Familial non-medullary thyroid carcinoma (FNMT): analysis of \textit{fPTC/PRN}, \textit{NMTC1}, \textit{MNG1} and \textit{TCO} susceptibility loci and identification of somatic \textit{BRAF} and \textit{RAS} mutations

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Abstract

Linkage analysis has identified four familial non-medullary thyroid carcinoma (FNMT) susceptibility loci: \textit{fPTC/PRN} (1p13.2-1q22), \textit{NMTC1} (2q21), \textit{MNG1} (14q32) and \textit{TCO} (19p13.2). To date, there is no evidence for the involvement of genes from the RAS/RAF signalling pathway in FNMT. The aim of our study was to evaluate the role of the four susceptibility loci, and RAS/RAF signalling pathway genes, in FNMT. In total, 8 FNMT families, and 27 thyroid lesions from family members (22 papillary thyroid carcinomas (PTCs): 11 classic, 10 of the follicular variant and 1 of the mixed variant; 4 follicular thyroid adenomas (FTAs) and 1 nodular goitre (NG)), were evaluated for the involvement of the four susceptibility regions, using linkage and loss of heterozygosity (LOH) analyses. \textit{BRAF} and \textit{H-, N- and K-RAS} mutations were also screened in the 27 lesions and patients. Linkage analysis in seven informative families showed no evidence for the involvement of any of the four candidate regions, supporting a genetic heterogeneity for FNMT. Twenty tumours (74%), of which 18 were PTCs, showed no LOH at the four susceptibility loci. The remaining seven tumours (four PTCs, two FTAs and one NG) showed variable patterns of LOH. Fourteen tumours (52%) had somatic mutations: \textit{BRAF}-V600E mutation was observed in 9 out of the 22 PTCs (41%); and \textit{H-RAS} and \textit{N-RAS} mutations were detected in 5 out of the 22 PTCs (23%). Our data suggest that the four candidate regions are not frequently involved in FNMT and that the somatic activation of \textit{BRAF} and \textit{RAS} plays a role in FNMT tumourigenesis.

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Introduction

Non-medullary thyroid carcinoma (NMTC) refers to neoplasms originating from the thyroid follicular cells and represents about 90% of all thyroid cancers. The two main variants of NMTC are the papillary thyroid carcinoma (PTC) and the follicular thyroid carcinoma (FTC), although PTC accounts for over 85% of the lesions reported (Farid et al. 1994, Schlumberger 1998). Approximately 5% of NMTC occurs on the background of a familial predisposition and in this case, it is referred to as familial NMTC (FNMT; MIM 188550; Malchoff & Malchoff 2006). FNMT is a clinical entity characterized by an earlier age of onset, higher degree of aggressiveness, and more frequent multifocal disease and recurrence compared with its sporadic counterpart (Malchoff & Malchoff 1999, Alsanea & Clark 2001, 2002).
Sturgeon & Clark 2005, Malchoff & Malchoff 2006, Sippel et al. 2007). High incidence of multinodular goitre (MNG) and follicular thyroid adenoma (FTA) are also common features in FNMT families (Musholt et al. 2000, Uchino et al. 2002). FNMT inheritance seems to be autosomal dominant and penetrance is incomplete and increases with age (Malchoff & Malchoff 2006).

No FNMT susceptibility genes have yet been cloned. Linkage analyses have mapped four different chromosomal regions that may harbour FNMT susceptibility genes. The ‘thyroid carcinoma with oxyphilia’ locus (TCO; MIM 603386) was mapped to chromosome 19p13.2 in a French family with an unusual form of NMTC with cell oxyphilia (Canzian et al. 1998). A MNG susceptibility locus (MNG1; MIM 138800) was mapped to chromosome 14q32 in a large Canadian family with MNG and low occurrence of NMTC (Bignell et al. 1997). Another locus predisposing to FNMT was identified on chromosome 1p13.2-1q22 in a U.S. family with recurrent PTC and papillary renal neoplasia (PRN) (PTC/PRN or PRN1; MIM 605642; Malchoff et al. 2000). A fourth susceptibility locus, named ‘non-medullary thyroid carcinoma 1’, was mapped by McKay et al. (2001) to chromosome 2q21 in a large Tasmanian family with high frequency of PTC (NMTC1; MIM 606240).

Tumour loss of heterozygosity (LOH) represents a rather frequent molecular alteration in sporadic FTA and FTC (Trovato et al. 2004) but not in PTC (Ward et al. 1998). Tumour-specific LOH is found in sporadic FTC, with and without oxyphilia, at both 19p13.2 and 2q21 (Stankov et al. 2004). Another study found LOH at 14q32 in sporadic FTA (22%) and FTC (43%; Sarquis et al. 2006). These findings suggest that tumour suppressor susceptibility genes lie in these chromosomal regions and that genetically inherited, or somatically acquired, disruptions of these genes may predispose to FNMT initiation and/or progression.

Although the majority of sporadic PTC, and a significant proportion of the FTA/FTC, harbours activating mutations in genes from the RAS/RAF pathway (Kimura et al. 2003, Vasko et al. 2003), no oncogenic germline mutations have been detected in the K-, N-, H-RAS, BRAF, MEK1 and MEK2 genes in FNMT cases (Xing 2005, Hou & Xing 2006). Nevertheless, the somatic contribution of RAS/RAF pathway genes in the development of FNMT tumours has not been investigated yet.

In the present study, we have performed allelotype and haplotype analyses on eight families with FNMT, and 27 associated thyroid tumours, in an effort to determine the significance of the genes located in the four candidate regions in the predisposition to FNMT. The role of BRAF and RAS oncogenic mutations in FNMT expression was also investigated.

Materials and methods
Families and tissue specimens
According to Charkes (2006), in families with three to five members affected with NMTC, more than 96% of the affected members have the familial trait. Therefore, in the present work, the criteria for eligibility of the FNMT families were that three (or more) first-degree family members had to be affected with NMTC. Benign thyroid lesions such as nodular goitre (NG) and FTA are thought to be features of FNMT, because a personal or family history of benign thyroid conditions is present in about 45% of patients with FNMT (Sippel et al. 2007). Thus, in the families selected for study (all having three or more cases with NMTC), the patients with NG or FTA were also considered affected (Musholt et al. 2000, Uchino et al. 2002, Sippel et al. 2007). NG was detected by palpation and latter confirmed by cytological and/or histological analyses.

A panel of eight Portuguese families with a total of 50 individuals affected with FNMT was identified (Fig. 1). When available, the samples of thyroid lesions from affected family members were collected. A total of 27 familial thyroid lesions, either fresh frozen in liquid nitrogen (n=3) or formalin-fixed paraffin embedded (FFPE; n=24), were obtained. Ten fresh frozen sporadic PTCs were also used in the present study. Histopathological classification of all tumour specimens was performed by one pathologist and confirmed by an independent reviewer, following the criteria described in WHO classification of thyroid tumours (DeLellis et al. 2004). Venous blood and/or tumour samples from patients and relatives were obtained following patients’ written informed consent, and this study was approved by our institution ethical committee.

DNA extraction
DNA was extracted from peripheral blood leukocytes and fresh frozen tumour samples using standard protocols, and from FFPE tumour samples using a method described by Imyanitov et al. (2001).

Linkage analysis
We isolated DNA, from peripheral blood leukocyte or normal thyroid tissue, from 76 individuals (39 affected, 30 asymptomatic and 7 spouses) from the eight FNMT families. Family 8, in whom DNA
was available from only one affected individual, was not suitable for linkage analysis.

Linkage of FNMTC to the four candidate loci was investigated in the seven informative families using 19 microsatellite markers: \( fPTC/PRN \) (1p13.2-1q22, tel-D1S418-D1S2811-cen-D1S2344-D1S2715-D1S305-tel); \( NMTC1 \) (2q21, cen-D2S2215-D2S112-D2S2256-D2S114-tel); \( MNG1 \) (14q32, cen-D14S617-D14S81-D14S1054-D14S1434-D14S265-tel) and \( TCO \) (19p13.2, tel-D19S912-D19S884-D19S391-D19S586-D19S535-cen). Physical positions and primer sequences were obtained from the ENSEMBL Genome Browser (http://www.ensembl.org). Fluorescently labelled primers were used to amplify the microsatellite polymorphic regions by PCR (conditions are available upon request). PCR products were analysed in an automated sequencer ABI Prism 310 (Applied Biosystems, Foster City, CA, USA) using the ABI PRISM GeneScan Analysis Software v3.1 (Applied Biosystems).

Figure 1 Family trees from the eight families with FNMTC.
Parametric multipoint linkage analysis was performed using the GeneHunter program-v2.1r5 with the easyLINKAGEPlus-v5.05 program (Lindner & Hoffmann 2005), assuming a dominant model of inheritance. We assumed a disease allele frequency of 0.001 and a penetrance of 85%. The same program was used for multipoint non-parametric analysis. Allele frequencies were calculated by the easyLINKAGEPlus-v5.05 program, using data from the analysed families.

LOH analysis

Overall, 27 thyroid lesions from 20 patients of the eight FNMTC families were analysed for LOH: 22 carcinomas (11 classic PTCs, 10 PTCs of the follicular variant (PTCfv) and 1 PTC of the mixed variant (PTC-mix)), 4 FTAs (2 follicular, 1 of the oxyphilic cell-type variant (FTAocv) and 1 of the microfollicular variant (FTAmicfv)) and 1 adenomatous NG. In all cases, >90% of the cells had the histological characteristics of the group to which that specimen was ascertained. DNA was extracted as indicated above. For each patient, tumour and matched leukocyte DNA were allelotyped, using 19 microsatellite markers from the four candidate loci, already described in the ‘linkage analysis’ section. Allelic loss was calculated using the method of Canzian et al. (1996). LOH was defined with the values of LOH index <0.6 or >1.67. All LOH-positive loci were reanalysed at least once.

Detection of RAS and BRAF mutations

Analysis of RAS and BRAF genes was undertaken in constitutional DNA from representative affected members of the eight FNMTC families, and in DNA from 27 familial tumour samples (the same series analysed for LOH). This analysis was also performed in ten sporadic PTCs (nine classic and one PTCfv). The Primers were designed to amplify the three mutational hot spots (codons 12, 13 and 61) of the N-, K- and H-RAS genes and the mutational hot spot (codon 600) of the BRAF gene. The oligonucleotide primer sequences and PCR conditions are available upon request. Sequencing analysis was performed using the same primers as for PCR, and the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems; assay conditions are available on request). Sequencing products were separated in an automated sequencer ABI Prism 310 (Applied Biosystems) and analysed using the Sequence analysis software version 3.4.1 (Applied Biosystems).

Rearrangements, frequently detected in sporadic NMTC tumours, such as RET-PTC or PAX8-PPARγ were not investigated in this tumour series because, in the majority of the cases, no RNA was available for the analysis.

Statistical analysis

We compared the mean age of diagnosis of familial PTC with the BRAF-V600E mutation with that of sporadic PTC with the mutation. Comparison between the two groups was performed using the unpaired t-test. The difference between the two means was considered significant at P<0.05.

Results

Families

In the present study, eight families with FNMC were identified, with a total of 50 affected members: 16 in family 1; 8 in family 2; 5 in families 4, 5 and 6; 4 in families 3 and 8; and 3 in family 7 (Fig. 1).

Among the 50 patients from the eight families, the majority (54%) had thyroid carcinomas and the remaining presented benign thyroid lesions (46%). Of the 27 patients with PTC, 13 had concomitant benign lesions (NG and FTA) or other malignant tumours (anaplastic thyroid carcinoma (ATC; Table 1)).

The age range at which each affected individual was diagnosed was 12–72 years (mean 35 years), which is in accordance with the previously published data (Malchoff & Malchoff 1999).

The mode of inheritance in these families appeared to be autosomal dominant.

Linkage analysis

Linkage to the four FNMTC susceptibility loci (1p13.2-1q22, 2q21, 14q32 and 19p13.2) was
evaluated in the seven informative families (families 1–7). Although FNMTC in these families had a possible dominant transmission, both parametric and non-parametric multipoint approaches were used in the linkage analysis. The logarithm of the odds scores obtained were either null or, in the majority of cases, negative at the four candidate regions (Table 2), weakening the hypothesis of the involvement of genes located at these four loci in thyroid tumour initiation, in these families. Similar results were obtained in the multipoint non-parametric analysis (data not shown). The examination of the segregation of the haplotypes (data not shown), taking into account the possibility that some cases with benign lesions could represent phenocopies, supported the previous analysis.

LOH studies

An extensive LOH study was pursued in 27 thyroid tumour/benign nodules, from 20 patients of the eight families, using 19 microsatellite markers located at the four FNMTC susceptibility loci (1p13.2-1q22, 2q21, 14q32 and 19p13.2; Fig. 2).

Twenty tumours (74%), of which 18 (67%) were PTC (10 classic, 7 PTCfv and 1 PTCmix), did not show LOH in any region. The remaining seven tumours (three PTCfv, one classic PTC, one FTA, one FTAocv and one NG) showed variable patterns of allelic losses in the candidate regions. A similar frequency of LOH was detected in the four susceptibility regions (mean = 14.8%), ranging from 11.1% (on chromosome 19p13.2) to 18.5% (on chromosome 14q32). However, most of the losses were detected in tumours from families 2 and 3 and were nearly absent in the tumours from the remaining families. In family 2, three of the six tumours shared LOH on the D14S617 locus from the 14q32 region. One of these tumours, an FTA of the oxyphilic cell-type variant, also showed LOH involving the entire 2q21 candidate region. In family 3, an ATC was detected in patient II.3, 9 years after the removal of a classic PTC, and it is thus interesting to note that this PTC showed allelic losses at the four loci analysed.

Table 2 Results of linkage analysis in seven families with FNMTC

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<th>Marker</th>
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The most informative microsatellite marker from each region of interest was chosen. LOD, logarithm of the odds.

BRAF and RAS mutations in FNMTC tumours

An analysis of the mutational hot spots from BRAF gene (codon 600) and N-, K- and H-RAS genes (codons 12, 13 and 61) was carried out in the DNA from 27 FNMTC thyroid lesions and in constitutional DNA from representative affected members of each family.

BRAF-V600E mutation was observed in nine PTCs (seven classic, one of the follicular variant and one of the mixed variant), from nine patients, of families 1, 3, 5, 6, 7 and 8 (Table 3). In particular, BRAF-V600E mutations were detected in 7 out of the 11 familial classic PTCs (64%). The absence of germline BRAF mutations in the nine patients indicated that the detected BRAF mutations were somatic. BRAF mutations were also absent in the constitutional DNA from representative affected patients from families 2 and 4.

In order to compare the mean age of diagnosis between familial and sporadic BRAF-V600E positive PTCs, ten previously analysed sporadic PTCs, which had the BRAF-V600E mutation, were selected for this study. Of the nine familial PTCs with BRAF-V600E mutation, we selected seven cases that had not been identified following a family screening. The mean age of diagnosis in the familial PTC with BRAF-V600E (41.4 ± 9.5) was lower than in the sporadic PTC with BRAF-V600E (56.7 ± 18.0; P = 0.05).

The analysis of the three RAS isoforms revealed that three PTCs (one classic and two of the follicular variant), from three patients of family 2, had H-RAS mutations (Table 3); and two PTCs (one classic and one of the follicular variant), from one patient of family 5, had N-RAS mutations (Table 3). The absence of germline RAS mutations in these four patients indicated that the mutations were somatic. RAS mutations were also absent in the constitutional DNA from representative affected patients from families 1, 3, 4, 6, 7 and 8.

Discussion

To date, no FNMTC predisposing genes have been identified. With the aim of further clarifying the genetic factors underlying FNMTC, eight Portuguese families...
**Figure 2** LOH analysis in 27 thyroid lesions from FNMT patients.

F, family; P, patient; PTC, papillary thyroid carcinoma; FTA, follicular thyroid adenoma; NG, nodular goitre; c, classic; fv, follicular variant; ocv, oxyphilic cell type variant; ad, adenomatous; mcv, microfollicular variant; mix, mixed variant. Microsatellites were informative in an average of 81% (range 59–96%) of the cases.

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- Loss of heterozygosity (LOH)
- Retention of heterozygosity
- Homozygosity
- Tumour not amplified
with FNMT, and 27 thyroid lesions from affected family members, were evaluated for the involvement of the four susceptibility regions (\textit{p}TC/\textit{PRN} (1p13.2-1q22), \textit{NMTC1} (2q21), \textit{MNG1} (14q32) and \textit{TCO} (19p13.2)), using as genetic strategies, linkage and LOH analysis. The role of \textit{RAS}/\textit{RAF} activation in FNMT tumourigenesis was also investigated.

Results of linkage analysis, following both parametric and non-parametric methods, performed in the seven informative families showed no evidence for the involvement of any of the four previously identified susceptibility regions in FNMT initiation, suggesting a heterogeneous genetic background for FNMT.

To our knowledge, the present series of 27 FNMT tumours lesions is, so far, the largest addressed in LOH studies. This analysis revealed that the vast majority (74\%) of the lesions, mainly PTC (67\%), had no LOH at the four susceptibility loci. These results suggest that the inactivation of tumour suppressor genes located at the candidate regions, by allelic loss, is not a common feature in familial PTC. These observations are in agreement with the studies of Brunaud et al. (2003) that found, by comparative genomic hybridization, chromosomal imbalances in only 24\% of familial PTC, involving chromosomes 1p, 2q and 19p in <12\% of the cases.

LOH was only detected in some tumours from families 3 and 4; however, the frequency and location of the losses were variable among tumours from the same family, showing no specific pattern. The losses on D14S617, observed in three tumours from family 2, are more likely involved in tumour progression, since this family showed no linkage to this region.

In the 50 patients from the eight FNMT families, PTC either isolated or associated with other thyroid lesions was the most common phenotype and was observed in 54\% of the cases. Thirteen patients (26\%) had more than one thyroid lesion, of different histological types, and not family specific. This evidence of tumour phenotype heterogeneity in the same individual, together with the observation that thyroid lesions from the same patients or families, showed different LOH rates and patterns, suggested that the histological type and molecular signatures of FNMT lesions might not be exclusively determined by an inherited molecular defect. We thus hypothesized that, in FNMT patients, an inherited defective gene could predispose the genome to acquire somatic mutations/rearrangements in crucial genes (e.g. in the \textit{RAS}/\textit{RAF} pathway), that would also be accountable for tumour clinicopathological features. In order to evaluate this hypothesis, we analysed \textit{H}-, \textit{N}-, \textit{K}-\textit{RAS} and \textit{BRAF} hotspots in our 27 tumour series.

Sequencing analysis identified \textit{BRAF}-V600E somatic activating mutations in 9 out of the 22 patients from the eight FNMT families.
PTCs (41%). In particular, BRAF-V600E mutations were detected in 64% of classic PTCs. RAS mutations were present in 5 out of the 22 PTCs (23%): H-RAS mutations (G12R and G13R) were detected in 3 PTCs from 3 patients of family 2, and the N-RAS mutation Q61R was detected in 2 PTCs from 1 patient of family 5. Overall, 14 out of the 27 FNMTC lesions (52%) had mutually exclusive BRAF and RAS mutations.

BRAF mutations are frequently detected in the sporadic forms of classic PTC (48%; Fugazzola et al. 2006). In contrast, sporadic mutations involving codons 12, 13 and 61 from H-RAS are rare in both follicular and papillary tumours (overall <5%; Bouras et al. 1998, Vasko et al. 2003). The herein reported H-RAS G12R mutation has only been reported once in an ATC and, to the best of our knowledge, H-RAS G13R has not been reported in thyroid cancer. However, both mutations have been detected by other authors in tumours from other tissues, thus confirming their oncogenic role (Forbes et al. 2006). The N-RAS Q61R mutation, herein identified in two familial tumours (one classic PTC and one PTCfv), has been described in 21–30% of sporadic PTCfv but not in classic PTC (Zhu et al. 2003, Di Cristofaro et al. 2006).

As all the families selected for the present study have three, or more, patients with thyroid carcinoma, the chances of analysing a sporadic NMTC case in our families is remote (<6%; Charkes 2006). Thus, the identification of somatic RAS/RAF mutations in familial thyroid carcinomas suggests that these defects are involved in FNMTC tumour progression. Accordingly, it is remarkable that all the affected members from family 7 had a somatic BRAF-V600E mutation in their PTC, and also that tumours from different patients of family 5 had BRAF or RAS somatic mutations. It is also noteworthy that in family 2, three tumours from different patients showed mutations only in H-RAS. Although this could be a fortuitous association, it is possible that in family 2, an inherited defective gene may specifically increase susceptibility to mutations in the H-RAS isoform, as it has been reported in colorectal carcinomas from patients with germline mutations in hMSH6, that specifically harbour the highest frequency of K-RAS mutations (Oliveira et al. 2004).

Nikiforova et al. (2003) showed a significant association of sporadic BRAF mutation with older age. In the present series of BRAF-positive familial PTC, the mean age of diagnosis (41.4 ± 9.5) was lower than that of the BRAF-positive sporadic PTC (56.7 ± 18.0), however, this difference was only marginally significant (P = 0.05).

In summary, our data show that the four candidate loci, fPTC/PRN, NMTC1, MNG1 and TCO are not frequently involved in FNMTC tumourigenesis, suggesting the contribution of further, yet unidentified, susceptibility loci. The present work also discloses the somatic activation of proto-oncogenes within the RAS/RAF pathway in FNMTC tumour progression.

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