A new form of familial multi-nodular goitre with progression to differentiated thyroid cancer

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Abstract

We report a kindred with euthyroid multi-nodular goitre (MNG) of adolescent onset. Two of the seven subjects with MNG have progressed to papillary thyroid cancer. One affected male had nodular kidney disease, and breast cancer occurred in one affected female. Genes that were candidates on the basis of the associated kidney (PAX8) and breast diseases (sodium iodide symporter (NIS)), were sequenced. No mutations were found in the coding region, intron/exon splice sites or in the promoter sequences (from –1248 relative to the translation initiation codon) of PAX8. Similar results were obtained for NIS. Subsequently, microsatellite analyses were performed on 14 informative family members. We used 2 to 3 markers per locus for 6 loci (on chromosomes 1,2,3,14,19,X) previously reported to predispose to MNG and/or familial non-medullary thyroid cancer (FNMTc). On the basis of non-significant logarithm of the odds ratio (LOD) scores or inheritance of different alleles in affected individuals, all loci have been excluded. Thyroidectomy specimens from three members of the kindred show multiple benign lesions, with papillary cancer in two. The morphological features do not resemble those seen in familial adenomatous polyposis, Cowden syndrome, or in multiple oxyphil lesions. From these findings and from the absence of any linkage to any of the known loci associated with MNG or FNMTc, we suggest that this represents a new form of inherited MNG with a significant risk of progression to papillary carcinoma.

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Introduction

Thyroid enlargement is common in iodine-deficient areas. It is not infrequent (in adults) in countries with an adequate iodine intake, as illustrated by the Whickham survey, which found a goitre in 23% of women and 5% of men, decreasing to 10% and 2% respectively at 20-year follow-up (Vanderpump *et al.* 1995). A history of benign thyroid disease is a significant risk factor for the development of thyroid cancer (Musholt *et al.* 2000), which affects 0.5–10 individuals per 100 000 per year.

The differentiated thyroid cancers, papillary and follicular, arise from follicular cells and account for 90% of cases of thyroid malignancy (reviewed in Ludgate & Evans 2004). They are familial in 7–9% of cases, and linkage to a range of loci has been established, but the genes involved are known

in only a small minority of these cases. In contrast, there has been much more progress in delineating the molecular defect in sporadic thyroid cancers. Chromosomal rearrangements which lead to the activation of the RET proto-oncogenes without the need for ligand binding have been identified in papillary thyroid carcinomas (Grieco *et al.* 1990). More recently, BRAF mutations have been described in a significant proportion of papillary carcinomas (Kimura *et al.* 2003, Soares *et al.* 2003), and the great majority of papillary carcinomas show either a BRAF mutation or a rearrangement, usually involving RET, rarely TRK. Translocations between chromosomes 2 and 3 have been detected in a series of follicular tumours, producing an in-frame fusion of PAX8 to peroxisome proliferator-activated receptor gamma (PPAR γ) (Kroll *et al.* 2000, Cheung *et al.* 2003). In addition, around 50% of both benign

and malignant follicular tumours display RAS activation (Nikiforova *et al.* 2003). All 3 RAS genes can be involved.

Familial non-medullary thyroid cancer (FNMTC) and its relationship with familial multinodular goitre (MNG) are now recognised as clinical entities. Linkage analysis using microsatellite markers has identified three MNG loci. MNG1 was mapped using a single large Canadian family with adolescent onset of euthyroid MNG in both males and females (Bignell *et al.* 1997). The locus is at 14q but does not include the gene for the thyrotrophin (TSH) receptor at 14q31. Study of 37 smaller FNMTC pedigrees indicated that only a small proportion of this disorder is attributable to MNG1. As MNG occurs approximately five times more frequently in women than in men, and in the absence of male-to-male transmission in an Italian kindred, Capon and colleagues investigated the X chromosome. They obtained a significant logarithm of the odds ratio (LOD) score for chromosome Xp22, referred to as MNG2 (Capon *et al.* 2000). Locus 3q26.1–q26.3 (MNG3) was identified by linkage analysis of two unrelated Japanese families with MNG (euthyroid but with elevated serum TSH) in several generations (Takahashi *et al.* 2001).

In the case of FNMTC, several further loci have been implicated. A susceptibility gene was located at 2q21 in a large Tasmanian family with the follicular variant of papillary carcinoma. The significance of the locus (which apparently does not contain the gene for PAX8) was confirmed by linkage analysis of 80 pedigrees that produced a LOD score of 3.07 (McKay *et al.* 2001). A gene predisposing to thyroid cancers with follicular and papillary architecture accompanied by cell oxyphilia has been identified at 19p13.2 (Canzian *et al.* 1998). More recently, a study of ten families with FNMTC found interaction between the 2q21 and 19p13.2 loci. Interestingly, the tumours in 9 of the 10 families showed cell oxyphilia (McKay *et al.* 2004). The linkage to the locus at 19p13.2 is not necessarily to the TCO gene, as a second gene (GRIM 19) at this locus has been found to be mutated in some sporadic oxyphil tumours (Maximo *et al.* 2005).

Locus 1q21 has also been linked with a phenotype of papillary thyroid cancer associated with renal neoplasia and occasional pre-menopausal breast cancer (Malchoff *et al.* 2000).

FNMTC is a group of discrete syndromes, each characterised by mutation in a different gene, and in most cases by differing morphology in the associated tumours. Furthermore, mutations in the

adenomatous polyposis coli gene (5q21–22) correlate with multiple tumours having a distinct morphology, which have been termed familial adenomatous polyposis (FAP)-associated thyroid carcinomas (Harach *et al.* 1994), and mutations in PTEN (phosphatase and tensin homologue, 10q23.31) that are associated with multiple non-oxyphil follicular adenomas, which may progress to carcinoma (Liaw *et al.* 1997).

We report our findings of a family with adolescent onset of MNG progressing to papillary thyroid cancer. Characterisation of the gene defect would have obvious benefits for the family in terms of promoting genetic counselling and health screening, as well as contributing to our understanding of thyroid neoplasia. We have therefore studied two candidate genes, and carried out linkage studies to ascertain whether this family represents a previously described type of MNG or FNMTC, or is a new entity.

Case reports and family history

Index patient

The index patient (IV 2), a 12-year-old girl born in 1990 and residing in an iodine-sufficient area, presented with MNG. Ultra-sound scan showed multiple hyper-echoic thyroid nodules (three in the left and one in the right lobe). Fine needle aspirate (FNA) histology showed no evidence of malignancy. Pre-operative thyroid function tests showed normal TSH (1.4 mU/l; normal range 0.35–5.5 mU/l) and normal free thyroxine (T₄) (16.2 pmol/l; normal range 9.8–23.1 pmol/l), confirming a euthyroid condition. TSH receptor (TSHR) antibodies, were negative, and thyroid peroxidase (TPO) antibodies were within the normal range (8.6 kU/l; normal range <32.0 kU/l). In addition, the thyroglobulin (TG) antibodies were 2.2 kU/l (normal range <46.0 kU/l). The karyotype was normal, as was the kidney scan. In view of the family history, a total thyroidectomy was carried out and multiple adenomas were found with no evidence of malignancy. The patient is currently euthyroid, receiving daily replacement with thyroxine.

Family history

There is a strong family history of MNG, progressing to papillary thyroid cancer in two members of the family, as shown in the family tree (Fig. 1).

Figure 1 Family tree showing four generations of affected individuals. The proband is individual IV 2.

Although physical examination of the thyroid of the girl's 18-year-old brother (IV 1) (born in 1984) was normal, a thyroid scan revealed 8 or 9 hyper-echoic cysts. His FNA showed changes suggestive of papillary thyroid cancer. He also underwent total thyroidectomy. In infancy, he had undergone nephrectomy for polycystic kidney disease. No other member of the family was demonstrated to have any abnormality on renal ultrasound examination.

Her 22-year-old sister (IV 5) (born in 1980) has MNG but a thyroid scan revealed small hypo-echoic cysts. She did not undergo thyroidectomy but will be carefully followed.

The girl's mother (III 2) (born in 1961) underwent partial thyroidectomy at the age of 17 for MNG. At the age of 27, she underwent a second partial thyroidectomy and at age 28 she had a total thyroidectomy for papillary carcinoma. She has recently developed breast carcinoma, at age 41.

One brother (IV 4) (born in 1989) and one sister (IV 6) (born in 1986) have no signs of thyroid or kidney lesions, both are post-puberty. A second brother (IV 7) (born in 1982) has not been examined; one sister is pre-pubertal (IV 3) (born in 1994).

A maternal uncle (III 3) and his family have no signs of thyroid or kidney lesions. By contrast, her maternal grandmother (II 2), great-grandmother (I 2) and mother's first cousin (III 5) underwent surgery for presumed benign thyroid disease.

The father's (III 1) side of the family has no history of MNG or thyroid dysfunction.

Materials and methods

Sample collection and preparation

EDTA blood samples were obtained from individuals (I 2; II 2, 7, 8; III 1, 2, 3, 5; and IV 1, 2, 3, 4, 5, 6) all with informed consent and ethical approval. Genomic DNA was extracted using the QIAamp blood kit, according to the manufacturer's instructions (Qiagen) and was quantified spectrophotometrically.

Candidate genes

PAX8 is a paired-box-containing transcription factor expressed in thyroid and kidney, and shown to be involved in the genesis of several Wilms' tumours (Poleev *et al.* 1992). Some types of unilateral polycystic disease in infants have a male

predominance (Lazebnik *et al.* 1999), suggesting that the associated thyroid and kidney disease could be due to mutations in PAX8. Sodium iodide symporter (NIS), expressed in thyroid, breast and some other tissues, results in the transport of iodide, which is essential for thyroid hormone production, and through a short loop feedback is postulated to have a direct effect on thyroid growth. Somatic mutations in the *NIS* gene in the thyroid in a family with a germline mutation in one *NIS* allele could give the affected follicular cells a growth advantage, potentially leading to the formation of a nodule. A similar mechanism has been postulated for the thyroid peroxidase gene (Krohn & Paschke 2001).

Sequencing studies

Genomic DNA was PCR amplified in a 50 µl reaction volume containing 3 µl DNA (100 ng/l), 5 µl PCR buffer (10×) (1.5 mM MgCl₂), 2 µl dNTP (10 mM) (Promega), 2 µl forward primer (10 pmol/l) (Invitrogen), 2 µl reverse primer (10 pmol/l) and 1 µl Taq polymerase (1 U/l) (Promega). Reactions were performed using a Genius Techne machine: 1 cycle, 95 °C for 2 min; 30 cycles, 94 °C for 1 min, X °C for 1 min, 72 °C for 1 min; 1 cycle, 72 °C for 5 min. For each primer pair, conditions were optimised for annealing temperature (X), MgCl₂ concentration and the presence/absence of DMSO.

Previously described (Macchia *et al.* 1998) PAX8 promoter and intronic primers were used. The *NIS* was amplified using primers designed using Primer 3 software (<http://frodo.wi.mit.edu/cgi-bin/primer3>) (Table 1).

PCR products were polyethylene glycol (PEG)-precipitated and sequenced in a 10 µl reaction

containing 2 µl Big Dye Terminator Cycle Sequencing Ready Reaction (ABI Prism, PE Biosystems, Foster City, CA, USA), 1 µl primer (10 pmol/l) and approximately 25 ng PEG-precipitated PCR. Sequencing reactions were run on a Genius (Techne) PCR machine, using the same annealing temperature for each individual primer as had been used for the primer combination. The products were sodium acetate-precipitated and analysed on an ABI Prism 377.

Microsatellite analysis

Primer pairs flanking microsatellites in the vicinity of the six known MNG/FNMTC loci were selected from the ABI Version 2.5 set using the genome browser gateway (www.genome.ucsc.edu). Details are shown in Table 2.

The genotyping commenced with PCR amplification in a 12 µl reaction volume, which contained 24 ng (in 2 µl) genomic DNA, 200 µM dNTPs (Amersham), 0.4–0.5 µl primer mix (5' fluorescent labelled) (Applied Biosystems) (at 10 pmol dilution), 1.5 mM MgCl₂ and 0.5 U Taq polymerase (Qiagen).

Sixteen PCRs (1 for each primer pair) were performed on the 14 individuals (3 duplicated) and the UK pool. The reactions were performed in 96-well plates (AB Gene, Epsom, Surrey, UK) using an MJ Engine Tetrad PCR machine. After initial denaturation and activation of the Taq at 95 °C for 15 min, we performed 3 cycles of touch-down PCR starting from 62 °C, followed by 30 cycles of 95 °C for 20 min, 58 °C for 30 min and 72 °C for 45 min. A final extension step at 72 °C for 10 min was performed.

The PCR amplicons underwent agarose gel electrophoresis to confirm that the expected size

Table 1 Primer sequences forward (F) and reverse (R) used to amplify the exons (1–15) of the sodium iodide symporter (NIS) and the size in base pairs (bp) of the resulting amplicons.

Exon	Sequence (5'–3')	Size bp
F1A/R1A	GCAGGACAGACAGACAGCAG/ATAGCGATAGGCCTCCGAC	550
F1B/R1B	CTTCGGAGCCTGGGACTAC/CTGTACAGCGGCCAAAG	418
F2/R2	CCCACCTAGAGAGCAGACCA/GCAGATAGGCCGGGTCTT	374
F/R3 & 4	CACCCTTCTGCCTCCTCTC/ CCAGAGTCTCCCCACAAGAG	386
F5/R5	GTCCTAGGCACCACTGAAGG/CTCAACTACCTGGGACCACA	477
F/R6 & 7	TCCCCTATAAGGTCCAGAAGC/TCCCTCTGCTAGGAATGCTC	553
F8/R8	TGCATATTAAATGCCGACTCTG/TCTCTAGGGATCAAGCAGGA	351
F/R9 & 10	GGAGGGGCAAATATCTCCTT/TGAGTTCTGCCACTGACACAT	513
F/R11 & 12	GTTCTTGAGGTCTCGCTTTC/ CACAGAGTGCAGGGCAAGTA	586
F13/R13	AATGGAGGTCTGTGCTAGGC/ACAGCACACAGTGGCAGTTT	368
F14/R14	GTGGGACTGGAGCCTGTTAG/ATGAGCGATGGAGAAAGGTG	351
F15/R15	AGATGAGCTGACACGGAACA/ GATCACACCACTGCACTCCA	572

Table 2 Microsatellite markers selected for the known multinodular goitre (MNG) and familial non-medullary thyroid cancer (FNMTc) loci indicating the labelling dye, location, size in base pairs (bp) of expected amplicons, and chromosomal intervals.

Locus	Markers	Dye	Location	Size (bp)	Intervals
MNG1	d14s280	Fam	90,072,907	243–263	
	d14s65	Ned	95,511,512	129–161	5 cM
	d14s985	Hex	99,386,856	244–285	4 cM
MNG2	dxs1226	Ned	22,208,848	200–381	
	dxs986	Fam	78,036,903	200–340	56 cM
	dxs1001	Hex	118,490,628	200–354	40 cM
MNG3	d3s1614	Ned	169,631,284	107–131	
	d3s1565	Fam	174,703,580	182–198	5 cM
	d3s1262	Fam	187,444,391	115–137	13 cM
FNMTc1	d19s884	Ned	7,955,955	97–117	
	d19s221	Hex	12,473,742	91–115	4 cM
	d19s226	Ned	14,394,399	241–273	2 cM
FNMTc2	d2s347	Fam	124,244,954	271–301	
	d2s112	Fam	133,319,465	79–95	9 cM
FNMTc3	d1s498	Ned	148,418,077	200–361	
	d1s484	Hex	157,884,102	276–290	9 cM

range of fragments had been obtained, and capillary electrophoresis to assess the peak height when compared with the ROX labelled molecular weight standards.

To minimise the number of reactions, 2–3 PCR products of different size ranges/differing dyes were pooled for analysis. A 10.5 µl multiplex reaction volume contained 2 µl pooled PCR products, 8 µl HiDi formamide (Applied Biosystems) and 0.5 µl Size standard (400 bp) (Applied Biosystems). The samples were size separated on an ABI 3100 Genetic Analyser, using the Genescan program (Applied Biosystems) for ‘allele calling’. A LOD score was calculated for each marker, based on 100% penetrance in women, 50% penetrance in males, dominant mode of transmission and no phenocopies.

Histopathological examination of thyroids

The thyroid tissues were formalin fixed and embedded in paraffin prior to the generation of 5–10 micron sections and standard haematoxylin and eosin staining.

Results

Sequencing candidate genes

The sequences of the coding region, intron/exon splice sites and promoter, from –1248 relative to the ‘start’ of PAX8 were identical in the unaffected father (III 1) and the affected son (IV 1). Furthermore, the sequences of both family members were

compared with those for PAX8 available in the NCBI database, www.ncbi.nlm.nih.gov (Accession numbers, NM_003466, BC001060, L19606.1, AB062505 and X69699). The comparison has detected three different polymorphisms, including in the promoter region. One version has four missing base pairs, GGGC or GCGG at position –479. Both members of the family tested were homozygous for this deletion polymorphism.

Similar results were obtained for NIS, with the sequences of the unaffected father (III 1) and the affected mother (III 2) being identical. Four reported sequences of NIS were identified in the NCBI database (Accession numbers, HSU66088, NM_000453.1, AC005796 and D87920.1). The first three, published by USA authors, are identical and match the sequences of NIS obtained in the two family members investigated. The fourth is published by Japanese authors and reports several polymorphisms, the most notable being (1) a G to C transition changing proline 102 to alanine; (2) an AC to CA transversion at 536 (in the 2nd and 3rd bases) which changes thymine to glutamine; (3) substitution of CAG by AGC at 556 which changes serine to glutamine; and (4) 120 bases missing from the 3′ non-coding region (the last part of exon 15) between 1206 and 1086 base pairs before the polyA tail.

Microsatellite analysis

Microsatellite analysis produced a non-significant LOD score for all six loci tested, as shown in Table 3.

Table 3 Non-significant LOD scores obtained for each marker; calculation based on 100% penetrance in women, 50% penetrance in males, dominant mode of transmission and no phenocopies.

MNG 1 (d14)			FNMTC1 (d19)			MNG2 (dx)			FNMTC2 (d2)			MNG3 (d3)			FNMTC3 (d1)		
Location	LOD	Marker	Location	LOD	Marker	Location	LOD	Marker	Location	LOD	Marker	Location	LOD	Marker	Location	LOD	Marker
0.000	-2.0480	D14s280	0.000	-0.5142	D19s884	0.000	-1.5101	dXs1226	0.000	-1.9196	D2s112	0.00	-1.92656	D3s1614	0.00	-2.0603	D1S498
6.150	-1.9844		4.925	-0.4719		30.095	0.1659		5.055	-1.7805		4.145	-1.8167		6.895	-0.5565	
12.300	-4.4044	D14s65	9.850	-0.5142	D19s221	60.190	-1.4323	dXs986	10.110	-1.9195	D2S347	8.290	-1.9293	D3s1565	13.79	-0.2464	D1S484
16.955	-3.9126		12.880	-0.5170		78.825	-0.6295					15.840	-1.446				
21.610	-4.3860	D14s985	15.910	-0.5531	D19s226	97.46	-3.5824	dXs1001				23.390	-1.9392	D3s1262			

Further evidence for the exclusion of these loci as containing genes predisposing to familial MNG or FNMTC was provided by detailed examination of the alleles inherited. In some instances affected individuals inherited different alleles from their affected mother. In contrast, the same maternal alleles were inherited by non-affected and affected children. FNMTC1 (d19) is used as an example to illustrate this in Fig. 2.

Histopathological examination of thyroid glands

Thyroid tissue was available from three members of the family. The findings in the thyroidectomy specimens from the propositus (IV 2), and the first specimen from her mother (III 2) were essentially similar, with multiple benign nodules composed of colloid-rich follicles often with some papillary infolding. The second specimen from the mother showed a papillary carcinoma (follicular variant) throughout the resected tissue, also present in the total thyroidectomy specimen. The thyroidectomy specimen from the brother (IV 1) showed benign adenomas similar to his sister, but no evidence of papillary carcinoma, as had been detected in the FNA. The nuclear features of papillary carcinoma that were present in the latter two specimens were absent from the benign lesions. The lesions arose in otherwise normal thyroid tissue, they were not oxyphil, and did not show the features of FAP-associated tumours.

Discussion

In a population with adequate iodine intake, nodular goitre in children is extremely rare. The fact that nodular goitre presented in childhood in this family, coupled with the uniformity of gland histology (in those cases where resected material was available) justifies the assumption that this is a genetically determined condition.

Initially, genes that were candidates on the basis of the accompanying kidney (PAX8) and breast diseases (NIS) were examined. In both PAX8 and NIS the sequences of an affected and a non-affected family member were identical, thereby eliminating these genes as a cause. Of interest, a number of polymorphic variations for each of the two genes were identified. In the case of PAX8, the promoter region varies by four base pairs. Promoter polymorphisms have been shown to influence transcription rates, although this is

FNMTC1

Figure 2 Microsatellite analysis of FNMTC1. Individuals III 3 and III 2 inherited the same maternal (II 2) allele; III 3 is not affected. Individuals IV 5 and IV 1 inherited different maternal (III 2) alleles; both are affected.

unlikely to be of relevance to the family. Some of the NIS polymorphisms have the capacity to alter the biological activity of the symporter or to modulate the stability of the mRNA. It would be interesting to compare the iodide trapping capabilities of the variant NIS forms.

Six loci were investigated using microsatellite analysis and in all cases we obtained LOD scores <1. This may be partly explained by the relatively small number of individuals analysed ($n = 14$). However, for each locus, different 'affected' maternal alleles were inherited by affected offspring or first degree relatives, providing a further exclusion criterion.

Although several thyroid-specific genes were eliminated by our investigations, linkage analysis of MNG1 did not exclude thyroid transcription factor 1 (TTF1) as a cause of the phenotype. TTF1 is located between 34,978,942 and 34,975,641 on chromosome 14, while the MNG1

loci are at 90,072,907 to 99,386,856 on the same chromosome.

Whilst these results were being analysed, four further loci for euthyroid MNG were reported on chromosomes 2q, 3p, 7q and 8p, based on a dominant model with reduced penetrance (Bayer *et al.* 2004). The study also highlighted several features of the disease including its heterogeneity and possible polygenic nature. The cohort comprised multiple unrelated families with benign disease, unlike the present study of a single kindred with a consistent morphological phenotype, implying a homogeneous pathogenetic process.

The role of previously reported somatic rearrangements and mutations identified in sporadic thyroid cancer was also considered. Karyotype analysis of two affected family members (IV 1 and 2) revealed no gross chromosomal abnormality, thereby eliminating translocations as the cause. We would expect germline mutations in RAS or

BRAF to produce a more severe phenotype, as these oncogenes are involved in a wide range of malignancies.

Future studies aimed at identifying the genetic cause of MNG and papillary thyroid carcinoma in the family could include whole-genome linkage analysis. This could be performed using microsatellite markers but recent reports suggest that more information and higher LOD scores can be obtained using single nucleotide polymorphisms to identify loci (Evans & Cardon 2004). Through a combination of genetic analysis and morphological criteria, we conclude that the gene(s) predisposing to MNG and FNMTC in this family are different from those described in other kindred.

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