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Development of a Comprehensive Mutational Panel as an Effective Tool for Personalized Diagnostic of Medullary Thyroid Carcinomas

Abstract

Medullary Thyroid Carcinoma (MTC) originates from mutations in Calcitonin-Producing Parafollicular C cells of the thyroid, is a rare malignancy, accounting for 3-4% of all thyroid carcinomas. It occurs in a hereditary form (HMTC, 25%) or in a sporadic form (SMTC, 75%). The prognosis for patients with MTC is poor, as the tumor metastasizes at early stages; and the only curative therapeutic option so far is radical surgery. Genetic analysis helps identify inherited cases at a stage where prophylactic surgery can be offered to carriers of such mutations to prevent the disease. This approach may also be used to determine better treatment options for patients who are already diagnosed with MTC.

The goal of this project was to develop the most comprehensive mutational panel for the detection of clinically relevant mutations in clinical MTC samples. A total of 143 mutations in 8 human genes were selected from numerous papers and public databases and included into the MTC mutational panel. The assay design was carried out using Sequenom's online design tools. The final file comprised from 115 assays corresponding to 143 mutations included in the MTC panel will be further processed using the SEQUENOM® Mass-ARRAY iPLEX® platform for DNA genotyping of clinical samples by the cancer research scientists at the Abramson Cancer Center of the University of Pennsylvania.

Introduction

Medullary Thyroid Carcinoma (MTC) that originates from calcitonin-producing parafollicular cells of the thyroid gland is a rare malignancy accounting for 3-4% of all thyroid carcinomas. It was first characterized by John B. Hazard in his paper entitled "Medullary (solid) carcinoma of the thyroid—a clinicopathologic entity" (Hazard et al. 1959). The prognosis for patients with MTC is very poor as the tumor metastasizes at early, and since the tumor's average age of onset is 21 ± 6, the only realistic curative therapeutic option so far is radical surgery (Alvandi et al. 2011).

MTC occurs in two forms: hereditary (HMTC in 25% of all cases) and sporadic (SMTC in 75% of all cases) (Jimenz et al. 2008). In 1961, John H. Sipple described the association between MTC and pheochromocytoma, an association that's known as multiple endocrine neoplasia type 2 (MEN 2) syndrome (Jimenz et al. 2008). There are two sub-types of MEN 2 syndrome, MEN 2A that is found in 20-50% of HMTC cases and only in 5-20% of HMTC cases when together with hyperparathyroidism. MEN 2B subtype is much more aggressive then MEN 2A and occurs in 50% of cases alongside marfanoid habitus and with mucosal and digestive neurofibromatosis (OMIM 162300). Familial MTC (FMTC) is not associated with any of the MEN syndromes, and it is least aggressive of the three HMTC (OMIM 155240). Genetic analysis has helped identify inherited cases at a stage where prophylactic surgery can be offered to carriers to prevent the disease. Activating mutations of the *RET* proto-oncogene are associated with the pathogenesis of MTC and have been demonstrated in nearly all hereditary and in 30-50% of SMTC cases (Cakir et al. 2009). Only 60% of SMTC cases were successfully attested

to mutations, and the rest remain unclassified. In the same time the SMTC is equally aggressive as its HMTC version.

Since gene mutations are the main suspect of cause of most types of cancer, mutational profiling of clinical tumor samples becomes very important as a guide for tumor classification, potential prediction of the patient outcomes and treatment options (). Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) mass spectrometry (MS) of DNA is in broad use for targeted single nucleotide polymorphism (SNP) and somatic mutations genotyping studies. Detection of somatic mutations requires a higher level of sensitivity than most standard SNP genotyping methods. Whereas germline and other genetic mutations are simply classified as heterozygote or homozygote alleles, somatic mutations, due to the fact that they are present in a portion of cells (typically in tumor cells surrounded by normal cells) require quantitative mutation frequency assessment.

MALDI-TOF MS is used as the base for the commercially available Sequenom MassARRAY platform for mutations genotyping. The Sequenom approach can detect mutations even if they are present only in 5% of the cell population and can give quantitative information on each mutation (www.sequenom.com).

The multiplex reaction (iPLEX) assay in this method is a single base primer extension assay. First, PCR amplifying fragments of about 100 base pairs (bp) with primers flanking the mutation is conducted in a multiplex reaction for several products. Next, extension primers designed immediately adjacent to the mutation site prompt extension by one nucleotide depending on the template sequence. The difference in mass

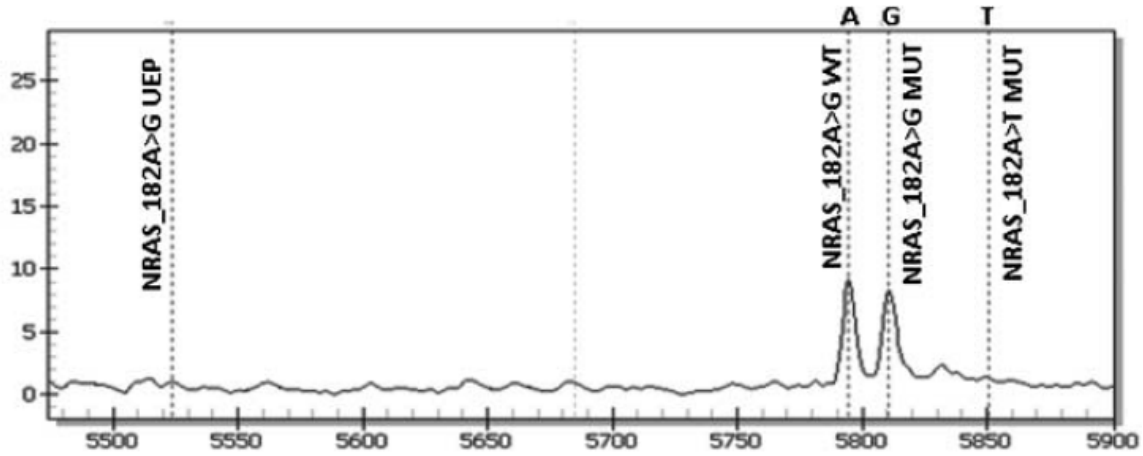
between extended products allows distinction of wild-type and mutant alleles (Gabriel S, et al, 2009; Millis M. 2011) (Figure 1).

The prime objective of this project was to develop a most comprehensive mutational panel to date for the detection of clinically relevant mutations in MTC samples. To this end a total of 143 mutations in 8 human genes were selected from numerous peer-reviewed publications as well as from the available public databases to be included into the MTC mutational panel. The selection criteria were based on the coding mutations (mutations that occur in the coding area of the genes) that were reported to occur in MTC patients and considered functionally relevant. Some of the mutations included into the MTC mutational panel (such as *BRAF* gene V600E mutation) were also described in other types of thyroid cancer, but most of the mutations were unique to MTC (such as all *RET* gene mutations). To this end a Mutational Assay Panel was designed for MALDI-TOF MS genotyping encompassing the most significant genes in this disease: total of 143 mutations in *RET*, *BRAF*, *KRAS*, *HRAS*, *SDHB*, *SDHD*, *VHL* and *CDKN1B* organized in the file consisting of 115 assays. The assay design was carried out using Sequenom's online design tools (ProxSNP and PreXTEND (https://www.mysequenom.com/Tools) and Assay Design software (v. 3.1)).

The developed assay file is fully compatible with the SEQUENOM® MassARRAY iPLEX® software and will be further used for DNA genotyping of clinical tumor samples.

FIGURE 1. A representative case of MALDI-TOF based genotyping. The Figure shows the MassARRAY spectrum for a NRAS mutation (c. 182 A>G) for which there is either a

wild-type allele (A) or mutation (G). This figure has been transcribed from the publication by Ricarte-Filho et al. 2009.



Materials and Methods

The list of mutations for the MTC mutational panel was generated based on results of annotation of scientific publications (source: PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/)) and screening of public biological databases including the Catalogue Of Somatic Mutations In Cancer (COSMIC) Database (http://www.sanger.ac.uk/genetics/CGP/cosmic/), University of Utah MEN2 Database (http://arup.utah.edu/database/MEN2/MEN2_display.php) and National Cancer Institute Database (http://www.cancer.gov/cancertopics/pdq/genetics/medullarythyroid/HealthProfessional/Table4). Human DNA sequences that flanking mutations selected were retrieved from COSMIC database, UCSC Genome Browser (http://genome.ucsc.edu/) and formatted with DNASTAR Lasergene software (v. 9). The assay design was carried out using Sequenom's online design tools (ProxSNP and PreXTEND (https://www.mysequenom.com/Tools) and Assay Design software (v. 3.1)).

Results and Discussion

Data Collection

As a first step, the list of genes mutations described in MTC was prepared. To date, 98% of mutations found in hereditary MTC belong to *RET* (Rearranged During Transfection) gene. *RET* proto-oncogene encodes one of the receptor tyrosine kinases, cell-surface molecules that transduce signals for cell growth and differentiation (OMIM 164761). RET mutations differ in the aggressiveness of the MTC (Abraham11_6–8), for example, RET mutations at amino acid position 918 and 883 are considered to be responsible for the most aggressive types of MTC (Cakir et al. 2009). They are found in over 95% of the MEN 2B cases and in most of the RET mutations of SMTC. (http://emedicine.medscape.com/article/1744824-overview). Overall, the mutation in position 918 is found in over 50% of the classified MTC cases. RET mutations in amino acid positions 609, 611, 618, 623, 630 and 634 ((http://emedicine.medscape.com/article/1744824-overview) are responsible for over 90% of MEN 2A and FMTC cases. Two percent of HMTC remain unclassified, with outlying mutations in genes such as Succinate Dehydrogenase (*SDHB* and *SDHD*), being found. Their contribution to MTC pathogenesis remains unknown.

SMTC is much more ambiguous then its hereditary counterpart, as only 40-60% of known SMTC cases can be attested to mutations. While being found in almost all known cases of HMTC, *RET* gene is found in only 20-40% (http://emedicine.medscape.com/article/1744824-overview) of SMTC. Mutations in genes such as *BRAF*, *KRAS*, *VHL*, *HRAS* and *CDKN1B* have also been found in SMTC, but altogether they only amount to 10-20% of the known cases of SMTC.

The selection criteria for the Mutational Panel were based on coding mutations that were reported to occur in MTC and are considered to be functionally relevant. The genes examined are represented in Table 1, and the complete list of mutations that was included in the panel is available in Table 2. The mutations were collected from Biological databases as well as a wide range of online publications found through Public Medical Database (PubMed). A grand total of 143 mutations that occur in MTC were included in the Mutational Panel.

Next, fragments of gene sequences (typically 200-250 bp in length) containing mutations selected were collected in the Excel file. To specify a mutation in the DNA sequence the following format was used:

1) For a single nucleotide variation

catc[A/T]tggt

2) For deletion

catc[TTC/--]tgggt

3) For insertion

catc[--/TTC]tgggt

Assay Development

The Excel file was converted to a .txt format, to be used as an input file for the ProxSNP, which, through the connection to SNP database, inspects the nucleotide sequences for any polymorphisms, and if too many potential polymorphisms are found, then the sequence is considered inadequate and the program rejects it, the reason being that software cannot find appropriate sequences for primer design s. The output file of ProxSNP is at the same time an input file for PreXTend, a program that highlights

sequences that are suitable for the design of primers (a fragment of DNA that can serve as a starting point for DNA synthesis) and makes sure that there will be no crosshybridization between them.

Subsequently, the output file of PreXTend was used as an input file for MassARRAY Assay Design Software, which in turn generated the Array File, compatible with Sequenom Software. The Array File includes sequences for two PCR primers (Primers that have 5' tag nucleotide sequences attached), the Unextended Extended Primers (UEP) and their mass, two Extended Primers and their mass; and additional information about each of 115 assays (which correspond to 143 mutations). This file contains 18 iPLEXs (W1-W18). The number of assays in each iPLEX varies at intervals of 1 to 15 (see Figure 2)

Conclusion

The prime objective of this project was to develop a most comprehensive mutational panel for the detection of clinically relevant mutations in MTC samples. The mutational panel developed and delivered by this applicant will be further processed using the SEQUENOM® Mass-ARRAY iPLEX® platform and ultimately used for mutations profiling of the clinical MTC samples by the cancer research scientists at the Abramson Cancer Center of the University of Pennsylvania.

FIGURE 2

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	
1	WELL	TERM	SNP_ID	2nd-PCRP	1st-PCRP	AMP_LEN	UP_CONF	MP_CONF	Tm(NN)	PcGC	PWARN	UEP_DIR	UEP_MASS	UEP_SEQ	EXT1_CAL	EXT1_MASS	EXT1_SEQ	EXT2_CALL	EXT2_MASS	E
2	W1	iPLEX	RET_Y806C_AG	ACGTTGGATG	ACGTTGGATGA	95	73.1	60	50.5	58.8	d	F	5137.3	CCTCCTCA	A	5408.6	CCTCCTCA	G	5424.6	C
3	W1	iPLEX	SDHD_H50R_AG	ACGTTGGATG	ACGTTGGATGC	88	98	60	55.2	61.1		R	5595.6	TGGTGGC	G	5842.8	TGGTGGCT	A	5922.7	T
4	W1	iPLEX	RET_V591I_GA	ACGTTGGATG	ACGTTGGATGT	99	88.3	60	60	68.4		R	5709.7	CCAGGCT	G	5956.9	CCAGGCTC	A	6036.8	C
5	W1	iPLEX	RET_G533C_GT	ACGTTGGATG	ACGTTGGATGT	96	94.6	60	55.3	63.2	D	F	6004.9	GGAGTGT	A	6276.1	GGAGTGTC	G	6292.1	G
6	W1	iPLEX	RET_V778I_GA	ACGTTGGATG	ACGTTGGATGA	101	93.5	60	55.1	55	D	R	6164	GTGGTTG	G	6411.2	GTGGTTGA	A	6491.1	G
7	W1	iPLEX	RET_S922F_CT	ACGTTGGATG	ACGTTGGATGC	97	100	60	46.1	30		F	6204.1	TAAATGG	C	6451.3	TAAATGGA	T	6531.2	T
8	W1	iPLEX	RET_S686N_TCAA	ACGTTGGATG	ACGTTGGATGT	108	75.1	60	59.8	66.7	d	F	6343.1	CAGGCCT	AA	6614.3	CAGGCCTT	TC	6670.2	C
9	W1	iPLEX	RET_S904C/F_CG/T	ACGTTGGATG	ACGTTGGATGT	95	87	60	45.4	33.3		F	6564.3	GAGATGT	C	6811.5	GAGATGTT	G	6851.5	G
10	W1	iPLEX	RET_T338I_CT	ACGTTGGATG	ACGTTGGATGC	103	90.7	60	59.8	59.1	d	F	6778.4	GTGGAAC	C	7025.6	GTGGAACA	T	7105.5	G
11	W1	iPLEX	RET_V202M_GA	ACGTTGGATG	ACGTTGGATGA	94	90.5	60	58.5	52.2	d	F	6959.5	AGTTCTT	A	7230.7	AGTTCTTG	G	7246.7	A
12	W1	iPLEX	SDHB_S163P_TC	ACGTTGGATG	ACGTTGGATGA	93	98.4	60	46.8	30.4	D	F	7159.7	CTTATTTG	C	7406.9	CTTATTTGA	T	7486.8	C
13	W1	iPLEX	RET_G321R_GA	ACGTTGGATG	ACGTTGGATGT	92	77.3	60	61.3	62.5	DH	R	7289.7	CCCCTGA	G	7536.9	CCCCTGAT	A	7616.8	C
14	W1	iPLEX	RET_D631A/G/V_AC/G/T	ACGTTGGATG	ACGTTGGATGA	98	87.2	60	57.7	60	d	F	7580.9	gaccaACC	C	7828.1	gaccaACCC	A	7852.1	g
15	W1	iPLEX	RET_M848T_TC	ACGTTGGATG	ACGTTGGATGT	100	84.3	60	62	60	ds	R	7701	CCAGGCA	T	7972.2	CCAGGCAA	C	7988.2	C
16	W1	iPLEX	RET_C618W_CG	ACGTTGGATG	ACGTTGGATGA	100	98.6	60	60.3	53.8	D	F	8060.2	AACTGCT	C	8307.4	AACTGCTT	G	8347.5	A
17	W2	iPLEX	RET_EL632-633DV_GCCG	ACGTTGGATG	ACGTTGGATGC	99	88.3	65.3	56	64.7	d	R	5180.4	GATCACC	CG	5427.6	GATCACCG	GC	5467.6	G
18	W2	iPLEX	RET_H665Q_CG	ACGTTGGATG	ACGTTGGATGT	94	87.1	65.3	51.4	58.8	d	R	5346.5	GAGGAG	G	5593.7	GAGGAGA	C	5633.7	G
19	W2	iPLEX	RET_D489N_GA	ACGTTGGATG	ACGTTGGATGT	100	98.5	65.3	53.8	61.1	D	R	5546.6	GCCTAGA	G	5793.8	GCCTAGAC	A	5873.7	G
20	W2	iPLEX	RET_G691S_GA	ACGTTGGATG	ACGTTGGATGA	97	87	65.3	51.5	57.9	D	F	5690.7	GGTCAGC	A	5961.9	GGTCAGCT	G	5977.9	G
21	W2	iPLEX	RET_T278N_CA	ACGTTGGATG	ACGTTGGATGC	100	92.2	65.3	63.9	78.9	DH	R	5750.7	CTCCACC	C	6037.9	CTCCACCA	A	6077.8	C
22	W2	iPLEX	RET_I852M_CG	ACGTTGGATG	ACGTTGGATGA	99	86.1	65.3	53.1	50	d	R	6175	GAGATCT	G	6422.2	GAGATCTG	C	6462.2	G
23	W2	iPLEX	RET_C620W_CG	ACGTTGGATG	ACGTTGGATGC	100	97.1	65.3	52.3	55	D	F	6262.1	GAGGAG	C	6509.3	GAGGAGG	G	6549.3	G
24	W2	iPLEX	RET_G911D_GA	ACGTTGGATG	ACGTTGGATGT	104	83.9	65.3	49.8	38.1		R	6389.2	ATCCATTT	G	6636.4	ATCCATTTA	G	6716.3	A
25	W2	iPLEX	RET_E805K_GA	ACGTTGGATG	ACGTTGGATGA	97	57.5	65.3	58.8	54.5	d	R	6806.4	AGGGAG	G	7053.6	AGGGAGC	A	7133.5	A
26	W2	iPLEX	RET_G550E_GA	ACGTTGGATG	ACGTTGGATGA	97	89	65.3	55.1	54.5	dH	R	6846.4	GGTGGAC	G	7093.6	GGTGGAGA	A	7173.5	G
27	W2	iPLEX	RET_R886W_CT	ACGTTGGATG	ACGTTGGATGA	99	98.6	65.3	49.9	39.1	D	R	6942.5	GAAATCC	T	7213.7	GAAATCCC	C	7229.8	G
28	W2	iPLEX	RET_E768D_GC	ACGTTGGATG	ACGTTGGATGT	91	99.7	65.3	62	60.9	D	F	7018.6	TCAGAGA	C	7265.8	TCAGAGAA	G	7305.8	T
29	W2	iPLEX	CDKN1B_V109G_TG	ACGTTGGATG	ACGTTGGATGA	99	90.1	65.3	67.6	70.8	Ds	F	7468.8	TGCCGGC	G	7756	TGCCGGCG	T	7795.9	T
30	W2	iPLEX	HRAS_A11/G12dup_GC	ACGTTGGATG	ACGTTGGATGC	99	97.3	65.3	63.7	60	d	R	7602.9	GATGGTC	G	7850.1	GATGGTCA	C	7890.1	G
31	W2	iPLEX	RET_Y791F_AT	ACGTTGGATG	ACGTTGGATGT	98	94.6	65.3	57.9	40.7	d	F	8147.3	TCAACCA	A	8418.5	TCAACCAC	T	8474.4	T
32	W3	iPLEX	HRAS_G12C_GT	ACGTTGGATG	ACGTTGGATGT	99	97.3	78.8	55.8	64.7	D	R	5051.3	ACTCTTG	G	5298.5	ACTCTTGC	T	5322.5	A
33	W3	iPLEX	RET_K666E_AG	ACGTTGGATG	ACGTTGGATGC	94	87.1	78.8	50.9	52.9	s	F	5099.3	ACCACAA	A	5370.5	ACCACAAC	G	5386.5	A
34	W3	iPLEX	RET_R770Q_GA	ACGTTGGATG	ACGTTGGATGT	91	99.7	78.8	50.1	52.9	d	R	5170.4	ACTCTGA	G	5417.6	ACTCTGAC	A	5497.5	A
35	W3	iPLEX	RET_C611F_GT	ACGTTGGATG	ACGTTGGATGA	101	96.1	78.8	53.3	55.6	d	F	5459.6	GCTATGG	G	5746.8	GCTATGGC	T	5786.7	G
36	W3	iPLEX	RET_C515S_GCCT	ACGTTGGATG	ACGTTGGATGT	98	94.6	78.8	55.3	66.7	Dg	R	5598.6	GACTGCA	CT	5869.9	GACTGCAC	GC	5885.9	G
37	W3	iPLEX	KRAS_Q61K_CA	ACGTTGGATG	ACGTTGGATGT	96	99.9	78.8	48.8	42.1	s	R	5704.7	ATTGCAC	C	5991.9	ATTGCACT	A	6031.8	A
38	W3	iPLEX	RET_E901K_GA	ACGTTGGATG	ACGTTGGATGA	98	96	78.8	47.4	42.1		F	5824.8	TTGTCCC	C	6072	TTGTCCCG	A	6096	T
39	W3	iPLEX	RET_V804L/M_c.2413GT/A	ACGTTGGATG	ACGTTGGATGA	93	63.3	78.8	59.8	70	d	F	5949.9	CAGGCCC	A	6221.1	CAGGCCCG	G	6237.1	C
40	W3	iPLEX	RET_C531R_TC	ACGTTGGATG	ACGTTGGATGT	96	94.6	78.8	67.7	80	Dg	R	6129	TGGGGAC	T	6400.2	TGGGGAGC	C	6416.2	T
41	W3	iPLEX	SDHD_G12S_GT	ACGTTGGATG	ACGTTGGATGA	94	89.2	78.8	60.8	71.4	Dh	R	6319.1	CCTCACC	G	6566.3	CCTCACCT	T	6590.3	C
42	W3	iPLEX	RET_A639G_CG	ACGTTGGATG	ACGTTGGATGC	91	95.8	78.8	56.8	70.6	d	F	6454.2	ttcgGTGCC	C	6701.4	ttcgGTGCC	G	6741.4	tt
43	W3	iPLEX	RET_E921K_GA	ACGTTGGATG	ACGTTGGATGT	97	100	78.8	45.1	28.6		R	6526.3	AGATATG	G	6773.5	AGATATGA	A	6853.4	A
44	W3	iPLEX	RET_Y791N_TA	ACGTTGGATG	ACGTTGGATGT	98	94.6	78.8	52.7	45.5	h	F	6623.3	CCACCCA	A	6894.5	CCACCCAC	T	6950.4	C
45	W3	iPLEX	RET_E843D_GT	ACGTTGGATG	ACGTTGGATGC	98	82.9	78.8	66.4	69.6	H	R	7041.6	GCCCATG	G	7288.7	GCCCATGG	T	7312.8	G
46	W3	iPLEX	HRAS_Q61K_CA	ACGTTGGATG	ACGTTGGATGT	81	91.7	78.8	66.2	65.4	Ds	R	7850.1	GTCCCGC	C	8137.3	GTCCCGCA	A	8177.2	G
47	W4	iPLEX	HRAS_G13R_GC	ACGTTGGATG	ACGTTGGATGC	99	97.3	76.3	54.1	64.7	D	R	5051.3	CGCACTC	G	5298.5	CGCACTCT	C	5338.5	C
48	W4	iPLEX	RET_R820C_CT	ACGTTGGATG	ACGTTGGATGT	108	75.4	76.3	57.4	70.6	D	R	5107.3	GCCAGGC	T	5378.5	GCCAGGCC	C	5394.5	G
49	W4	iPLEX	RET_C609F/S/Y_G/T/C/A	ACGTTGGATG	ACGTTGGATGT	82	95.2	76.3	54.2	58.8	D	F	5186.4	AGCTGGC	G	5473.6	AGCTGGCT	T	5513.5	A
50	W4	iPLEX	RET_A510V_CT	ACGTTGGATG	ACGTTGGATGC	97	94.5	76.3	63.6	83.3	d	R	5437.5	GGGCAGC	T	5708.7	GGGCAGCC	C	5724.7	G
51	W4	iPLEX	RET_L881V_CG	ACGTTGGATG	ACGTTGGATGT	97	100	76.3	58.8	63.2	D	R	5644.7	TTCCGCC	G	5891.9	TTCCGCCC	C	5931.9	T
52	W4	iPLEX	KRAS_Q61L/R_AT/G	ACGTTGGATG	ACGTTGGATGT	96	99.9	76.3	51.2	45	s	R	5993.9	TCATTGC	G	6241.1	TCATTGCA	T	6265.1	T

Table 1.

Gene	Amount of mutations	Protein function/ Signaling pathway or Process
RET	130	A member of the cadherin superfamily, encodes one of the receptor tyrosine kinases/cell growth and differentiation
BRAF	1	Raf/mil family of serine-threonine protein kinase/ MAPK-signaling pathway
VHL	3	A tumor-suppressing gene. The protein products of VHL play a major role in the oxygen sensing pathways.
KRAS	3	A member of the small GTPase superfamily/ MAPK-signaling pathway
HRAS	5	A member of the small GTPase superfamily/ MAPK-signaling pathway
SDHD	2	Complex II of the respiratory chain, which is specifically involved in the oxidation of succinate, carries electrons from FADH to CoQ. The subunit D protein is one of two integral membrane proteins anchoring the complex to the matrix side of the membrane.
SDHB	1	Complex II of the respiratory chain, which is specifically involved in the oxidation of succinate, carries electrons from FADH to CoQ. The iron-sulfur subunit is highly conserved and contains three cysteine-rich clusters which may comprise the iron-sulfur centers of the enzyme.
CDKN1B	1	This gene encodes a cyclin-dependent kinase inhibitor, which shares a limited similarity with CDK inhibitor CDKN1A/p21. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1.

Table 2

Gene	Protein Mutation	Germline/Somatic
RET	RET_A510V_CT	g
	RET_A639G_CG	s
	RET_A640G_CG	g
	RET_A641R_GCCG	s

	RET_A641S_GT	g/s
	RET_A883F_GCTT	g/s
	RET_A883T_GA	g/s
	RET_A919V_ CT	s
	RET_C515S_GCCT	g
	RET_C531R_TC	g
	RET_C609F/S/Y_G/T/C/A	g
	RET_C609G/R/S_T/G/C/A	g/s
	RET_C611F_GCTT	g
	RET_C611F_GT	g
	RET_C611G/R/S_TG/C/A	g
	RET_C611S_GC	g
	RET_C611S_GCCT	g
	RET_C611W_CG	g
	RET_C611Y_GA	g
	RET_C611Y_GCAT	g
	RET_C618F/S/Y_GT/C/A	g/s
	RET_C618G/R/S_TG/C/A	g/s
	RET_C618W_CG	g
	RET_C620F/S/Y_GT/C/A	g
	RET_C620G/R/S_TG/C/A	g/s
	RET_C620W_CG	g
	RET_C630A_TGGC	s
	RET_C630F/S/Y_G/T/C/A	g
	RET_C630G/R_TG/C	g/s
	RET_C634F/S/Y_G/T/C/A	g/s
	RET_C634G/R/S_TG/C/A	g/s
	RET_C634T_TGAC	s
	RET_C634W_CG	s
	RET_D489N_GA	s
	RET_D631-R635dup_GAg	s
	RET_D631A/G/V_AC/G/T	g
	RET_D631D/E_CT/A 71	g
	RET_D631N/Y_GA/T	g
	RET_E511K_GA	g
	RET_E623K_GA	g
	RET_E632-L633del_GT	s
	RET_E632K_GA	g
	RET_E768D_GC	g/s
	RET_E805K_GA	g
	RET_E818K_GA	g
	RET_E843D_GT	g
	RET_E884K_GA	s
	RET_E901K_GA	s
	RET_E921K_GA	s
	RET_EL632-633DV_GCCG	g

	RET_F619F_CT	g
	RET_G321R_GA	g
	RET_G533C_GT	g/s
	RET_G550E_GA	g/s
	RET_G691S_GA	g
	RET_G911D_GA	s
	RET_G911F_GGTT	s
	RET_H665Q_CG	g
	RET_I852M_CG	g
	RET_K603E_AG	g
	RET_K666E_AG	g
	RET_K907E_AG	g
	RET_K907M_AT	g
	RET_L790F_GT	g
	RET_L881V_CG	g
	RET_M700L_AT	g
	RET_M848T_TC	g
	RET_M918T_TC	g/s
	RET_M918V_AG	g/s
	RET_N777S_AG	g
	RET_P766S_CT	s
	RET_P841L_CT	s
	RET_P841P-GA	g
	RET_Q781R_AG	g
	RET_R600Q_GA	g
	RET_R635G_CG	g
	RET_R770Q_GA	g
	RET_R820C_CT	s
	RET_R833C_CT	g
	RET_R844Q_GA	g
	RET_R886W_CT	g
	RET_R908K_GA	s
	RET_R912P_GC	g
	RET_S649L_TCCT	g
	RET_S686N_TCAA	g
	RET_S819I_GT	g
	RET_S891A_TG	g
	RET_S904C/F_CG/T	g/s
	RET_S922F_CT	s
	RET_S922P_TC	s
	RET_S922Y_CA	g
	RET_T278N_CA	s
	RET_T338I_CT	g
	RET_T930M_CT	s
	RET_V202M_GA	s
	RET_V292M_GA	g

	RET_V591I_GA RET_V648I_GA RET_V778I_GA RET_V804L/M_c.2413GT/A RET_Y791F_AT RET_Y791N_TA RET_Y806C_AG	s g/s g g g g g
SDHD	SDHD_G12S_GT SDHD_H50R_AG	g g
SDHB	SDHB_S163P_TC	g
HRAS	HRAS_A11/G12dup_GC HRAS_G12C_GT HRAS_G13R_GC HRAS_G13V_GT HRAS_Q61K_CA	s s s s s
KRAS	KRAS_Q61K_CA KRAS_Q61L/R_AT/G	s s
VHL	VHL_N78I VHL_F76_del(TTC) VHL_P59_del(C)	s s s
BRAF	BRAF_V600E_	s
CDKN1B	CDKN1B_V109G_TG	s

References:

1. Millis, M. (2011, Summer). Medium-Throughput SNP Genotyping Using Mass Spectrometry: Multiplex SNP Genotyping Using the iPLEX® Gold Assay. Springer Protocols, 700. Retrieved August 20, 2012, from http://link.springer.com/protocol/10.1007%2F978-1-61737-954-3_5

This paper discusses and explains the basics of genotyping with the using MALDI-TOF Mass Spectrometry. This was the first paper that I have read right after being given the project. After fully interpreting this paper, I realized that I am going to be able to finish the project on my own. This paper served as a guide to me throughout the process of

doing this project, as well as while writing the entire research report. In addition, this paper was used to make sure that I am not saying something that is factually incorrect.

2. Gabriel, S., Ziaugra, L., & Tabbaa, D. (2009, January 1). UNIT 2.12 SNP Genotyping Using the Sequenom MassARRAY iPLEX Platform. Current Protocols in Human Genetics.

This paper describes in details the SNP genotyping method based on the Sequenom MassARRAY platform. It includes two step protocol (initial locus-specific PCR reaction, followed by single base extension using mass-modified dideoxynucleotide terminators) an assay structure and how using MALDI-TOF mass spectrometry identify the SNP allele. The paper is mentioned in the corresponded section of the Introduction.

3. Ricarte-Filho, J., Ryder, M., Ghossein, R., Fagin, J., Chitale, D., Rivera, M., et al. (2009, June 1). Mutational Profile of Advanced Primary and Metastatic Radioactive Iodine-Refractory Thyroid Cancers Reveals Distinct Pathogenetic Roles for BRAF, PIK3CA, and AKT1. CANCER RESEARCH. Retrieved July 1, 2012, from http://cancerres.aacrjournals.org/content/69

The paper describes profiling of 111 mutations in RET, BRAF, NRAS, HRAS, KRAS, PIK3CA, AKT1 genes in clinical poorly differentiated, anaplastic and radioactive iodine-refractory differentiated thyroid cancers. The genotyping method is based on the Sequenom MassARRAY platform. It was shown that RAS mutations were prevalent in primary PDTC, whereas BRAF was more common in metastatic PDTC and ATC.

PIK3CA or AKT1 mutations were rare. The paper is mentioned in the corresponded section of the Introduction.

4. MEN2 Database. (n.d.). AURP Scientific Resource for Research and Education.. Retrieved July 12, 2012, from http://arup.utah.edu/database/MEN2/MEN2_display.php
This database was only used for its mutations list during the collection phase of the project.

5. Human BLAT Search. (n.d.). UCSC Genome Browser. Retrieved August 17, 2012, from http://genome.ucsc.edu/cgi-bin/hgBlat
The Human BLAT database was used for alignment of nucleic sequences. The sequences retrieved from COSMIC were inputted in the BLAT Database to be aligned with the rest of the nucleic sequence, as only a small part of it could be gathered from COSMIC.

6. Catalogue of Somatic Mutations in Cancer - COSMIC. (n.d.). Wellcome Trust Sanger Institute. Retrieved July 10, 2012, from http://www.sanger.ac.uk/genetics/CGP/cosmic/
This database was used to retrieve the nucleic sequences that were used as the base to be inputted in the BLAT database. As it is impossible to use BLAT database with only knowing the position of the mutation, COSMIC was used to retrieve the minimal part of the sequence required to find the full nucleic sequence for any particular gene.

7. Genetics of Endocrine and Neuroendocrine Neoplasias (PDQ®). (n.d.). National Cancer Institute. Retrieved July 12, 2012, from http://www.cancer.gov/cancertopics/pdq/genetics/medullarythyroid/HealthProfessional/Table4

This database was only used for its mutations list during the collection phase of the project.

8. OMIM Entry - # 171400 - MULTIPLE ENDOCRINE NEOPLASIA, TYPE IIA; MEN2A . (n.d.). OMIM - Online Mendelian Inheritance in Man . Retrieved July 19, 2012, from http://omim.org/entry/171400

This entry was used as the basis of understanding of the MEN 2A syndrome. It talks about the relationship between MTC and diseases such as pheochromocytoma, and about the basics of what MEN 2A syndrome actually is. In addition, it talks about what mutations are associated with the syndrome. It was specifically used for comparison with the MEN 2B and FMTC.

9. Jimenez, C., Hu, M. I., & Gagel, R. (2008, Spring). Management of Medullary Thyroid Carcinoma. Elsevier Saunders, ?, 15.

This MTC review was the first of many that I have read in the duration of this project. This review provided me with the basic information about MTC without which any attempt at actually finishing this project would have been obsolete. Many parts of the introduction are referred to this paper, as it was very influential. Similarly to many other sources, mutations were taken from this publication during the first phase of the project.

10. MacConaill, L Profiling Critical Cancer Gene Mutations in Clinical Tumor Samples. PLoS ONE (2009). http://www.plosone.org/article/info:doi/10.1371/journal.pone.0007887

This publication was one of many that were used only for its mutations. No part, except for the abstract, which contributed to the overall idea of the research report, has been read.

11. Ehsan Alvandi, Seyed Mohammad Akrami, Mohsen Chiani, Mehdi Hedayati, Babak Noori Nayer, Mohammad Reza Mohajeri Tehrani,et al. (2011, April 5). Molecular Analysis of the RET Proto-Oncogene Key Exons in Patients with Medullary Thyroid Carcinoma: A Comprehensive Study of the Iranian Population. Thyroid, 1. Retrieved September 1, 2012, from http://online.liebertpub.com/doi/abs/10.10

This publication was one of many that were used only for its mutations, and or one small piece of information. No part, except for the abstract, which contributed to the overall idea of the research report, has been read.

12. Moura, M., Cavaco, B., Pinto, A., & Leite, V. (2011, February 16). High Prevalence of RAS Mutations in RET-Negative Sporadic Medullary Thyroid Carcinomas. JCEM ONLINE, 95, 6.

This paper has shown a study where 64% of the patients that had Sporadic MTC were found to have a BRAF mutation in position 600. This is very unusual as this mutation is considered to be PTC specific. In addition, this publication has given additional context to this research report. Similarly to many other sources, mutations were taken from this publication during the first phase of the project.

13. OMIM Entry - # 162300 - MULTIPLE ENDOCRINE NEOPLASIA, TYPE IIB; MEN2B . (n.d.). OMIM - Online Mendelian Inheritance in Man . Retrieved June 19, 2012, from http://omim.org/entry/162300

This entry was used as the basis of understanding of the MEN 2B syndrome. It talks about the relationship between MTC and diseases such as pheochromocytoma, and about the basics of what MEN 2B syndrome actually is. In addition, this entry talks about what mutations are associated with the syndrome. It was specifically used for comparison with the MEN 2A and FMTC.

14. hybridization, f. i., & (1989), I. e. (n.d.). OMIM Entry - + 164761 - REARRANGED DURING TRANSFECTION PROTOONCOGENE; RET . OMIM - Online Mendelian Inheritance in Man . Retrieved July 19, 2012, from http://omim.org/entry/164761

This entry was used as the basis of understanding MTC, and why the mutations RET gene are found in so many cases of MTC. Unfortunately, as with many other publications, no definitive answer was given. This entry was also used as a guide, to make sure that what I say about mutations in RET gene is factually correct.

15. RT-PCR., & (2003), M. e. (n.d.). OMIM Entry - # 155240 - THYROID CARCINOMA, FAMILIAL MEDULLARY; MTC . OMIM - Online Mendelian Inheritance in Man. Retrieved July 19, 2012, from http://omim.org/entry/155240

This entry was used as the basis of understanding of the FMTC syndrome. It talks about the relationship between MTC and diseases such as pheochromocytoma, and about the basics of what FMTC syndrome actually is. In addition, this entry talks about what mutations are associated with the syndrome. It was specifically used for comparison with the MEN 2B and MEN 2A.

16. Hazard, J., Hawk, W., & Crile, G. (1959, January 1). MEDULLARY (SOLID) CARCINOMA OF THE THYROID—A CLINICOPATHOLOGIC ENTITY. JCEM, 19. Retrieved June 26, 2012, from http://jcem.endojournals.org/content/19/1/152

This publication was the first time MTC was classified. The paper itself was not read by this applicant, however, due to its historic relevance, it was referred to in the first paragraph of the introduction.

17. Cakir, M., & Grossman, A. (2009, May 25). Medullary Thyroid Cancer: Molecular Biology and Novel Molecular Therapies. Neuro Endocrinology, 25.

This publication, alongside many MTC reviews, was used as the guideline for this research report. Many facts, such as information about Sporadic and Hereditary MTC were confirmed by this publication. Similarly to many other sources, mutations were taken from this publication during the first phase of the project.