RAS/*BRAF* mutational status in familial non-medullary thyroid carcinomas: A retrospective study

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Abstract. There are contrasting views on whether familial non-medullary thyroid carcinomas (FNMTCs) are characterized by aggressive behavior, and limited evidence exists on the prognostic value of *BRAF* and *RAS* mutations in these tumors. Thus, in the present study, clinicopathological features were analyzed in 386 non-medullary thyroid carcinomas (NMTCs), subdivided in 82 familial and 304 sporadic cases. Furthermore, the *RAS* and *BRAF* mutational statuses were investigated in a subgroup of 34 FNMTCs to address their clinical and biological significance. The results demonstrated that, compared with sporadic NMTCs, FNMTCs are characterized by significantly higher rates of multicentricity and bilaterality and are more frequently associated with chronic autoimmune thyroiditis. Notably, a statistically significant difference in the rates of multicentricity was observed by subgrouping familial tumors according to the number of relatives involved; those with ≥3 affected relatives were more likely to be multicentric. Furthermore, the FNMTC cohort exhibited higher rates of tumors >4 cm in size with extrathyroidal or lymph node involvement. However, no significant difference was observed. Similarly, no differences were observed with respect to the age of onset or the patient outcome. The mutational profiling exhibited a rate of 58.8% for *BRAF* V600E mutations in familial tumors, which is at the upper limit of the mutational frequency observed in historical series of sporadic thyroid cancer. A high rate of *NRAS* mutations (17.6%) was also observed, mostly in the follicular variant histotype. Notably, compared with *BRAF*/*RAS*-wild type FNMTCs, the familial carcinomas bearing *BRAF* or *NRAS* mutations exhibited slightly higher rates of bilaterality and multicentricity, in addition to increased frequency of locally advanced stage or lymph node involvement. The present data support the theory that FNMTCs are characterized by clinicopathological features that resemble a more aggressive phenotype and suggest that *RAS*/*BRAF* mutational analysis deserves to be further evaluated as a tool for the identification of FNMTCs with a potentially unfavorable prognosis.

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Introduction

Approximately 95% of thyroid malignancies develop from thyroid epithelial cells and are classified as non-medullary thyroid carcinoma (NMTC). Of these, 3-6% are estimated to have a familial origin, being thus identified as hereditary non-medullary thyroid cancers (1). Hereditary thyroid tumors can be associated with other established hereditary syndromes or, more frequently, are non-syndromic familial NMTCs (FNMTCs) (1). While the clinical features and genes involved in thyroid tumors associated with hereditary syndromes are well characterized, FNMTCs represent a condition with uncertain clinical and molecular characteristics (1).

A major challenge in the clinical management of FNMTCs is whether they are characterized by a more aggressive biological and clinical behavior compared with sporadic NMTCs, as suggested by several studies (2-7). These studies describe various prognostic features indicative of the biological aggressiveness of FNMTCs, including earlier age of onset, higher rate of multifocality, increased lymph node involvement,

extrathyroidal invasion, higher incidence of tumor recurrence and poor overall survival compared with sporadic NMTCs (2-7). By contrast, other studies have failed to identify major differences in the pathological and clinical behavior between FNMTCs and NMTCs (8-12). This controversy likely indicates the biological heterogeneity of tumors diagnosed as FNMTCs, since they are defined based on the presence of a well-differentiated thyroid cancer of follicular cell origin in ≥2 first degree relatives, in the absence of other predisposing hereditary or environmental causes (1). Therefore, certain studies have indicated that the diagnostic criteria for familial origin must be more stringent, since families with 2 affected members are likely to be enriched by the casual occurrence of 2 sporadic thyroid tumors in 1 family (13,14). Based on this premise and on the observation that genetic profiling may be useful in indicating those tumors with more aggressive biological behavior and poor clinical outcome within the heterogeneous cohort of human NMTCs (15), *RAS* and *BRAF* mutations were investigated in a series of FNMTCs to address their clinical and biological significance.

Materials and methods

Patients. A cohort of 82 patients with FNMTCs was selected from 2 independent Italian Academic Institutions, including the Endocrinology Unit, Department of Medical and Surgical Sciences, University of Foggia (Foggia, Italy) and the Section of Internal Medicine, Endocrinology, Andrology and Metabolic Diseases, University of Bari Aldo Moro (Bari, Italy). FNMTCs were selected based on the presence of a well-differentiated thyroid cancer of follicular cell origin in ≥2 first degree relatives (1). Anamnestic features were carefully reviewed to exclude known hereditary syndromes, and the presence of other predisposing hereditary or environmental causes in the families. Within the cohort of 82 patients with FNMTCs, DNA samples for mutational analysis were obtained from a subgroup of 34 patients. Paraffin-embedded specimens from these 34 thyroid cancer and peritumoral non-infiltrated normal thyroid glands were collected from the Pathology Unit, Department of Clinical and Experimental Medicine, University of Foggia; the Section of Pathology, Department of Emergency and Organ Transplantation, University of Bari Aldo Moro; and the Pathology Unit, IRCCS, Referral Cancer Center of Basilicata, Rionero in Vulture (Potenza, Italy) from patients who had undergone surgical resection of the primary tumor between 1987 and 2013. Paraffin-embedded sections were analyzed for the percentage of cancer cells prior to performing separate manual microdissection of the tumor and normal thyroid peritumoral areas. Written informed consent for the use of the biological specimens for investigational procedures was obtained from all patients prior to the study. As the control, a group of 304 patients with sporadic NMTCs who had undergone surgical removal of the thyroid gland between 1984 and 2013 at the University of Foggia were selected. Anamnestic features were carefully collected from each patient/family to exclude the presence of relatives with thyroid carcinoma. Tumors were grouped according to the TNM classification of malignant tumors (16). Presence of thyroiditis was defined based on positivity for anti-thyroglobulin and thyroperoxidase antibodies and ultrasound imaging. Anti-thyroglobulin and thyroperoxidase were evaluated, respectively, using the Anti-Thyroglobulin Human ELISA Kit (catalog no. ab178631) and the Anti-Thyroid Peroxidase Human ELISA Kit (catalog no. ab178632; Abcam, Cambridge, MA, USA). Clinicopathological characteristics of the patient cohort are described in Table I.

Table I. Baseline characteristics of 386 patients with NMTCs divided into 2 cohorts: FNMTCs and sporadic NMTCs.

Characteristics	FNMTC	Sporadic NMTC
Patients	82	304
Age (years)	44.39±12.85	45.14±13.87
Gender	N (%)	N (%)
Female	15 (18.3)	224 (73.7)
Male	67 (81.7)	80 (26.3)
Histology	N (%)	N (%)
Papillary	50 (60.9)	164 (54)
Papillary, follicular variant	17 (21)	84 (27.6)
Follicular	2 (2.5)	21 (6.9)
Papillary and follicular	6 (7.4)	12 (3.9)
Papillary, tall cell variety	3 (3.7)	7 (2.3)
Sclerosing papillary	4 (4.9)	8 (2.6)
Hurtle cell carcinoma	0 (0)	7 (2.3)
Insular carcinoma	0 (0)	1 (0.3)
Tumor stage	N (%)	N (%)
T1	48 (58.5)	188 (61.8)
T2	6 (7.3)	33 (10.9)
T3	18 (22)	56 (18.4)
T4	10 (2.2)	27 (8.9)
N0	60 (73.2)	237 (78)
N1	22 (26.8)	67 (22)
M0	81 (98.8)	293 (96.4)
M1	1 (1.2)	11 (3.6)

NMTC, non-medullary thyroid carcinoma; FNMTC, familial NMTC.

RAS and BRAF mutational analysis. Pyrosequencing analysis of *KRAS*, *BRAF*, and *NRAS* mutations were performed using anti-EGFR monoclonal antibody response kits for *KRAS*, *BRAF*, or *NRAS* status (Diatech Pharmacogenetics SRL, Ancona, Italy). Codons 12, 13, 61 and 146 of the *KRAS* gene, codons 464, 466, 469 and 600 of the *BRAF* gene and codons 12, 13 and 61 of the *NRAS* gene were pyrosequenced as previously reported (17). *HRAS* mutational status (codons 12, 13 and 61) was analyzed by Sequenom mass spectroscopy technology by Diatech Labline (Ancona, Italy), an external service (Diatech Pharmacogenetics SRL). Based on the evidence that *BRAF* and *RAS* mutations are mutually exclusive in thyroid cancer (18), specimens were initially analyzed for *BRAF* mutations, and *BRAF* wild type samples were subsequently analyzed for *NRAS*, *KRAS* and *HRAS* mutations. Tumors were selected for mutational analysis

Table II. Clinicopathological characteristics of 386 patients with NMTCs subdivided into 2 cohorts: FNMTCs and sporadic NMTCs.

Characteristics	FNMTC (n=82)	Sporadic NMTC (n=304)	P-value
Age (years)	44.39±12.85	45.14±13.87	0.645
Gender			
Female	67 (81.7%)	224 (73.7%)	0.086
Male	15 (18.3%)	80 (26.3%)	
Multicentricity	41 (50.6%)	93 (30.7%)	0.001
Bilaterality	26 (31.7%)	66 (22.0%)	0.049
Thyroiditis	32 (39.0%)	54 (17.8%)	<0.0001
T	28 (34.1%)	83 (27.3%)	0.141
N	22 (26.8%)	67 (22.0%)	0.220
Rates of relapse	10 (12.2%)	44 (14.5%)	0.372
Second surgery	2 (2.4%)	16 (5.3%)	0.224
Second radioiodine therapy	10 (12.2%)	38 (12.5%)	0.556

NMTC, non-medullary thyroid carcinoma; FNMTC, familial NMTC; T, tumor >4 cm (T3) or with extrathyroidal involvement (T4); N, metastatic lymph nodes.

Table III. Clinicopathological characteristics of 386 patients with NMTCs divided into 3 groups: Sporadic NMTC, FNMTCs with 2 relatives diagnosed and FNMTCs with ≥3 relatives diagnosed.

Characteristics	Sporadic NMTCs, n (%)	FNMTCs with 2 relatives diagnosed, n (%)	FNMTCs with ≥3 relatives diagnosed, n (%)	P-value
Total	304	69	13	
Age (years)	45.14±13.87	44.22±13.35	45.31±10.20	0.876
Gender				
Female	224 (73.7)	57 (82.6)	10 (76.9)	0.140
Male	80 (26.3)	12 (17.4)	3 (23.1)	
Multicentricity	93 (30.7)	34 (50.0)	7 (53.8)	0.010
Bilaterality	66 (22.0)	21 (30.4)	5 (38.5)	0.255
Thyroiditis	54 (17.8)	29 (42.0)	3 (23.1)	<0.0001
T	83 (27.3)	24 (34.8.0)	4 (30.8)	0.245
N	67 (22.0)	17 (24.6)	5 (38.5)	0.897
Rates of relapse	44 (14.5)	9 (13)	1 (7.7)	0.887
Second surgery	16 (5.3)	2 (2.9)	0 (0)	0.533
Second radioiodine therapy	38 (12.5)	9 (13)	1 (7.7)	0.806

NMTCs, non-medullary thyroid carcinomas; FNMTCs, familial NMTC; T, tumor >4 cm (T3) or with extrathyroidal involvement (T4); N, metastatic lymph nodes.

based on the availability of paraffin-embedded specimens. The *BRAF*/*RAS* mutational analysis was also performed on DNA samples obtained from the peritumoral normal thyroid gland in selected cases in which the tumor specimen presented with mutated *BRAF* or *NRAS*.

Statistical analysis. Data are reported as the mean ± standard deviation. The mean differences were compared by unpaired Student's t- or 1-way test. The differences between categorical variables were tested by Pearson's χ^2 test. The statistical package SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA) was used for the analysis. P<0.05 was considered to indicate a statistically significant difference. Kaplan Meyer curves for overall and recurrence-free survival were not calculated, since the number of events was not sufficient.

Results

Clinicopathological features. Major differences between the clinicopathological features of the 82 FNMTCs and 304 sporadic NMTCs are reported in Table II. Notably, a statistically significant difference in the frequency of multicentricity and bilaterality was observed between the 2 subgroups; FNMTCs were demonstrated as more likely to be multicentric

Table IV. *BRAF* and *RAS* mutational statuses in 34 familial non-medullary thyroid carcinomas.

Patients	*BRAF*	*NRAS*	*KRAS*	*HRAS*
1	V600E	-	-	-
2	V600E	-	-	-
3	V600E	-	-	-
4	V600E	-	-	-
5	V600E	-	-	-
6	V600E	-	-	-
7	V600E	-	-	-
8	V600E	-	-	-
9	V600E	-	-	-
10	wt	Q61R	-	-
11	wt	Q61R	-	-
12	V600E	-	-	-
13	wt	Q61R	-	-
14	wt	Q61R	-	-
15	wt	wt	wt	wt
16	V600E	-	-	-
17	wt	wt	wt	wt
18	V600E	-	-	-
19	V600E	-	-	-
20	V600E	-	-	-
21	V600E	-	-	-
22	wt	wt	wt	wt
23	wt	Q61R	-	-
24	V600E	-	-	-
25	V600E	-	-	-
26	wt	Q61R	-	-
27	V600E	-	-	-
28	wt	wt	wt	wt
29	wt	wt	wt	wt
30	wt	wt	wt	wt
31	V600E	-	-	-
32	wt	wt	wt	wt
33	wt	wt	wt	wt
34	V600E	-	-	-

wt, wild type.

and bilateral (Pearson's χ^2 test, P=0.001 and 0.049, respectively). Furthermore, the prevalence of associated chronic autoimmune thyroiditis was significantly higher in FNMTCs than in sporadic thyroid tumors (Pearson's χ^2 test, P<0.0001). In order to address the relevance of autoimmune thyroiditis in multicentricity and bilaterality of FNMTCs, the relationship between these variables was further analyzed. Indeed, the rates of multicentricity and bilaterality were similar in patients with FNMTC with or without associated autoimmune thyroiditis (48 vs. 53.3% for multicentricity and 32.7 vs. 30% for bilaterality), Furthermore, the odds ratios for multifocality and bilaterality were consistently higher in FNMTCs vs. NMTCs, regardless of thyroiditis [OR, 1.81 (CI 95%, 1.07-3.29), P=0.027; and 2.5 (CI 95%, 1.49-4.19), P=0.001, respectively].

Table V. Clinicopathological characteristics of 34 patients with familial non-medullary thyroid carcinoma divided in 2 groups: *BRAF*/*NRAS*-mutated and *BRAF*/*RAS*-wild type tumors.

Characteristics	*BRAF*/*NRAS*-mutated, n (%)	*BRAF*/*RAS*-wt, n (%)	P-value
Total	26	8	
Age (years)	44.77±14.04	50.38±13.50	0.329
Gender			
Female	22 (84.6)	6 (75.0)	0.438
Male	4 (15.4)	2 (25.0)	
Multicentricity	17 (65.4)	4 (50.0)	0.352
Bilaterality	10 (38.5)	2 (25.0)	0.402
Thyroiditis	7 (26.9)	4 (50.0)	0.213
T	11 (42.3)	1 (12.5)	0.130
N	6 (23.1)	0 (0)	0.171
Rates of relapse	5 (19.2)	0 (0)	0.236
Second radio-iodine therapy	5 (19.2)	0 (0)	0.236

wt, wild type; T, tumor >4 cm (T3) or with extrathyroidal involvement (T4); N, metastatic lymph nodes.

A slight trend was observed with respect to gender, with a higher incidence of FNMTCs observed in females (P=0.086). The frequency of tumors >4 cm in size (T3, according the TNM classification), with extrathyroidal involvement (T4), or with metastatic lymph nodes (N1) were slightly higher in FNMTCs, although these differences were not observed to be statistically significant (Table II). No differences were observed with respect to age of onset, rates of relapse and second surgery/radioiodine therapy between the 2 groups.

It has been previously suggested that families with <3 members diagnosed with thyroid cancer of follicular origin may be enriched by sporadic carcinomas that occur within the same family (13,14), likely due to identical polymorphisms (19). Thus, a subgroup analysis was performed in the current study according to the numbers of affected relatives (Table III). Notably, progressive increases in the frequencies of multicentric tumors (P=0.010) and thyroiditis (P<0.0001) were observed from sporadic tumors to FNMTCs with 2 and ≥3 relatives diagnosed. Similar trends of progressive increases in the rates of bilaterality and lymph node involvement were also observed by comparing the 3 subgroups, although these differences were not indicated to be statistically significant (Table III).

RAS/*BRAF mutational analysis*. The mutational analysis of the *BRAF*, *KRAS*, *NRAS* and *HRAS* genes was performed in a subgroup of 34 cases of FNMTC (Table IV). Notably, 58.8% (20/34 cases) of tumors exhibited the V600E *BRAF* mutation, and 17.6% (6/34 cases) the *NRAS*-mutated phenotype (mostly in the follicular variant histotype) whereas none of the tumors showed mutation in the *KRAS* and *HRAS* genes, with 23.6% (8/34 cases) of tumors presenting a wild type phenotype for all of *BRAF*, *NRAS*, *HRAS* and *KRAS* genes. In order to exclude that the *BRAF*/*NRAS* mutations observed in the tumors were inherited germline mutations (20,21), the mutational

Table VI. Clinicopathological characteristics of 34 patients with familial non-medullary thyroid carcinomas divided into 3 groups: *BRAF*-mutated, *NRAS*-mutated and *BRAF*/*RAS*-wt tumors.

Characteristics	*BRAF*-mutated, n (%)	*NRAS*-mutated, n (%)	*BRAF*/*RAS*-wt, n (%)	P-value
Total	20	6	8	
Age (years)	43.6±12.2	48.5±19.8	50.3±13.5	0.474
Gender				
Female	18 (90.0)	4 (66.7)	6 (75.0)	0.156
Male	2 (10.0)	2 (33.3)	2 (25)	
Multicentricity	12 (60)	5 (83.3)	4 (50)	0.458
Bilaterality	8 (40)	2 (33.3)	2 (25)	0.628
Thyroiditis	6 (30)	1 (16.7)	4 (50)	0.826
T	10 (50.0)	1 (16.7)	1 (12.5)	0.063
N	5 (25.0)	1 (16.7)	0 (0)	0.380
Rates of relapse	4 (20.0)	1 (16.7)	0 (0)	0.561
Second radioiodine therapy	4 (20.0)	1 (16.7)	0 (0)	0.561

wt, wild type; T, tumor >4 cm (T3) or with extrathyroidal involvement (T4); N, metastatic lymph nodes.

analysis was extended to the peritumoral non-infiltrated thyroid tissue in 8 cases whose tumor presented a mutation in *BRAF* or *NRAS* gene (6 *BRAF*- and 2 *NRAS*-mutated), observing a wild type phenotype in all normal thyroid tissues surrounding the *BRAF*/*NRAS*-mutated tumors (data not shown).

The prognostic significance of the *BRAF*/*NRAS* mutational statuses were further investigated with the aim of validating their value in the selection of familial tumors with poor prognostic factors. Indeed, the frequency of multicentricity and bilaterality was higher in *BRAF*/*NRAS*-mutated familial tumors vs. the wild type subgroup (65.4 and 38.5 vs. 50.0 and 25.0%, respectively; Table V). Furthermore, compared with wild type FNMTCs, mutated tumors were more common in a locally advanced stage (T3 or T4, according to TNM classification, 42.3 vs. 12.5%; Table V) or with lymph node involvement (23.1 vs. 0.0%; Table V). The prevalence of thyroiditis was higher in *BRAF*/*NRAS* wild type tumors compared with that of mutated familial cancer cases (26.9 vs. 50.0%; Table V). However, these differences were not observed to be statistically significant, probably due to the limited number of tumors that were profiled. To address independently the relevance of *BRAF* and *NRAS* mutations, familial tumors were further analyzed according to their specific mutation (*BRAF*- vs. *NRAS*-mutated vs. *RAS*/*BRAF*-wild type). Consistently, *BRAF*- and *NRAS*-mutated FNMTCs displayed slightly higher prevalence of multicentricity, bilaterality, T and N stage than wild type tumors (P>0.05; Table VI).

Discussion

Major challenges in the clinical management of FNMTCs include the lack of agreement on whether these carcinomas are characterized by an unfavorable outcome compared with sporadic thyroid carcinomas, and whether any specific molecular tools are able to aid in selecting, in a clinical setting, those tumors with a potential biologically aggressive phenotype. Certain previous studies have suggested that FNMTCs are characterized by unfavorable pathological and clinical features that are responsible for higher recurrence rates, poor overall survival, increased frequency of multifocality and lymph node/extrathyroidal invasion (2-7). However, contrasting conclusions were reported in other studies that did not observe major differences in the prognostic factor profile nor in the clinical outcomes of FNMTCs and NMTCs (8-12). These different conclusions are likely to be due to the different length of the follow-up in these studies and/or the dissimilar criteria used to define FNMTCs (2 vs. 3 affected family members) (13,14), which generally reflects the biological and clinical heterogeneity of FNMTCs (1). The present study confirmed the higher rate of multicentricity and bilaterality in familial vs. sporadic NMTCs, thus refining the criteria for the diagnosis of patients with FNMTCs. Indeed, multicentric tumors were significantly more frequent in the 3-member cohort, although the 2-member cohort also presented an increased rate of multicentricity vs. the sporadic NMTC subgroup. Furthermore, a trend towards a gradual increase of bilaterality and lymph node involvement was observed from sporadic to 2-member and 3-member subgroups. However, it is important to note that, aside from these differences in the prognostic factor distribution, major differences in terms of patients outcome were not observed. Overall, a low rate of tumor relapse and no tumor-associated mortalities were observed, regardless of the familiarity of the tumor. This represents a major difference between the current study and others that previously demonstrated that different prognostic factor profiles between familial and sporadic thyroid tumors result in a significantly different prognosis (2-7). It is possible that the more intense follow-up generally applied to familial over sporadic NMTCs is responsible for the overall excellent prognosis observed in the present cohort of patients. The length of the current follow-up observation (median follow up, 86.5 and 84.7 months for FNMTCs and NMTCs, respectively) suggests that the prognosis of familial thyroid tumors may not be, in the long term, significantly different from that of sporadic carcinomas.

Partially unexpected is the observation that FNMTCs are characterized by higher rates of chronic autoimmune thyroiditis.

While other studies reported an increased rate of benign nodules associated with FNMTC (22), the higher prevalence of thyroiditis in the present cohort may be partially explained by the higher frequency of familial tumors in females (Table II). However, it is intriguing to speculate that chronic autoimmune thyroiditis, associated with lymphocyte infiltration and cytokine production (23,24), may represent a favorable milieu for the development of thyroid malignancies, as suggested for sporadic carcinomas (25). Since specific genetic loci have been associated with the risk of developing chronic autoimmune thyroiditis (26), the present data suggest that common genetic predisposing factors may be involved in either autoimmune thyroiditis or thyroid carcinomas. Further studies are required to address this hypothesis. However, the evidence that thyroiditis is not associated with multifocality and bilaterality in the present FNMTC cohort suggests that chronic inflammation may not contribute to the unfavorable prognostic profile of familial thyroid carcinomas, but it may be involved in the regulation of key cellular processes for cancer onset and progression (25).

To address the relevance of genetic profiling in the selection of FNMTCs with an unfavorable outcome, *BRAF* and *RAS* mutational analysis was performed in a subgroup of familial tumors. A major limitation of the present study is the low number of tumor samples available for the molecular profiling (mostly due to the low rate of FNMTCs among thyroid epithelial tumors), which significantly attenuates the strength of the current data, as suggested by the lack of statistical significance in specific analyses. Thus, no definitive conclusions can be drawn from this part of the study, although there are specific issues that deserve consideration, since they may be relevant to the design of further studies. Notably, a high frequency of *BRAF* mutations was observed at the upper limit of the mutation frequency observed in sporadic thyroid tumors (27). As expected, *NRAS* mutations were prevalent in follicular carcinomas (28), but no *KRAS* or *HRAS* mutations were observed in the present cohort. Furthermore, although certain comparisons between the groups were not identified as statistically significant, they strongly suggest that the majority of multifocal FNMTCs are characterized by *BRAF*/*NRAS* mutations, and that *BRAF*- or *NRAS*-mutated familial carcinomas exhibit a more advanced T and N stage and a higher risk of relapse (Tables V and VI). By contrast, *BRAF*/*RAS* wild type tumors presented an increased frequency of associated thyroiditis, suggesting that thyroiditis is not a feature associated with nor responsible for the unfavorable prognostic profile of these tumors. Indeed, the present data must be taken with caution and should be interpreted in the perspective of the unsolved controversy on whether *BRAF* and *NRAS* mutations are prognostic in sporadic thyroid carcinomas (28-31). However, the present genetic profiling suggests that the mutational status of specific genes deserves to be further and more extensively evaluated in FNMTCs, since it may aid in the identification of those tumors with a more aggressive clinical phenotype.

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