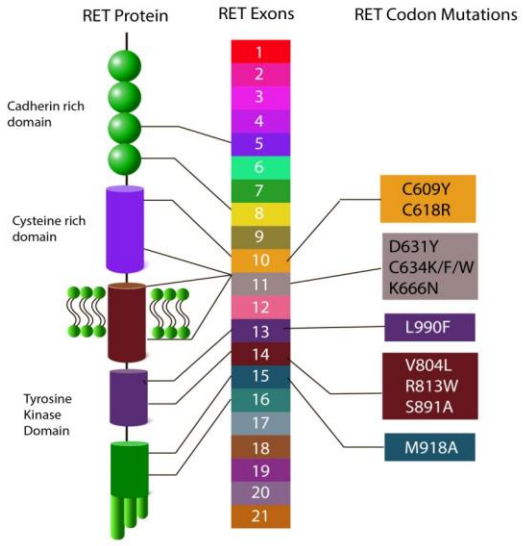
RET mutation stability alterations were predicted using MAESTRO, a sophisticated technique. If Gpred is smaller than 0.0, which indicates the overall predicted change in stability (kcal/mol), a stabilizing mutation is suggested. For the cpred reliability estimation, a value is provided that ranges from 0.0 (unreliable) to 1.0. This algorithm detected 04 stabilizing mutations such as D631Y with predicted score of -0.129, C634R with predicted score of -0.07, K666N with predicted score of -0.1299 and R813W with predicted score of -0.084, and rest of them were predicted as destabilizing mutations with the threshold score of “greater than 0”, that affected the function of RET protein.

UCSF-Chimera was used to predict highly pathogenic variants. A study was done to see how the changed residue interacted with its neighboring amino acid residues in order to look into the effects of mutations. Predictions were created based on the VDW radii. The interactions are most likely not beneficial for the protein to function normally, indicating that unfavorable interactions could have a significant impact on the structure.A substitution of the amino acid "C609Y" revealed the formation of 20 contacts, "C618R" revealed the formation of 17 clashes, "C634R" revealed the formation of 3 clashes, "C634F" revealed the formation of 8 clashes, "C634W" revealed the formation of 1 clash, "L790F" revealed the formation of 22 clashes, "V804L" revealed the formation of 3 contacts, and "R813W" revealed the formation of 36 contacts with the surrounding residues. The substitutions of amino acids “D631Y”, “K666N”, “S891A” and “M918A” showed no clashes with the surrounding residues. Unfavorable encounters could result from

these contacts.TheMutationMapper tool reported the high prevalence of the C634R/F/W mutation in the thyroid cancer population. The survival of patients with thyroid cancer was strongly impacted by the downregulated *RET.*

This study collectively revealed that “C609Y”,"C618R", “C634R”,"C634F","C634W","L790F, "V804L" and "R813W" were predicted to be the most harmful/damaging mutations in *RET* gene associated with thyroid cancer on the basis of molecular simulation. In molecular simulation, all of the variants showed unfavorable condition by creating clashes/contacts in the RET protein structure. This interpretation enables the next generation of precision medicine solutions and offers straightforward and very helpful tools for clinical settings.

Conclusion



Graphical representation of conclusion of this study; The proto-oncogene *RET* is composed of 21 exons located on chromosome 10 (10q11.2) and encodes for a transmembrane receptor tyrosine kinase for members of the glial cell line–derived neurotrophic factor family (GDNF) and associated ligands. The *RET* extracellular segment contains four cadherin like domains, followed by a domain containing cysteine residues involved in the formation of intermolecular disulfide bonds.This study revealed that C609Y, C618R, D631Y, C634R, C634F, C634W, K666N, L790F, V804L, R813W, S891A and M918T are highly associated with thyroid cancer. The survival of patients with thyroid cancer was strongly impacted by the downregulated *RET.* Variants namely; “C609Y”,"C618R", “C634R”,"C634F","C634W","L790F, "V804L" and "R813W" were predicted to be the most harmful/damaging mutations in *RET* gene on the basis of molecular simulation. The research investigation using bioinformatics tools enables the next generation of precision medicine solutions and offers straightforward and very helpful tools for the treatment of thyroid cancer.

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