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Supplemental Information

Genetic Landscape of Somatic Mutations in a Large Cohort of Sporadic Medullary Thyroid Carcinomas Studied by Next-Generation Targeted Sequencing

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SUPPLEMENTAL INFORMATION

No	No in the report	Genetic Variation	VAF (%)
1	6	RET c.2753T>C; p.M918T (s)	43,7
2	11	RET c.2753T>C; p.M918T (s)	40,1
3	14	RET c.2753T>C; p.M918T (s)	32.03
4	15	RET c.2753T>C; p.M918T (s)	11,8
5	22	RET c.2753T>C; p.M918T (s)	42,5
6	23	RET c.2753T>C; p.M918T (s)	50,8
7	43	RET c.2753T>C; p.M918T (s)	92,7
8	50	RET c.2753T>C; p.M918T (s)	44,7
9	51	RET c.2753T>C; p.M918T (s)	28,9
10	53	RET c.2753T>C; p.M918T (s)	38,6
11	62	RET c.2753T>C; p.M918T (s)	62,2
12	63	RET c.2753T>C; p.M918T (s)	41,0
13	85	RET c.2753T>C; p.M918T (s)	44,0
14	89	RET c.2753T>C; p.M918T (s)	45,8
15	90	RET c.2753T>C; p.M918T (s)	55,5
16	92	RET c.2753T>C; p.M918T (s)	27,4
17	93	RET c.2753T>C; p.M918T (s)	48,1
18	94	RET c.2753T>C; p.M918T (s)	59,0
19	96	RET c.2753T>C; p.M918T (s)	35,7
20	101	RET c.2753T>C; p.M918T (s)	44,8
21	105	RET c.2753T>C; p.M918T (s)	20,3
22	110	RET c.2753T>C; p.M918T (s)	42,3
23	118	RET c.2753T>C; p.M918T (s)	17,0
24	121	RET c.2753T>C; p.M918T (s)	34,6
25	123	RET c.2753T>C; p.M918T (s)	39,0
26	125	RET c.2753T>C; p.M918T (s)	37,4
27	127	RET c.2753T>C; p.M918T (s)	18,0
28	131	RET c.2753T>C; p.M918T (s)	46,6
29	134	RET c.2753T>C; p.M918T (s)	41,0
30	135	RET c.2753T>C; p.M918T (s)	28,9
31	136	RET c.2753T>C; p.M918T (s)	38,3
32	139	RET c.2753T>C; p.M918T (s)	41,5
33	142	RET c.2753T>C; p.M918T (s)	37,0
34	170	RET c.2753T>C; p.M918T (s)	36,6
35	185	RET c.2753T>C; p.M918T (s)	46,5
36	191	RET c.2753T>C; p.M918T (s)	42,4
37	199	RET c.2753T>C; p.M918T (s)	29,6
38	203	RET c.2753T>C; p.M918T (s)	39,7
39	204	RET c.2753T>C; p.M918T (s)	64,6
40	205	RET c.2753T>C; p.M918T (s)	22,6
41	211	RET c.2753T>C; p.M918T (s)	33,0
42	238	RET c.2753T>C; p.M918T (s)	40,6
43	247	RET c.2753T>C; p.M918T (s)	43,1
44	248	RET c.2753T>C; p.M918T (s)	46,3
45	249	RET c.2753T>C; p.M918T (s)	19,3
46	257	RET c.2753T>C; p.M918T (s)	4,4
47	258	RET c.2753T>C; p.M918T (s)	19,0
48	262	RET c.2753T>C; p.M918T (s)	38,1
49	303	RET c.2753T>C; p.M918T (s)	39,6
50	305	RET c.2753T>C; p.M918T (s)	27,7
51	313	RET c.2753T>C; p.M918T (s)	29,7
52	314	RET c.2753T>C; p.M918T (s)	44,7
53	316	RET c.2753T>C; p.M918T (s)	31,5
54	317	RET c.2753T>C; p.M918T (s)	32,4
55	88	RET c.2753T>C; p.M918T (s)	19,7
		KRAS c.389C>T; p.A130V (g)	44,9
56	41	RET c.2753T>C; p.M918T (s)	47,3
		RET c.2774A>C; p.D925A (s)	46,7
57	242	RET c.2753T>C; p.M918T (s)	32,2

		RET c.890G>A; p.R297H (s)	11,8
58	196	RET c.2753T>C; p.M918T (s)	12,3
		RET c.2497C>T; p.R833C (s)	25
		RET c.2671T>G; p.S891A (s)	20,35
		MET c.3029C>T; p.T1010I (nv)	18,6
59	39	RET c.2753T>C; p.M918T (s)	3,0
		HRAS c.182A>G; p.Q61R (s)	34,4
60	140	RET c.2753T>C; p.M918T (s)	39,0
		KRAS c.544A>G; p.K182E (nv)	20
61	178	RET c.1900T>C; p.C634R (s)	38,4
62	184	RET c.1900T>C; p.C634R (s)	36,0
63	208	RET c.1900T>C; p.C634R (s)	20,6
64	241	RET c.1900T>C; p.C634R (s)	15,8
65	261	RET c.1900T>C; p.C634R (s)	43,3
66	263	RET c.1900T>C; p.C634R (s)	28,3
67	310	RET c.1900T>C; p.C634R (s)	35,3
68	83	RET c.1902C>G; p.C634W (s)	24,6
69	111	RET c.1902C>G; p.C634W (s)	33,0
70	126	RET c.1902C>G; p.C634W (s)	60,7
71	177	RET c.1902C>G; p.C634W (s)	24,5
72	188	RET c.1902C>G; p.C634W (s)	26,9
73	176	RET c.1902C>G; p.C634W (s)	17,3
		NRAS c.53C>T; p.A18V (s)	7,5
74	253	RET c.1902C>G; p.C634W (s)	11,6
		MET c.3029C>T; p.T1010I (s)	50,1
75	12	RET c.1901G>A; p.C634Y (s)	43,2
76	91	RET c.1901G>A; p.C634Y (s)	37,3
		RET c.644G>T; p.R215L (g)	49,9
77	124	RET c.1901G>T; p.C634F (s)	39,0
78	304	RET c.1900T>A; p.C634S (s)	28,7
79	54	RET c.2694_2705delTGTTTATGAAGA; p.D898_E901del (s)	27,9
80	141	RET c.2694_2705delTGTTTATGAAGA; p.D898_E901del (s)	54,1
81	215	RET c.2694_2705delTGTTTATGAAGA; p.D898_E901del (s)	38,7
82	201	RET c.2694_2705delTGTTTATGAAGA; p.D898_E901del (s)	26,6
		PPM1D c.1405A>G; p.K469E (g)	37,5
83	132	RET c.2694_2705delTGTTTATGAAGA; p.D898_E901del (s)	30,9
		RET c.2710T>C; p.S904P (s)	30,8
84	128	RET c.1908_1909insTGCCGCACG; p.T636_V637delinsCRT (s)	35,4
85	61	RET c.1899_1900delGTinsTG; p.C634G/L633L (s)	43,2
86	186	RET c.1894_1899delGAGCTG; p.E632_L633del (s)	95,2
87	302	RET c.1894_1902delGAGCTGTGC; p.E632_C634del (s)	42,9
89	66	RET c.1894_1904delGAGCTGTGCCG; c.1912_1918delATCGCAG; c.1908G>T; p. E632_A639delinsHR (s)	48,0
88	122	RET c.1886_1891delTGTGCG; p.L629_D631delinsH (s)	38,4
90	119	RET c.2647_2648delGCinsTT; p.A883F (s)	13,8
91	97	RET c.2647_2648delGCinsTT; p.A883F (s)	31,1
92	309	RET c.2647_2648delGCinsTT; p.A883F (s)	14,9
93	27	RET c.1858T>C; p.C620R (s)	14,4
94	100	RET c.1858T>C; p.C620R (s)	55,5
95	169	RET c.1858T>C; p.C620R (s)	41,5
		MET c.3029C>T; p.T1010I (nv)	6,7
96	75	RET c.2671T>G; S891A (s)	47,0
97	82	RET c.2671T>G; S891A (s)	20,8
98	137	RET c.1852T>C; C618R (s)	31,4
99	3	RET c.1852T>G; C618G (nv)	40,5
		TP53 c.847C>T; p.R283C (nv)	48,1
101	175	RET c.1888T>C; C630R (s)	45,1
100	252	RET c.3071C>T; p.S1024F (s)	17,6
102	7	HRAS c.182A>G; p.Q61R (s)	50.7
103	10	HRAS c.182A>G; p.Q61R (s)	28,0
104	17	HRAS c.182A>G; p.Q61R (s)	41,8
105	26	HRAS c.182A>G; p.Q61R (s)	39,8
106	44	HRAS c.182A>G; p.Q61R (s)	26,2
107	47	HRAS c.182A>G; p.Q61R (s)	38,2
108	57	HRAS c.182A>G; p.Q61R (s)	19,0

109	67	HRAS c.182A>G; p.Q61R (s)	41,3
110	73	HRAS c.182A>G; p.Q61R (s)	39,7
111	87	HRAS c.182A>G; p.Q61R (s)	46,0
112	95	HRAS c.182A>G; p.Q61R (s)	41,1
113	112	HRAS c.182A>G; p.Q61R (s)	29,6
114	182	HRAS c.182A>G; p.Q61R (s)	22,0
115	189	HRAS c.182A>G; p.Q61R (s)	16,4
116	207	HRAS c.182A>G; p.Q61R (s)	36,6
117	210	HRAS c.182A>G; p.Q61R (s)	43,8
118	250	HRAS c.182A>G; p.Q61R (s)	7,5
119	315	HRAS c.182A>G; p.Q61R (s)	59,6
120	20	HRAS c.182A>G; p.Q61R (s)	41,7
		MET c.3029C>T; p.T1010I (g)	51,4
121	60	HRAS c.181C>A; p.Q61K (s)	37,4
122	70	HRAS c.181C>A; p.Q61K (s)	25,3
123	99	HRAS c.181C>A; p.Q61K (s)	47,9
124	133	HRAS c.181C>A; p.Q61K (s)	38,7
125	246	HRAS c.181C>A; p.Q61K (s)	31,5
126	307	HRAS c.181C>A; p.Q61K (s)	31,4
127	74	HRAS c.182A>T; p.Q61L (s)	44,9
128	19	HRAS c.37G>C; p.G13R (s)	42,5
129	56	HRAS c.37G>C; p.G13R (s)	36,1
130	65	HRAS c.37G>C; p.G13R (s)	41,4
131	214	HRAS c.37G>C; p.G13R (s)	35,2
132	251	HRAS c.37G>C; p.G13R (s)	23,2
		MET c.3029C>T; p.T1010I (s)	8,3
133	5	KRAS c.34G>C; p.G12R (s)	40,0
134	69	KRAS c.34G>C; p.G12R (s)	42,7
135	71	KRAS c.34G>C; p.G12R (s)	29,0
136	172	KRAS c.34G>C; p.G12R (s)	42,8
137	179	KRAS c.34G>C; p.G12R (s)	42,5
138	190	KRAS c.34G>C; p.G12R (s)	44,2
139	197	KRAS c.34G>C; p.G12R (s)	30,2
140	212	KRAS c.34G>C; p.G12R (s)	24,0
141	244	KRAS c.34G>C; p.G12R (s)	45,2
142	21	KRAS c.182A>G; Q61R (s)	50,3
143	239	KRAS c.183A>T; Q61H (s)	10,5
144	174	KRAS c.437C>T; p.A146V (s)	36,0
145	8	NRAS c.181C>A; p.Q61K (s)	20,6
146	52	TSHR c.1888A>C; p.I630L (s)	31,0
		TP53 c.472C>T; p.R158C (g)	52,8
147	195	EIF1AX c.404G>C; G135A (*)	41,4
148	198	CHK2 c.341G>A; W114* (nv)	10,1

Table S1: Related to Figure 1. List of genetic variations found in 148 sMTC

(**s**) verified somatic; n.v. no detectable by direct sequencing; (*****) validated as somatic in tissue but blood not available for germline validation

TRANSPARENT METHODS

Patient cohort

We studied surgically removed tumoral tissues from 209 sMTC cases: 175/209 (83.7%) were primary tumors, 33/209 (15.8%) were lymph-node metastases and 1/209 (0.5%) showed tumor recurrence. All patients were diagnosed and followed at the Unit of Endocrinology of the Department of Clinical and Experimental Medicine of the University of Pisa. The diagnosis of sMTC was based on the absence of germline *RET* mutations, absence of a familial history of the disease, and negative clinical and laboratory data for the presence of other endocrine neoplasia. Informed consent forms for *RET* genetic screening and other clinical procedures were signed by each of the investigated subjects, and the present study was approved by the Institutional Review Board.

The clinical and pathological data of the patients were collected in a database and were available for correlation with the molecular data. Of 209 sMTC patients, 86 were male and 123 female with a mean age at the diagnosis of 54.4 yrs. [median: 56; range 20-87 yrs.]. According to the AJCC Cancer Staging System, 7th Edition (Edge and Compton, 2010), 63/209 (30.1%) were at stage I, 13/209 (6.2%) at stage II, 30/209 (14.4%) at stage III and 74/209 (35.4%) at stage IV; for 29/209 (13.9%), the data were not available. The mean tumor size was 2.07cm [median: 1.6; range: 0.1-9.5 cm] calculated on 157/209 (75.1%) patients.

According to their clinical status, defined based on both the serum calcitonin levels and imaging results (i.e., neck ultrasound, computed tomography scan and bone scintigraphy), patients were classified into three groups: A) “disease free” patients [n= 85/209 (40.7%)]; B) patients with “persistent disease” only at the biochemical level [n= 24/209 (11.5%)]; C) patients with evidence of metastatic disease and/or dead patients [n=69/209 (33.0%)]. The data on outcome were not available in 31/209 (14.8%) cases. At the time of the present study, the mean follow-up was 84.93 months [median: 65; range 3–324 months].

RET gene somatic mutation analysis by Sanger direct sequencing in exons 10, 11, 13, 14, 15 and

16 and exons 2, 3, 4 of was previously performed on 148 sMTC; moreover, in 65 of these cases we sequenced exons 2, 3, 4 of *HRAS*, *KRAS* and *NRAS* mutations following standard screening procedures in our laboratory (Ciampi et al., 2013; Romei et al., 2011).

Tissue samples were collected at surgery and snap-frozen at -80° when patients were operated in Pisa; for the others, formalin-fixed paraffin-embedded (FFPE) tissues were used. Blood samples were collected in EDTA and were available for most of the cases studied.

Nucleic acid isolation from tumoral tissues

Snap-frozen tumoral tissue was available in 159 cases, while FFPE tissue was available for the other 50 cases.

For most cases, genomic DNA was extracted using the automated method Maxwell16® (Promega, Madison, WI, USA) using the following kits: Maxwell® 16 FFPE Plus LEV Blood DNA Purification kit for frozen tissues and Maxwell® 16 FFPE Plus LEV DNA Purification kit for FFPE tissues. Approximately 40mg of internal tumoral tissue was used in frozen samples, while the tumoral areas with more than 50% of tumoral cells were collected from four 4-µm unstained slides; DNA was finally eluted in 300 µl of DNase and RNase-free water for blood DNA and 50 µl for tissue DNA and quantified using the Qubit 3 fluorometer (Invitrogen, Calsbad, CA, USA) and the Qubit™ dsDNA HS Assay kit.

Ion S5 targeted sequencing

NGS libraries were obtained using a thyroid-specific custom panel designed using the AmpliSeq Designer tool available from Thermo Fisher (https://www.ampliseq.com/). The DNA panel contained 212 couples of primers that can amplify 20.34 kb of the genome including the following regions: *RET* (entire CDS + 5'- and 3'-UTR), *HRAS* (entire CDS), *KRAS* (entire CDS), *NRAS*

(entire CDS), *TP53* (exons 5-9), *GNAS* (exons 8-9), *EIFA1X* (exons 1, 2, 5, 6), *AKT1* (exon 3), *MET* (exon 14), *CHEK2* (exons 4, 5, 7, 12, 14), *BRAF* (exon 15), *CTNNB1* (exon 3), *STK11* (exons 1, 7, 8), *PTEN* (exons 5-8), *PIK3CA* (exons 9, 20), *PPM1D* (exons 5, 6), and*TSHR* (exon 10).

Twenty-five nanograms of tumoral genomic DNA were amplified with specific primers for the above-described panel using the Ion AmpliSeq library kit 2.0 (Life Technologies, Calsbad, CA, USA)following manufacturer's instructions; different samples were barcoded using the Ion Xpress Barcode Adapters kit (Life Technologies, Calsbad, CA, USA). DNA library quantification was performed using the Qubit™ dsDNA HS Assay (Life Technologies), and 100pM dilutions were pooled together. The number of samples pooled together was calculated according to the desired vertical coverage of reads (2000X). Emulsion clonal PCR was performed using the OneTouch (OT2) System (Life Technologies), and enrichment of Ion Sphere Particles (ISP) was performed using the Enrichment System (ES) (Life Technologies, Calsbad, CA, USA). Finally, massively parallel sequencing was performed on a 520 Chip in an Ion S5 deep sequencer (Ion Torrent; Applied Biosystem, Calsbad, CA, USA) following the manufacturer's instructions.

NGS Data analysis

Raw sequencing data analysis was analyzed using Torrent Suite Software v.5.6 (Life Technologies) and included alignment to the hg19 human reference genome, quality score assignment, variant calling and coverage analysis. The data were further analyzed by Ion Reporter v.5.6 (https://ionreporter.thermofisher.com) with a bioinformatics workflow that calculated the Phred Quality score and performed annotation to dbSNP, COSMIC, and ExAC databases; data were finally filtered to several *in silico* prediction tools of pathogenicity (PhyloP, SIFT, Grantham, PolyPhen and FATHMM).

The system could evaluate the variant allele frequency (VAF) of the mutated allele within the sample. The VAF value for the heterozygous germline variations was 50% while, for somatic variations, it was variable depending on the abundance of the tumor cells with respect to normal

cells in the analyzed sample, as well as on the heterogeneity of mutations in tumoral cells (Li et al., 2017). We arbitrarily set the clinical sensitivity of VAF $\geq$ 10% for variations already known to be drivers (i.e., *RET, HRAS, KRAS*); clinical sensitivity was lowered to VAF $\geq$ 5% for putative additional mutations as previously suggested (Nikiforov et al., 2014). Nevertheless, driver alterations in typical hotspots (such as *RET* M918T mutation) with a lower VAF value were considered positive if validated by Sanger direct sequencing.

With a few exceptions, all variants detected were validated in the same tissue DNA by direct sequencing using specific conditions and primers, while novel and unconventional variations were tested on blood genomic DNA to assess their somatic or germline origin.

TERT promoter mutation analysis

Direct sequencing analysis of the *TERT* gene promoter hotspot mutations C228T and C250T was performed on all cases studied. Amplification of DNA obtained from frozen samples was performed using F: 5'-CTGGCGTCCCTGCACCCTGG-3' and R: 5'-ACGAACGTGGCCAGCGGCAG-3' as previously described (Romei et al., 2018), while oligos for FFPE sample analysis were newly designed: F: 5'-CCCTTCACCTTCCAGCTCC-3', R: 5'-CAGCGCTGCCTGAAACTC-3'. Sanger direct sequencing analysis was performed using the AbiPrism 3130xl Genetic Analyzer (Applied Biosystems, Calsbad, CA, USA).

Whole exome sequencing

Six sMTC, 4 negative at the targeted sequencing analysis for any mutation and 2 positive only for *RET* somatic mutation were further analyzed by whole exome sequencing (WES) through an external service (IGA Technology Services Srl, Udine, Italy) to look for any other possible gene alteration not detected with our gene mutation panel.

Statistical analysis

Statistical analysis and graphs were generated using Prism GraphPad (version 8) (https://www.graphpad.com/). The different prevalence values of categorical data within the different mutational status were analyzed by Chi-squared test. The differences in the outcome and mutational status categories were evaluated by 1-way ANOVA and unpaired Student's t-test with Welch's correction in the case of significant differences between variances. The correlation between the VAF and tumor size was evaluated by a linear regression curve, while differences in survival between *RET*- and *RAS*-mutated sMTC cases were analyzed by Kaplan-Meier curves with the log-rank test. Differences were considered statistically significant when the *P* value was less than 0.05.

SUPPLEMENTAL REFERENCES

Ciampi, R., Mian, C., Fugazzola, L., Cosci, B., Romei, C., Barollo, S., Cirello, V., Bottici, V., Marconcini, G., Rosa, P.M., Borrello, M.G., Basolo, F., Ugolini, C., Materazzi, G., Pinchera, A., Elisei, R., 2013. Evidence of a low prevalence of RAS mutations in a large medullary thyroid cancer series. Thyroid 23, 50–7.

Edge, S.B., Compton, C.C., 2010. The american joint committee on cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. Ann. Surg. Oncol. https://doi.org/10.1245/s10434-010-0985-4

Li, M.M., Datto, M., Duncavage, E.J., Kulkarni, S., Lindeman, N.I., Roy, S., Tsimberidou, A.M., Vnencak-Jones, C.L., Wolff, D.J., Younes, A., Nikiforova, M.N., 2017. Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer. J. Mol. Diagnostics. https://doi.org/10.1016/j.jmoldx.2016.10.002

Nikiforov, Y.E., Carty, S.E., Chiosea, S.I., Coyne, C., Duvvuri, U., Ferris, R.L., Gooding, W.E., Hodak, S.P., LeBeau, S.O., Ohori, N.P., Seethala, R.R., Tublin, M.E., Yip, L., Nikiforova, M.N., 2014. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by thyroseq V2 next-generation sequencing assay. Cancer. https://doi.org/10.1002/cncr.29038

Romei, C., Cosci, B., Renzini, G., Bottici, V., Molinaro, E., Agate, L., Passannanti, P., Viola, D., Biagini, A., Basolo, F., Ugolini, C., Materazzi, G., Pinchera, A., Vitti, P., Elisei, R., 2011. RET genetic screening of sporadic medullary thyroid cancer (MTC) allows the preclinical diagnosis of unsuspected gene carriers and the identification of a relevant percentage of hidden familial MTC (FMTC). Clin Endocrinol 74, 241–247.

Romei, C., Tacito, A., Molinaro, E., Piaggi, P., Cappagli, V., Pieruzzi, L., Matrone, A., Viola, D., Agate, L., Torregrossa, L., Ugolini, C., Basolo, F., De Napoli, L., Curcio, M., Ciampi, R., Materazzi, G., Vitti, P., Elisei, R., 2018. Clinical, pathological and genetic features of anaplastic and poorly differentiated thyroid cancer: A single institute experience. Oncol. Lett. https://doi.org/10.3892/ol.2018.8470