Single nucleotide polymorphisms act as modifiers and correlate with the development of medullary and simultaneous medullary/papillary thyroid carcinomas in 2 large, non-related families with the RET V804M proto-oncogene mutation

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Background. *Single nucleotide polymorphisms (SNPs) may function as modifiers of the RET proto-oncogene, resulting in the expression of medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma (PTC). We present 2 non-related Italian-American families (Family 1,* n *= 107; Family 2,* n *= 31) with the RET V804M mutation. We have correlated the presence of specific SNPs and the rare RET V804M mutation to MTC, C-cell hyperplasia (CCH), and PTC.*
Methods. *Sequencing was performed on exons 10, 11, and 13-16 of the RET proto-oncogene. The presence of MTC, CCH, and PTC were correlated to specific SNPs.*
Results. *In both families, 3 SNPs in exon 11 (G691S), exon 13 (L769L), and exon 15 (S904S) were detected in 100% of patients with overt MTC. The SNP L769L was present in all patients including patients with PTC, MTC, and CCH.*
Conclusion. *SNP analysis revealed a similar pattern between the 2 families. SNPs in exon 11 (G691S) and exon 15 (S904S) appear to influence the development of MTC. A SNP in exon 13 (L769L) may serve as a modifier in the development of simultaneous MTC and PTC, as well as presentation of MTC, in patients with the RET V804M mutation. (Surgery 2010;148:1274-81.)*

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MEDULLARY THYROID CARCINOMA (MTC) is a highly aggressive malignant neoplasm of the thyroid gland that arises from the thyroid C cell and occurs either sporadically or as a part of the inherited autosomal dominant multiple endocrine neoplasia (MEN) type 2A (MEN 2A), and MEN type 2B (MEN 2B), and familial medullary thyroid carcinoma (FMTC) syndromes. Classic MEN 2A syndrome consists of MTC in up to 90% of the patients, pheochromocytoma in 57%, and primary hyperparathyroidism (PHPT) in 15% to 30% of patients. MEN 2B consists of MTC, pheochromocytoma, a marfanoid habitus, and ganglioneuromas of the intestinal tract mucosa.

MEN and FMTC syndromes develop as result of a missense mutation in the REarranged during Transfection (RET) proto-oncogene, which encodes for a tyrosine kinase receptor. This mutation results in the development of C-cell hyperplasia (CCH), which then progresses to invasive MTC.[1,2] RET mutations were classified based on risk for

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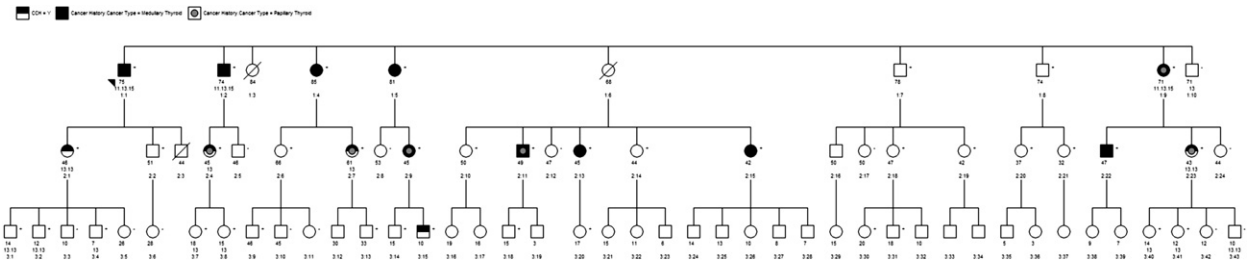

Fig 1. Pedigree of Family 1. Females are represented with circles; males are represented with squares. Family members tested and found to carry the REarranged during Transfection (RET) V804M mutation are indicated with a plus sign (+) in the upper right-hand corner of their corresponding symbol; those members who tested negative are represented with a negative sign (–). The absence of a symbol in the same location indicates that the individual has not been tested to date. The symbols of members affected with medullary thyroid carcinoma (MTC) are solid black (circle or square, depending on sex); if they have C-cell hyperplasia (CCH) the upper half of their symbols are solid black. Primary hyperparathyroidism (PTC) is represented with a gray dot in the center of the symbol. A hash mark (/) through a symbol indicates the person is deceased. The ages of the family members are located immediately below their symbol. The second number below their symbol represents the presence of specific SNPs: 11 = G691S, 13 = L769L, and 15 = S904S. The third number below their symbol represents the generation: person number.

aggressive MTC at the Seventh International Workshop on MEN.[3] More recently, the American Thyroid Association (ATA) stratified RET mutations into 1 of 4 risk levels (A–D), in which level D mutations carry the greatest risk.[2] The RET mutation in exon 13 codon 804 that leads to substitution of normal valine with methionine (V804M) was first described by Fink et al in 1996[4] and is rated an ATA level A mutation (lowest risk).

Several reports,[1,2] however, have shown that there is phenotypic variability in patients who carry the V804M mutation. We recently described a family with a variable presentation of MTC ranging from CCH in a 10-year-old, third-generation patient to late presentation of aggressive MTC in several first-generation patients. This family was also noted to have a high rate of PTC in first- and second-generation patients who underwent operation for MTC (6 of 15 operated patients).[5]

Papillary thyroid carcinoma (PTC) is the most prevalent type of thyroid cancer and arises from the thyroid follicular cells. Overall, PTC is less aggressive than MTC and has not been reported as part of the MEN syndromes, although 1 series[6] reported a 9.1% frequency of PTC in patients with germline RET mutations in exons 13 and 14. PTC has been associated with several somatic mutations including RAS, BRAF, MET, TSH-R, Gsa, and p53.[7] PTC is also associated with radiation-induced DNA double-strand breaks, which cause RET/PTC gene fusions.[7]

Single nucleotide polymorphisms (SNPs) are allelic variants of a gene that occur when 1 nucleotide is replaced without alteration of functional activity of the encoded protein.[8] SNPs have been reported to function as genetic modifiers of RET proto-oncogene mutations, resulting in the expression of MTC and PTC.[9-17] Specifically, SNP G691S in exon 11 and SNP S904S in exon 15 have been described in association with MTC in patients with germline RET proto-oncogene mutations.[8,11,13,18] SNP L769L in exon 13 has been described in association with both MTC and PTC.[7,15,19]

We have recently reported a high rate (40%) of simultaneous MTC and PTC, associated with PHPT and absence of pheochromocytoma, in a large family with the RET V804M mutation (Fig 1).[5] We now present a second, nonrelated RET V804M family, with simultaneous development of MTC and PTC (Fig 2). We detected 3 SNPs that were common in both families and have correlated them to the age of onset and development of MTC, CCH, and PTC in both families.

PATIENTS AND METHODS

Patients. Two nonrelated Italian-American families with 107 members (Family 1) and 31 members (Family 2) with the RET V804M mutation were retrospectively evaluated for presence of MTC, CCH and PTC. Operative records, pathology reports, blood calcitonin levels, and RET testing results analyzed under Institutional Review Board–approved protocol. All patients who were operated on for symptoms (elevated calcitonin level, presence of thyroid nodules) and diagnosed with MTC or CCH and PTC were studied. DNA results were further evaluated to identify the presences of SNPs.

Genetic testing. Standardized blood testing for the RET proto-oncogene mutations and SNPs were performed at the Mayo Clinic's Department of

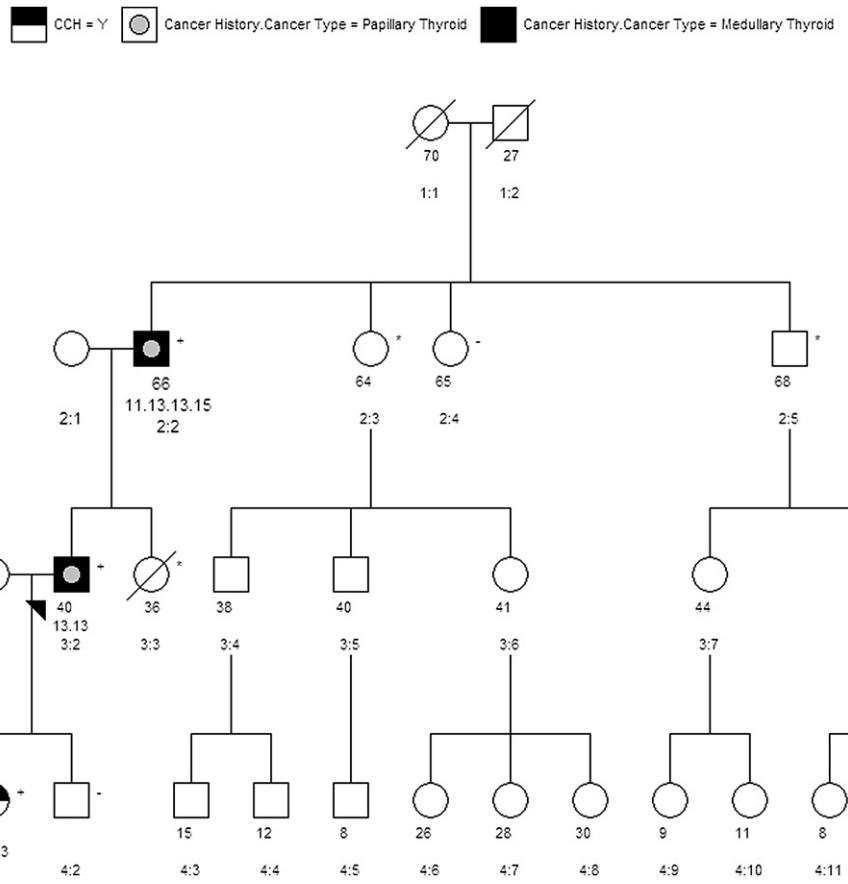

Fig 2. Pedigree of Family 2. Females are represented with circles; males are represented with squares. Family members tested and found to carry the REarranged during Transfection (RET) V804M mutation are indicated with a plus sign (+) in the upper right-hand corner of their corresponding symbol; those members who tested negative are represented with a negative sign (–). The absence of a symbol in the same location indicates that the individual has not been tested to date. The symbols of members affected with medullary thyroid carcinoma (MTC) are solid black (circle or square, depending on sex); if they have C-cell hyperplasia (CCH) the upper half of their symbols are solid black. Primary hyperparathyroidism (PTC) is represented with a gray dot in the center of the symbol. A hash mark (/) through a symbol indicates the person is deceased. The ages of the family members are located immediately below their symbol. The second number below their symbol represents the presence of specific SNPs: 11 = G691S, 13 = L769L, and 15 = S904S. The third number below their symbol represents the generation: person number.

Laboratory Medicine and Pathology (Rochester, MN), Quest Diagnostics (San Juan Capistrano, CA), or Laboratory Corporation of America (Raritian, NJ). Sequencing was completed on exons 10, 11, 13, 14, 15, and 16 of the RET proto-oncogene through amplification of genomic DNA using polymerase chain reaction followed by nucleotide sequence analysis on an automated capillary DNA sequencer. Sequence analysis allows for the identification of mutations and SNPs in all exons studied. A pedigree was constructed for both families and analyzed for haplotypes (Figs 1 and 2). Genetic counseling was provided to select members of both families treated in our facilities.

Operative pathology. All operative specimens were processed using standardized methods. Conventional hematoxylin and eosin and immunohistochemical staining of thyroid tissues for thyroglobulin and calcitonin were performed. The diagnoses of MTC and PTC were based on World Health Organization standards.

Evaluation of SNPs and statistical analysis. Categorical variables were analyzed using the Fisher exact test. Continuous variables were analyzed using the Wilcoxon rank sum test. The reported *P* values are two-sided.

RESULTS

A total of 43 patients were found to be positive for the RET V804M proto-oncogene mutation: 40 of the 107 members in Family 1 and 3 of the 31 members in Family 2. The variability in

RET-positive patients between the 2 families is because, to date, we have obtained the RET mutation status from only 5 members in the second family, 3 of whom were positive. The rest of the members in Family 2 have not been tested yet.

Of the 107 members in Family 1, we obtained RET mutation status from 79 with the following results: 40 members were positive for the RET mutation, 9 tested negative for the RET mutation, 30 members who were offspring of RET-negative patients were not tested, and 28 members have not been tested yet for different reasons (some of them refused testing).

In calculating the incidence of mutation carriers in those individuals who were tested in Family 1 (40/79) and Family 2 (3/5), the percentages were similar. We compared the 2 families because both show the cosegregation of the V804M mutation with SNPs over 3 generations. It is remarkable that Family 1 and Family 2 reveal the exact same SNPs and segregation of SNPs from the first to the second and then to the third generation.

Of the 40 RET-positive patients in Family 1, a total of 16 underwent total thyroidectomy. The sequencing data of 14 RET-positive and 5 RET-negative family members were evaluated for the presence of SNPs (Table I). Thyroidectomy was performed in 8 of 14 RET-positive patients, and their sequencing data were evaluated for the presence of SNPs. The remaining 6 of these 14 RET-positive patients were also tested for SNPs; they had no clinical evidence of MTC and, to date, have not undergone thyroidectomy. The 5 RET-negative patients tested for SNPs included 1 first-generation patient, 3 second-generation patients, and 1 third-generation patient in Family 1. Of the 3 members in Family 2 (n = 31) who were RET V804M–positive, 1 had MTC and PTC, 1 had CCH with micro-MTC (0.1 cm) and PTC, and 1 had CCH. All 3 were tested for SNPs.

A total of 3 SNPs were detected in both families: G691S in exon 11, L769L in exon 13, and S904S in exon 15. All 3 SNPs were present in 100% of patients with overt MTC in the first generation. At least 1 copy of a SNP in exon 13 (L769L) was present in all RET-positive and RET-negative patients. SNPs in exon 11 (G691S) and exon 15 (S904S) were present in patients with overt MTC and absent in patients with micro-MTC (0.1 cm) or CCH. We also detected a S836S SNP in 1 first-generation, RET-negative member of Family 1, and 2 of his 3 RET-negative children.

Lastly, we identified an intronic RET polymorphism (intron 14, IVS14(-24)G>A) in 2 RET-positive, third-generation members of Family 1, both without evidence of MTC (one had thyroidectomy without disease, another had a normal calcitonin level). When we assessed the association between the SNPs and pathology results in patients with the V804M mutation, the presence of G691S was associated with MTC (P = .0004) (Table II). All patients with G691S have MTC, and all patients without G691S do not have MTC. The same association was found for SNP S904S.

DISCUSSION

MTC is described in up to 90% of the patients with MEN 2A syndrome. This percentage varies depending on the specific RET mutation.[1,2] Phenotypic variations in the clinical presentation of MEN 2A are common within families with the same RET mutation. There are also variations in the age of onset of MTC and development of PHPT or pheochromocytoma.[20] RET mutations in codon 804 are categorized by the ATA as low-risk level A mutations and characterized by a later onset of MTC and less aggressive behavior. Conflicting reports,[21,22] however, have described the development of CCH in these patients as early as the age of 5 years and metastatic MTC by the age of 6 years. Consistent with these findings, we identified a 10-year-old with CCH in Family 1.[5]

We believe that the variability of MTC in patients with the RET V804M mutation may be attributed to SNPs. SNPs are thought to act as modifiers in phenotypic expression of the disease.[9-17] Specifically, SNP G691S in exon 11 and SNP S904S in exon 15 are believed to be responsible for the development of MTC in patients with germline RET proto-oncogene mutations.[8,11,13,18] It also has been reported that SNPs G691S and S904S appear to influence the age of onset of MTC in patients with MEN 2A syndrome. Several reports have shown that patients homozygous for these polymorphisms were, on average, diagnosed with MTC 10 years earlier than patients with MEN 2A bearing heterozygous or wild-type haplotype.[11,12]

The RET V804M mutation is rare, and studies examining an association of SNPs with this mutation are limited. We are aware of only 1 study by Magalhães et al[19] describing an L769L polymorphism associated with the RET V804M mutation. In their study, the absence of the L769L polymorphism resulted in the late presentation of MTC in a 60-year-old patient, while her daughter, who had 1 copy of the L769L polymorphism, developed MTC at the age of 32 years.[19] These data suggested that the L769L polymorphism may contribute to an earlier development of MTC in patients with

Table I. Single nucleotide polymorphisms (SNPs) analysis in correlation to pathology results in 2 families

No.	Patients (generation/age (y)/sex)	Pathology	Calcitonin level (pg/mL)	*RET* exon 14 2410 G>A V804M	*SNP* exon 11 c. 2071 G>A G691S	*SNP* exon 13 c. 2307 G>T L769L	*SNP* exon 15 c. 2712 C>G S904S	*SNP* exon 13 c.2508 G>T S836S	*SNP intron 14* IVS14(-24) G>A
Family 1									
1	I/75/M	MTC	670	+	+	+	+		
2	I/74/M	MTC	33	+	+	+	+		
3	I/71/F	MTC, PTC	400	+	+	+	+		
4	I/71/M	N/A	N/A	–		+		+	
5	II/46/F	CCH	2	+		++			
6	II/45/F	CCH, PTC, PHPT	10	+		+			
7	II/61/F	CCH, PTC	23	+		+			
8	II/43/F	CCH, PTC, PHPT	2	+		++			
9	II/42/F	N/A	N/A	–		++			
10	II/44/F	N/A	N/A	–		+		+	
11	II/41/F	N/A	N/A	–		+		+	
12	III/14/F	N/A	2	+		+			+
13	III/12/F	Normal	4.8	+		+			+
14	III/14/M	N/A	2	+		++			
15	III/12/M	N/A	9	+		++			
16	III/7/M	N/A	8	+		+			
17	III/15/F	N/A	2	+		+			
18	III/18/F	N/A	5	+		+			
19	III/10/M	N/A	N/A	–		++			
Family 2									
1	I/66/M	MTC, PTC	7	+	+	++	+		
2	II/40/M	CCH, PTC, 0.1 cm MTC	4	+		++			
3	III/15/F	CCH	2	+		++			

Age, age at diagnosis; *RET,* REarranged during Transfection; *I,* first generation; *II,* second generation; *III,* third generation; *M,* male; *F,* female; *MTC,* medullary thyroid carcinoma; *PTC,* papillary thyroid carcinoma; *PHPT,* primary hyperparathyroidism; *N/A,* not available (no surgery performed/no labs completed); *CCH,* C-cell hyperplasia; +, heterozygous; ++, homozygous; negative symbol - no mutation detected; *empty cell,* no SNP detected.

the RET V804M mutation. We found the L769L polymorphism was present in a patient who developed CCH at the age of 10 years, which is consistent with these findings. We also believe that the SNP L769L may be in linkage disequilibrium with V804M in the 2 families in our study through haplotype analysis of the pedigrees; however, at least 1 copy of the SNP L769L was present in all family members that we evaluated, both those members who were RET positive and RET negative.

L769L has been also reported in association with the development of PTC.[15,16] Some studies have demonstrated a "weak association" of PTC with the presence of the SNP L769L in exon 13.[7,19] We found a high incidence of PTC in our first series.[5] Lesueur et al[15] observed that some SNPs within the RET proto-oncogene represent low penetrant alleles for the PTC phenotype, and found the strongest association of PTC with SNPs L769L and G135A. The development of PTC is complex, and multiple mechanisms are involved in tumorgenesis.[7] A combination of the RET V804M mutation and the SNP L769L might trigger such an event or lead to a predisposition for PTC.

We previously reported a large RET V804M family with a high rate (40%) of simultaneous MTC and PTC associated with PHPT and the absence of pheochromocytoma.[5] We now present a second, non-related family with the RET V804M mutation. In this study, we evaluated SNPs in 8 of 16 members in 1 family, all of whom underwent operation as well as 6 members of this family's third generation, who were not operated. In the second family included in this study, SNPs were

Table II. Impact of SNP presence on the positive pathology results (only V804M + subjects: 17 subjects)

SNP	Pathology [odds ratio (P value)*]			Age at Diagnosis [years (P value)]
	MTC	CCH	PTC	
G691S	Infinite (0.0004)	Infinite (0.10)	2.1 (0.58)	71.5 vs. 26.3 (0.004)
L769L**	0.41 (0.60)	2.4 (0.62)	1.5 (1.00)	33.7 vs. 39.2 (0.70)
S904S	Infinite (0.0004)	Infinite (0.10)	2.1 (0.58)	71.5 vs. 26.3 (0.004)

*The odds ratio of "Infinite" is the result of 0 count in any of the categories. All of those "Infinite" results represent significant association between the SNP presence and the positive pathology results.
**Compare "++" with "+".

evaluated in all 3 members who underwent operation. In both of these non-related families with the RET V804M mutation, we found 3 SNPs: G691S in exon 11, L769L in exon 13, and SNP S904S in exon 15. An interesting finding was that all family members who developed MTC were in the first generation of both families and all shared the exact same SNPs (G691S, L769L, and S904S). Most of these persons in the first generation had metastatic disease versus members from the second and third generations of both families who only had CCH.

Previous studies have found that G691S and S904S are in linkage disequilibrium and cosegregate 100% of the time.[8,11,12] SNP S904S does not lead to an amino acid alteration but appears to be linked, due to a founder effect, with G691S. G691S is a missense mutation and does have genetic modifying potential shown through protein studies.[23] Additional functional studies will need to be performed on the G691S to further substantiate these findings.

In our series, no single RET-positive patient with only 1 SNP L769L developed overt MTC; however, 1 patient had micro-MTC, the rest had CCH, and some also had PTC. We found a high correlation between the development of overt MTC and the presence of all 3 SNPs in patients with the RET V804M mutation, suggesting that these SNPs may influence MTC tumorgenesis. Similar findings were described by Robledo et al[11] and reviewed by de Groot et al,[7] who hypothesized that G691S and S904S modify RET and affect the age of MEN 2A presentation. Another study[24] did not support these findings and found no impact of SNPs on the development of CCH or MTC, especially in patients with sporadic MTC. This finding indicates that the presence of SNPs alone may not necessarily influence the development of MTC, but the presence of a germline RET mutation with a specific SNP may lead to MTC.

Future studies are needed to evaluate additional somatic mutations and other RET variations that could influence the development of PTC in patients with the RET V804M mutation. We realize that our sample size is limited and hope to collect data from additional families with the V804M RET mutation through collaboration with other researchers. SNP studies of the RET proto-oncogene have revealed conflicting data. It is speculated that the discrepancies are the result of small sample sizes when looking at gene variations present in high frequencies but with low penetrance.[25]

In conclusion, we present 2 non-related families with the RET V804M mutation that do not present in a "low-risk" manner and exhibit a high incidence of simultaneous MTC and PTC. DNA analysis revealed a SNP pattern that correlated with phenotypic expression. SNPs in exon 11 (G691S) and exon 15 (S904S) were found in 100% of patients with overt MTC. A SNP in exon 13 (L769L) was present in all RET-positive and RET-negative patients that were tested. Based on our findings, the potential protein-modifying quality of the G691S missense mutation, and a survey of the published literature on SNPs found in the RET gene, we believe that these 3 SNPs alone, or in conjunction with the RET V804M mutation, may serve as modifiers in the development of MTC and simultaneous MTC and PTC in patients with the RET V804M mutation.

Determining the role of SNPs as potential genetic modifiers in the development of MTC and PTC in patients with germline RET V804M proto-oncogene mutation will help to predict phenotypic expression and the timing of the onset of MTC and/or PTC. This information will impact the management of patients, including the timing and extent of surgery in patients with the RET V804M mutation. Additional large genome-wide association studies are needed to support these findings.

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DISCUSSION

Dr Martha A. Zeiger (Baltimore, MD): Very nice study, interesting question, and it's wonderful that you are addressing it. My question is, what is your control group? In other words, you may be just looking at something that's a hereditary aspect, but has nothing to do with the disease process.

Two, could you reiterate what numbers have a particular SNP and with an associated phenotype versus those without the phenotype?

Dr Alexander L. Shifrin (Neptune, NJ): I think the first question is very difficult to answer. We don't have controls; we only have two large families. They were described within the last two years and we go as we detected them. We only can compare two families between themselves. We can compare within the family, the RET positive with the RET negative. And we can compare, within the family, between those who developed carcinoma versus those who did not have measured papillary thyroid carcinoma.

The second question was about specific SNPs.

We looked at two independent families, not related to each other, in two different regions. The SNPs which were detected were detected in a common region in the RET proto-oncogene , the same region which is analyzed in the major laboratories that perfrom RET mutation testing. All the SNPs which were detected in both family members were reported on this table. It's on exon 11 (G691S), exon 13 (L769L), and exon 15 (S904S).

Those are all SNPs on this particular person and this table. So the first person had medullary thyroid carcinoma. We detected these three SNPs. And patients without medullary thyroid carcinoma had only one SNP.

We also detected other SNPs, which were reported in the literature in correlation with medullary thyroid carcinoma, but we did not find this correlation between 836 and the two introns which we found. I think only one paper reported an association between these introns and the development ofmedullary thyroid carcinoma. We didn't find this correlation. But we did find a correlation between those three exons: 15, 13, and 11.

And what was interesting, when we got the second family, the second family had the same association. They didn't have more SNPs than reported in this table. This is all that we found. It's not that we were specifically looking for those SNPs. No, we were specifically looking in exons 10, 11, 13, and 16, as the most common exons reported by most of the laboratories. I think only three or four laboratories in the whole country reported whole RET proto-oncogene sequencing of all 21 exons, and several in Europe.

Unfortunately, insurance companies are not going to cover it if it's outside the States. We are trying to work on that and determine if we can get more data, looking at the entire RET proto-oncogene, looking for the entire distribution of the SNPs. So far, we looked only in these exons. So in this table, for example, this person had only two SNPs.

Dr Tobias Carling (New Haven, CT): Thanks to the fact that you have such a large family, you can look at some of these issues. Just a comment to the previous question, I think if you used the International HapMap Consortium Web site, you can actually detect what the overall general frequency of these SNPs are in the general population. And that way, you will at least have some sort of control population, even though this is a specific family.

But my question is whether you have done any functional analysis of these SNPs that you did detect and whether they altered the amino acid sequence, or if they are silent but still have some sort of functional alteration of the gene?

Dr Alexander L. Shifrin (Neptune, NJ): That's a very good question and I completely agree with your first statement. There was no specific functional analysis, but they did not alter the protein. Also, 691 was reported as more commonly associated with mutation. And maybe, in the future, it will be reported as changing the protein; but so far, no.

Dr Jeffrey F. Moley (St. Louis, MO): I congratulate you on a very nice study. These studies are very difficult to do. These patients with these 804 mutations, it's a very interesting spectrum of disease that they have. And it's very important to do these kinds of experiments to figure out what the modifier genes and polymorphisms are that can help direct therapy in the era of personalized medicine and preventative surgery.

My question has to do with the problem that all of us who do this kind of thing are encountering now, which is the burdensome regulatory requirements that one needs to wade through in order to complete these studies: IRB approval, consenting patients, dealing with issues of children and minors.

I was wondering how you all did such a nice job of getting these data and how you delegated individuals to do your regulatory requirements.

Dr Alexander L. Shifrin (Neptune, NJ): It was a struggle for us to get all the data because of those issues. We have an IRB approved protocol in Jersey Shore Hospital, which covers both families. And we have a collaborative project with Pittsburgh here, with Michael Stang and Jennifer Rabaglia, who was here before.

One of the genetic counselors works with the family. She is in close contact with both families and the genetic counselors from the other places, as well as the counselors who are involved in these studies.

We obtained a consent form for each single test. The tests were not released to us without consent from the person from this family. For example, if one SNP is already detected, we had to go here in Pittsburgh or in New Jersey to get a family member and ask them to sign the consent and send it to the laboratory.