An InDel in Phospholipase-C-B-1 is linked with euthyroid multinodular goiter

*Ameen D Bakhsh,[1] *Ioannis Ladas,[1] Marian L Hamshere,[2] Martyn Bullock,[3] George Kirov,[2] Lei Zhang,[1] Peter N Taylor,[1] John W. Gregory,[1] David Scott-Coombes,[4] Henry Völzke,[6] Alexander Teumer,[6] Kiran Mantripragada,[2] E Dillwyn Williams,[5] Roderick J Clifton-Bligh,[3] Nigel M Williams,[2] Marian E Ludgate,[1].

* Authors contributed equally [1]**Division of Infection & Immunity, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK.** [2]**Institute of Psychological Medicine & Clinical Neurosciences, School of Medicine, Cardiff University, Cardiff CF24 4HQ. UK.** [3]**Kolling Institute of Medical Research and Dept Endocrinology, University of Sydney, Royal North Shore Hospital, Sydney, NSW 2065, Australia.** [4]**Department of Endocrine Surgery, University Hospital of Wales, Cardiff CF14 4XW, UK.** [5]**Strangeways Research Laboratory, Cambridge CB1 8RN, UK.** [6]**Institute for Community Medicine, Study of Health in Pomerania, Ernst Moritz Arndt University, D-17475 Greifswald, Germany.**

Corresponding Author & address for reprints:
Dr. Ioannis Ladas
Harvard Institutes of Medicine,
Department of Radiation Oncology,
Dana Farber Cancer Institute,
4 Blackfan Circle, Lab 342 - Boston, MA
Phone +1 9786895751; email Ioannis_Ladas@dfci.harvard.edu

Abbreviated title: PLCB1 Intronic deletion linked with MNG

Key Words: Multinodular goiter; genome-wide linkage analysis; copy-number variation; next generation sequencing

Word Count: 3731 [excluding abstract, references & legends]

Abstract

Euthyroid multinodular goiter (MNG) is common but little is known about the genetic variation conferring predisposition. Previously we reported a family with MNG of adolescent onset in which some family members developed papillary thyroid carcinomas (PTC). We conducted a genome-wide linkage analysis and next generation sequencing to identify genetic variants that may confer disease predisposition. A multipoint nonparametric LOD score of 3.01 was obtained covering 19 cM on chromosome 20p. Haplotype analysis reduced the region of interest to 10 cM; analysis of copy number variation identified an intronic InDel (~1000 bp) in the *PLCB1* gene in all 8 affected family members and carriers (an unaffected person who has inherited the genetic trait); this InDel is present in ~1% of 'healthy' Caucasians. Next generation sequencing of the region identified no additional disease-associated variant, suggesting a possible role of the InDel. Since *PLCB1* contributes to thyrocyte growth regulation, we investigated the InDel in relevant Caucasian cohorts. It was detected in 0/70 PTC but 4/81 unrelated subjects with MNG [3 F, age at thyroidectomy 27-59 years, no family history of MNG/PTC]. The InDel frequency is significantly higher in MNG subjects compared with controls; $X^2 = 5.076$, $p = 0.024$. *PLCB1* transcript levels were significantly higher in thyroids with the InDel than without ($p<0.02$).

The intronic *PLCB1* InDel is the first variant found in familial multiple papilloid adenomata-type MNG and in a subset of patients with sporadic MNG. It may function through over-expression and increased PLC activity has been reported in thyroid neoplasms. The potential role of the deletion as a biomarker to identify MNG patients more likely to progress to PTC merits exploration.

Introduction

Euthyroid multinodular goiter (MNG) is common and affects at least 4% of the population, although the prevalence varies with ethnicity and the detection method employed (1). Furthermore, nodular goiter is far more prevalent in iodine deficient regions (2). Although solitary nodules are considered a risk for thyroid cancer (3) the situation for MNG is more controversial (4); the reported increase in the incidence of some thyroid cancers (5) may, in part, be due to increased use of diagnostic tools (6). *BRAF* mutations causing constitutive activation are the most frequent driver of papillary thyroid cancer (PTC) (7). Several genetic variations lead to sporadic thyroid cancers including, among others, *RET* chromosomal re-arrangements (8), translocations between chromosome 2 and 3 generating a PPARγ-PAX8 fusion protein (9), mutations in *RAS* genes (10) and poly-alanine tract length variation in *FOXE1* (11, 12).

Familial non-medullary thyroid cancers account for about 5% of thyroid cancers and have a younger age of onset than sporadic disease. They are associated with 4 susceptibility loci (13-16) on chromosomes 19p13.2, 2q21, 1q21 and 10q23 (*PTEN*). There is some overlap with familial goiter in which 8 predisposing loci have been identified (12, 17-20) on chromosomes Xp22, 3q26, 2q, 3p, 7q, 8p 14q13.3 and 14q32, the last two including the *NKX2.1* (21) and the RNAse *DICER1* genes respectively (22). A role for the predisposing loci on chromosomes 2q.35, 5q.24, 8p.12 and 14q.13 has been confirmed in Chinese families (23). Genes implicated in familial goiter and cancer generally differ from those in sporadic disease, with the exception of *NKX2.1* (21) and *FOXE1* (24).

Previously, we reported a family (25) exhibiting a type of euthyroid MNG with papillary adenomas of adolescent onset affecting 8 individuals in 4 generations to date. MNG is known to have progressed to PTC in 2 of the 8 affected family members. We applied microsatellite analysis to exclude loci described above on chromosomes 14q, Xp, 3q 9p, 2q and 1q. Since one family member had co-existing breast cancer and another co-existing kidney disease we investigated genes co-expressed in these tissues and the thyroid, *NIS* and *PAX8* respectively. Sanger sequencing revealed no abnormality in either gene. Subsequently, the *PTEN* gene has been fully sequenced in the family member with breast cancer and no mutations were detected.

The aim of this study was to apply genome-wide linkage analysis (GWLA) and next generation sequencing to identify the gene variant(s) responsible for the observed phenotype in this family. We then aimed to assess the frequency of any variant(s) detected in other relevant cohorts.

Subjects and Methods

Genome-Wide Linkage Analysis (GWLA)

We undertook a GWLA of the family described in (25) and summarized in figure 1.

All patient samples were obtained with informed consent and Local Research Ethics Committee (LREC) approval. Genomic DNA was extracted from whole blood from 18 family members (those labelled in the tree) of whom 8 were affected (7 females, 1 male), according to the manufacturer's instruction (Qiagen) and quantified using a Nanodrop. Samples (250 ng) were processed following the manufacturer's protocol and the DNA integrity monitored by agarose gel electrophoresis before being hybridized at 48°C for 18 hours to Affymetrix Genechip™ Human Mapping 10K 2.0 Arrays. The chips were scanned using an Affymetrix GeneChip scanner 3000; data were acquired using GCOS and analyzed using GTYPE software respectively.

Two quality control steps were performed; the first eliminated SNPs showing 'no call' in more than 4 individuals. The second step would have eliminated data from any individual with >10% 'no calls', but this did not apply and the data of all 18 family members were retained. Graphical Representation of Relationships (GRR) software was used to determine how many alleles are shared [identity by state (IBS)] at each locus. Mendelian errors were tested using PedCheck software. PLINK, was used to merge family data (founders) with HapMap to investigate ethnicity. Multidimensional scaling (MDS) was performed on the family merged with HapMap data from 60 European individuals (CEU), 90 Chinese (CHB) & Japanese (JPT), & 60 Yoruba (YRI). The family were closest to the European cluster (data not shown) thus allele frequencies were based on CEU HapMap data. Using MERLIN software, the primary analysis was multi-point non-parametric and the secondary analysis multipoint parametric dominant mode assuming 90%

penetrance in females, 50% in males and age of onset later than 12 years (based on clinical information summarized in figure 1). Single point analyses were also used to support the findings of multipoint analysis. Since data are derived from a single large family, there is considerable allele sharing and hence the Kong and Cox exponential (--exp) model was used (for non-parametric analysis) (26).

Haplotype Analysis

MERLIN software (--best) was also used to perform a haplotype analysis in the region of maximum LOD score on chromosome 20. The haplotype was also confirmed manually.

Copy Number Variation Analysis (CNV)

Genomic DNA for CNV analysis of the index patient was quantified and prepared for hybridization to Illumina Human 660W-Quad BeadChips according to the manufacturer's instructions. Data were analyzed using PennCNV (27) software; CNVs were required to be 1 kb long and cover at least 10 consecutive markers (SNP or cnvi) to be considered positive. We focused on the region with a high LOD score identified in the GWLA.

Next Generation Sequencing (NGS)

Primer pools for preparation of DNA libraries were designed using Ampliseq 3.0.1 software (https://ampliseq.com/) according to the manufacturer's protocol. A total of 429 primers were designed generating 100-300 bp amplicons. The primer pools (details in supplemental table 2) covered the exome sequences (all coding regions, intron/exon boundaries, proximal promoters and 3' untranslated regions) of a region spanning from chr20: 8113337 to 11907302. Approximately 10 ng of the genomic DNAs of interest were amplified according to the manufacturer's instructions. The amplified samples were partially digested by FuPa reagent (Life Technologies) and ligated with barcode/adapter mix. DNA libraries were then purified using Agencourt AMPure XP beads (Beckman Coulter), quantified by qPCR and adjusted to a final concentration of 100 pM, combined and prepared for Emulsion PCR with Ion OneTouch 2 (Life

Technologies). Following enrichment, the ion sphere particles were loaded onto an Ion PI Chip V2 and sequenced by Ion Torrent Proton sequencer. Sequencing data were analyzed by Ion Torrent Suite software (4.4.2), using the plug-in variant caller (v 4.2.10) and configuration with generic Personal Genome Machine (PGM) germ line settings and high stringency analysis mode.

NGS was performed on 98 individuals, all 18 family members plus 80 unrelated subjects with MNG (please see below).

Other variants identified in the family using NGS were interrogated in the SHIP cohort (Study of Health in Pomerania) (28). Relevant genotyping data were available from 986 individuals who were either unaffected or presented with diffuse goiter (as defined in (29)) and/or MNG (nodules identified by ultrasound). Figure 2 details the filtering steps and evaluations undertaken to assess whether detected variants might be linked with disease.

Defining deletion frequency

Primers within and flanking the deleted region were designed using Primer 3 software (supplemental table 2) for PCR amplification of genomic DNA from all family members and 105 unrelated euthyroid individuals from the UK. PCR amplicons were analyzed by agarose gel electrophoresis and PEG precipitated for Sanger sequencing using Big Dye Terminator Cycle Sequencing Ready Reaction (ABI Prism, PE Biosystems) and analysis on an ABI 3100 Genetic Analyser.

Tissues from patients recruited in Australia (snap frozen and stored in liquid nitrogen) were also studied and consisted of 70 PTC and 81 MNG patients. [Ethics approval from the Northern Sydney Area Health Service Human Research Ethics Committee]. To avoid population stratification, only subjects with self-reported white European ancestry were included; patient data and tissues were collected between 1992 and 2012 at the Kolling Institute of Medical Research. Genomic DNA for genotyping was obtained from thyroid tissue using Qiagen kits and analyzed by PCR and Sanger sequencing as described above; these samples also underwent NGS.

High Throughput Screening of PLCB1 InDel, analysis of additional cohorts.

We developed a qPCR based genotyping tool using primers within and flanking the *PLCB1* InDel as described above (Supplementary table 2). The genotyping tool was used to screen 200 breast cancer patients. Initial optimization experiments revealed that greatest specificity was obtained using primers flanking the InDel. The qPCR obtained a difference of approximately 10 Ct for samples with and without the InDel. The qPCR was performed with approximately 100 ng Genomic DNA Input, 1x SyBR green master qPCR mix (Invitrogen) and 100 nM of each primer in a 25 µl reaction. QPCR conditions included an initial hold step at 50°C for 2 minutes, then 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds then a hold step at 95°C for 1 minute, 55°C for 30 seconds and 95°C for 30 minutes. Samples found to harbor the InDel by qPCR were confirmed by Sanger sequencing.

Transcript measurements of PLCB1 isoforms

Thyroid tissue was obtained from 3 affected family members heterozygous for the InDel and five subjects undergoing thyroidectomy for autoimmune thyroid disease expressing two normal *PLCB1* alleles (all confirmed by genotyping). Thyroid RNA was extracted, reverse transcribed using standard protocols and qPCR (SYBR Green incorporation measured on a Stratagene MX 3000) was used to measure transcript levels and evaluate proportions of *PLCB1-a* and *PLCB1-b* isoforms (primers in supplemental table 2, wild type amplicon identity confirmed by Sanger sequencing). Comparison with standard curves for transcript levels of isoform 1a and 1b permitted calculations of absolute values for each sample. Transcripts for a housekeeping gene (*APRT*) were also measured and values were expressed relative to this (transcripts/1000 *APRT*). In a single qPCR experiment, all measurements were made in duplicate; the standard curve was also run in each reaction. Transcript levels of the various *PLCB1* isoforms were compared between deletion affected and non-affected thyroids using the Mann Whitney U test and differences where $p<0.05$ taken to be significant.

Results

Genome wide linkage, haplotype & copy number variation analyses

We obtained a multipoint nonparametric LOD score of 3.01 over 19.5 cM on chromosome 20p (figure 3 and supplementary figure 1). In secondary analysis, the same region gave a multipoint dominant LOD score of 2.16, based on a disease model with 0.01 allele frequency, 50% penetrance for males and 90% for females, both age >12. LOD scores on the remaining 21 autosomes and X chromosome were all below 1 (figure3). Single-point analyses supported the multipoint data for both nonparametric and model-based linkage on all chromosomes (supplementary table1).

Haplotype analysis was employed to identify a possible disease locus and reduced the region of interest to 8.73 cM (3.7 Mbp), which includes 10 genes (supplemental figure 2 and 3). The haplotype was not found in 503 individuals from the 1000 genome European dataset, although one individual missed only the last marker suggesting a shorter version of the haplotype (red highlight in supplementary figure 3a).

Analysis of copy number variation in an affected individual revealed a deletion of ~900 bp located in the 3rd intron in one copy of *phospholipase-C B1 (PLCB1)* in the region of interest (supplementary figure 4; the log R ratio mean was -0.451, over 14 markers, with at least one marker below -1.00).

Defining the deletion frequency in the family and selected cohorts

The length of the deletion was confirmed to be 1077 bp by standard PCR and Sanger sequencing, using primers flanking and within the deletion, to reveal one copy of full-length and one deleted allele in all affected and obligate carrier II-3 but only the full-length product in family members free of any sign of MNG. The sequence of the allele bearing the deletion corresponds to that immediately upstream and downstream of the deleted region but with an additional 'ATAA' inserted at the junction, hence it is an InDel.

Standard PCR was applied to genotype a selected cohort of 105 Caucasians in whom thyroid function testing was clinically indicated because of general fatigue. A woman in her forties, with no history of thyroid disease, was heterozygous for the InDel. Further *in silico* analyses, using the database for genomic variants (30) identified a report which detected the InDel (variation 67651, LRR -0.645) in 2 of 180 Caucasians but none in more than 450 people of other ethnicities (31). Combining our genotyping data with that of Conrad *et al*. (31) reveals 3 in 285 Caucasians harboring the InDel, suggesting that it is relatively rare (~1%).

Subsequently, genomic DNA was extracted from thyroid tissue from 70 patients undergoing surgery for non-familial PTC and an additional 81 operated for non-familial MNG. We used PCR analysis to test for the InDel, as described above. The InDel was not detected in any of the PTC patients but 4 of the 81 MNG were heterozygous for the InDel and sequencing revealed the same ATAA insertion at the junction. Comparison of the frequency of the InDel in the general population with that in MNG gives a X^2 value of 5.076 (1 degree of freedom), p= 0.024 (two-tailed). The 4 MNG patients (3 women, 1 man) are unrelated and with no apparent family history of MNG or PTC at the time of their surgery. The age at thyroidectomy was between 27 to 59 years and the pathology is variously described as ‘oncocytic neoplasm with variable patterns of growth’ to ‘cystic degeneration with calcification’. We also investigated whether the *PLCB1* InDel might be implicated in breast cancer using the qPCR-based screening protocol. Prevalence in this cohort was similar to that of the general population, i.e. 1%, since just 2 breast cancer patients harbored the *PLCB1* InDel.

Next Generation Sequencing of the Chr20 high LOD score region

The Proton Sequencer generated 9.9 Gbp of data, achieving 98% accurately mapped sequences with >88% of the percentage of target bases covered by at least 0.2 times the average base read depth.

A total of 181 sequence variants between Chr20 8113405 and 11907285 were identified in the family with the minor allele being on the disease risk haplotype in 12 of these. Given the rarity of PTC and the expected high penetrance, we expect a pathogenic variant to have a very low population frequency. After referring to the UCSC genome browser, only 1 of the 12 variants was found to have a minor allele frequency <1%; its presence in affected family members was confirmed by Sanger sequencing. The variant is at Chr20

10036484 (rs56234782) with T (98.8%) or C (1.2%) in the 3' UTR of the *ANKRD5* gene. To investigate whether it is implicated in goiter and/or thyroid nodule formation, we investigated its frequency in the SHIP cohort. However, even though the minor allele was more prevalent in the entire cohort, the prevalence in the affected population (goiters 1.9% and nodules 2.54%) was lower than in the unaffected populations (2.79% and 2.85% respectively), thereby excluding a role for it in MNG.

The MNG cohort was also submitted to NGS analysis. This identified more than 300 different sequence variants across the 80 patients, however, all were also present in the 1000 genomes cohort at a population frequency >1%. We therefore considered it unlikely that any of these variants are pathologically relevant to MNG, thereby confirming the relevance of the InDel.

Transcript measurements of PLCB1 & effect of knock-down on thyroid growth

Having confirmed that the InDel may contribute to the pathogenesis for MNG (perhaps in combination with other factors), we investigated how it might promote thyrocyte proliferation. The InDel is in the large 3rd intron of *PLCB1*, the phosphoinositide-specific enzyme which generates IP3 and DAG leading to PKC activation and also links signaling between the MAPK cascade and G protein coupled receptors (32). *PLCB1* is present in several isoforms including *PLCB1-a* and *PLCB1-b*, with the latter having a predominantly nuclear location (33). To test the hypothesis that the InDel causes preferential transcription of certain *PLCB1* isoforms, RNA was extracted from thyroids from the original family and from subjects undergoing thyroidectomy for benign disease. In all cases genomic DNA from the donor thyroid was tested for the *PLCB1* deletion.

QPCR analysis of InDel-affected thyroids did not indicate altered expression of the major *PLCB1* isoforms a and b (sequenced to confirm they were wild type, data not shown). However, qPCR measurements indicated significantly higher *PLCB1* transcript levels ($p < 0.02$) in thyroids from family members with the InDel, compared with those from benign thyroid disease who do not harbor the variant (figure 5). Lack of thyroid tissue precluded analyzing PLCB1 protein levels.

Discussion

Our GWLA led to the identification of an InDel in the family with a type of MNG, located in the large third intron of *PLCB1*, a gene encoding an enzyme with a central role in several signaling cascades involved in regulating thyrocyte growth. Subsequent NGS in the family failed to identify any other disease-linked variant, thus supporting a role for the *PLCB1* InDel in the pathogenesis of MNG in this family.

The InDel comprises the loss of 1077 bp with an ATAA inserted at the junction in all affected family members and the 4 unrelated patients with MNG. We suggest that this may indicate a 'cut and paste' event indicating transposon activity. Interestingly, a 11-kb transposon cluster has been identified immediately upstream of the 3.7 Mbp section on chr 20 displaying a non-parametric LOD score of 3.01 in the current study (34). Of note the LOD score of 3.01, whilst at the lower limit to be considered significant, is higher than the maximum estimated for a kindred having 8 affected individuals (35).

We detected the same InDel in 1 subject of a selected cohort of 105 people in whom measuring thyroid function was clinically indicated. We also consulted the database of genomic variants and found several reports of relevance. Conrad et al. found the deletion in 2 of 180 Caucasians but insufficient detail is provided to know whether it is a simple CNV or the same InDel identified in our studies. Combining our genotyping data with that of Conrad et al. reveals that 3 in 285 Caucasians harbor the deletion, suggesting that it is rare (31). Several other authors did not observe this deletion, but aware of the difficulty in detecting small CNVs, we did not include these in our calculation. In addition, 200 patients with breast cancer have been screened for the InDel with only two harboring this deletion. Hence, the prevalence was similar to the general population suggesting that there is no connection of the InDel with breast cancer.

We then considered how the deletion or novel *PLCB1* InDel might exert its effects. The region was explored using the Encyclopedia of DNA elements (ENCODE) (although compiled without inclusion of thyroid tissue or cell lines) (36), which revealed the existence of a binding site for the estrogen receptor alpha (ERα) within the deletion. This is of potential importance since all thyroid diseases are more prevalent in women than men (1). The incidence of thyroid disorders increases in the years immediately following puberty and *in vitro* studies have demonstrated that estrogen can promote thyrocyte proliferation (37) by several mechanisms. The *PLCB1* InDel is located in an intron; while many functional transcription factor binding sites are found in promoters, a systematic search for ERα binding sites in the human genome identified >1000 with >95% of them residing in introns and not promoters (38).

We also conducted experiments to determine whether the deletion alters the ratio of *PLCB1-a* and *PLCB1-b*, which are generated by alternative splicing. Differences in their C terminal sequence mean that only *PLCB1-a* has a nuclear export signal. We found no alteration in the ratio of *PLCB1-a* and b isoforms but in all cases transcript levels for *PLCB1* were higher in thyroids from people heterozygous for the InDel than in thyroids with two full-length copies. This suggests that the InDel may contribute to MNG development through overexpression of *PLCB1*. Furthermore, total PLC enzyme activity is elevated in thyroid neoplasms (39) but unfortunately PLC inhibitors lack the specificity required to identify which isoform is responsible. Increased *PLCB1* expression has also been reported in small cell lung carcinoma (40) and expression of *PLCB2* is substantially increased in breast cancer and is used as a prognostic marker (40).

As mentioned above, PLC enzymes activate PKC and genes implicated in this signal pathway are upregulated in euthyroid MNG (41). They also link signaling via Gq (which can also be activated via the thyrotropin receptor) to the MAPK cascade and in the thyroid disruption of this pathway, by thyrocyte-targeted Cre/Lox P knock-down of the Gqα subunit, produces mice which are resistant to goiter formation when fed a goitrogenic diet (42). However, when we performed western blots with protein extracts of thyroid tissue from family members with the *PLCB1* InDel we were surprised to observe that pMAPK levels were substantially lower than in thyroid tissue from patients with autoimmune thyroid disease or MNG without the *PLCB1* InDel (Supplementary Figure 5).

In conclusion, the *PLCB1* InDel identified in this family with MNG also occurs in a proportion of sporadic MNG, and may provide a biomarker to identify MNG patients more likely to progress to PTC. The *PLCB1* InDel appears to predispose to goiter formation, possibly by increasing *PLCB1* transcription with subsequent downstream effects.

Supplemental Data

The supplemental data comprises 5 figures and 2 tables;

Supplemental Figure 1; LOD scores of all Chromosomes

Supplementary Figure 2; Genes in high LOD score region chromosome 20

Supplemental Figure 3; Haplotype Frequency in 1000 genomes European dataset

Supplementary Figure 4; Copy number variation in high LOD score region chromosome 20

Supplementary Figure 5; Densitometry ratios for pERK/total ERK

Supplemental Table 1; Single point LOD scores all chromosomes

Supplemental Table 2; Primers used for NGS and to define deletion frequency

Web Resources

The March 2006 human reference sequence (NCBI Build 36.1) produced by the International Human Genome Sequencing Consortium, was used as a reference genome (UCSC Genome Browser;http://genome-euro.ucsc.edu/cgi-bin/hgGateway?hgsid=192302910&clade=mammal&org=Human&db=hg18).

Acknowledgements

We express our sincere gratitude to the members of the family who participated in this research.

The work was part funded by the Government of Saudi Arabia (ref A390), by the Medical Research Council and the Onassis Foundation.

SHIP is part of the Community Medicine Research Network of the University Medicine Greifswald, Germany (www.community–medicine.de).

Genomic DNA from patients with breast cancer was provided by Dr Florentia Fostira from the National Center for Scientific Research Demokritos (Athens, Greece).

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

References

1. Vanderpump MP, Tunbridge WM, French JM, Appleton D, Bates D, Clark F, Grimley Evans J, Hasan DM, Rodgers H, Tunbridge F 1995 The incidence of thyroid disorders in the community: a twenty-year follow-up of the Whickham Survey. Clin Endocrinol 43:55-68.

2. Carle A, Krejbjerg A, Laurberg P 2014 Epidemiology of nodular goitre. Influence of iodine intake. Best Pract Res Clin Endocrinol Metab 28:465-479.

3. Frates MC, Benson CB, Doubilet PM, Kunreuther E, Contreras M, Cibas ES, Orcutt J, Moore FD, Jr., Larsen PR, Marqusee E, Alexander EK 2006 Prevalence and distribution of carcinoma in patients with solitary and multiple thyroid nodules on sonography. J Clin Endocrinol Metab 91:3411-3417.

4. Fiore E, Rago T, Provenzale MA, Scutari M, Ugolini C, Basolo F, Di Coscio G, Berti P, Grasso L, Elisei R, Pinchera A, Vitti P 2009 Lower levels of TSH are associated with a lower risk of papillary thyroid cancer in patients with thyroid nodular disease: thyroid autonomy may play a protective role. Endocr Relat Cancer 16:1251-1260.

5. Kilfoy BA, Zheng T, Holford TR, Han X, Ward MH, Sjodin A, Zhang Y, Bai Y, Zhu C, Guo GL, Rothman N, Zhang Y 2009 International patterns and trends in thyroid cancer incidence, 1973-2002. Cancer Causes Control 20:525-531.

6. La Vecchia C, Negri E 2017 Thyroid cancer: The thyroid cancer epidemic - overdiagnosis or a real increase? Nat Rev Endocrinol 13:318-319.

7. Kimura ET, Nikiforova MN, Zhu ZW, Knauf JA, Nikiforov YE, Fagin JA 2003 High prevalence of BRAF mutations in thyroid cancer: Genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res 63:1454-1457.

8. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Dellaporta G, Fusco A, Vecchio G 1990 PTC is a novel rearranged form of the RET proto-oncogene and is frequently detected in-vivo in human thyroid papillary carcinomas. Cell 60:557-563.

9. Kroll TG, Sarraf P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA 2000 PAX8-PPAR gamma 1 fusion in oncogene human thyroid carcinoma. Science 289:1357-1360.

10. Lemoine NR, Mayall ES, Wyllie FS, Farr CJ, Hughes D, Padua RA, Thurston V, Williams ED, Wynfordthomas D 1988 Activated RAS oncogenes in human thyroid cancers. Cancer Res 48:4459-4463.

11. Gudmundsson J, Sulem P, Gudbjartsson DF, Jonasson JG, Sigurdsson A, Bergthorsson JT, He H, Blondal T, Geller F, Jakobsdottir M, Magnusdottir DN, Matthiasdottir S, Stacey SN,

Skarphedinsson OB, Helgadottir H, Li W, Nagy R, Aguillo E, Faure E, Prats E, Saez B, Martinez M, Eyjolfsson GI, Bjornsdottir US, Holm H, Kristjansson K, Frigge ML, Kristvinsson H, Gulcher JR, Jonsson T, Rafnar T, Hjartarsson H, Mayordomo JI, de la Chapelle A, Hrafnkelsson J, Thorsteinsdottir U, Kong A, Stefansson K 2009 Common variants on 9q22.33 and 14q13.3 predispose to thyroid cancer in European populations. Nat Genet 41:460-464.

12. Bullock M, Duncan EL, O'Neill C, Tacon L, Sywak M, Sidhu S, Delbridge L, Learoyd D, Robinson BG, Ludgate M, Clifton-Bligh RJ 2012 Association of FOXE1 polyalanine repeat region with papillary thyroid cancer. J Clin Endocrinol Metab 97:E1814-1819.

13. Canzian F, Amati P, Harach HR, Kraimps JL, Lesueur F, Barbier J, Levillain P, Romeo G, Bonneau D 1998 A gene predisposing to familial thyroid tumors with cell oxyphilia maps to chromosome 19p13.2. Am J Hum Genet 63:1743-1748.

14. Malchoff CD, Sarfarazi M, Tendler B, Forouhar F, Whalen G, Joshi V, Arnold A, Malchoff DM 2000 Papillary thyroid carcinoma associated with papillary renal neoplasia: genetic linkage analysis of a distinct heritable tumor syndrome. J Clin Endocrinol Metab 85:1758-1764.

15. McKay JD, Lesueur F, Jonard L, Pastore A, Williamson J, Hoffman L, Burgess J, Duffield A, Papotti M, Stark M, Sobol H, Maes B, Murat A, Kaariainen H, Bertholon-Gregoire M, Zini M, Rossing MA, Toubert ME, Bonichon F, Cavarec M, Bernard AM, Boneu A, Leprat F, Haas O, Lasset C, Schlumberger M, Canzian F, Goldgar DE, Romeo G 2001 Localization of a susceptibility gene for familial nonmedullary thyroid carcinoma to chromosome 2q21. Am J Hum Genet 69:440-446.

16. Frisk T, Foukakis T, Dwight T, Lundberg J, Hoog A, Wallin G, Eng C, Zedenius J, Larsson C 2002 Silencing of the PTEN tumor-suppressor gene in anaplastic thyroid cancer. Genes Chromosom Cancer 35:74-80.

17. Capon F, Tacconelli A, Giardina E, Sciacchitano S, Bruno R, Tassi V, Trischitta V, Filetti S, Dallapiccola B, Novelli G 2000 Mapping a dominant form of multinodular goiter to chromosome Xp22. Am J Hum Genet 67:1004-1007.

18. Takahashi T, Nozaki J, Komatsu M, Wada Y, Utsunomiya M, Inoue K, Takada G, Koizumi A 2001 A new locus for a dominant form of multinodular goiter on 3q26.1-q26.3. Biochem Biophys Res Commun 284:650-654.

19. Bayer Y, Neumann S, Meyer B, Ruschendorf F, Reske A, Brix T, Hegedus L, Langer P, Nurnberg P, Paschke R 2004 Genome-wide linkage analysis reveals evidence for four new susceptibility loci for familial euthyroid goiter. J Clin Endocrinol Metab 89:4044-4052.

20. Bignell GR, Canzian F, Shayeghi M, Stark M, Shugart YY, Biggs P, Mangion J, Hamoudi R, Rosenblatt J, Buu P, Sun S, Stoffer SS, Goldgar DE, Romeo G, Houlston RS, Narod SA, Stratton MR, Foulkes WD 1997 Familial nontoxic multinodular thyroid goiter locus maps to chromosome 14q but does not account for familial nonmedullary thyroid cancer. Am J Hum Genet 61:1123-1130.

21. Barnett CP, Mencel JJ, Gecz J, Waters W, Kirwin SM, Vinette KMB, Uppill M, Nicholl J 2012 Choreoathetosis, congenital hypothyroidism and neonatal respiratory distress syndrome with intact NKX2-1. Am J Med Genet Part A 158A:3168-3173.

22. Frio TR, Bahubeshi A, Kanellopoulou C, Hamel N, Niedziela M, Sabbaghian N, Pouchet C, Gilbert L, O'Brien PK, Serfas K, Broderick P, Houlston RS, Lesueur F, Bonora E, Muljo S, Schimke RN, Bouron-Dal Soglio D, Arseneau J, Schultz KA, Priest JR, Nguyen V-H, Ruben Harach H, Livingston DM, Foulkes WD, Tischkowitz M 2011 DICER1 Mutations in Familial Multinodular Goiter With and Without Ovarian Sertoli-Leydig Cell Tumors. J Am Med Assoc 305:68-77.

23. Liao S, Song W, Liu Y, Deng S, Liang Y, Tang Z, Huang J, Dong D, Xu G 2013 Familial multinodular goiter syndrome with papillary thyroid carcinomas: mutational analysis of the associated genes in 5 cases from 1 Chinese family. BMC Endocr Disord 13:48.

24. Tomaz RA, Sousa I, Silva JG, Santos C, Teixeira MR, Leite V, Cavaco BM 2012 FOXE1 polymorphisms are associated with familial and sporadic nonmedullary thyroid cancer susceptibility. Clin Endocrinol 77:926-933.

25. Bakhsh A, Kirov G, Gregory JW, Williams ED, Ludgate M 2006 A new form of familial multi-nodular goitre with progression to differentiated thyroid cancer. Endocr Relat Cancer 13:475-483.

26. Kong A, Cox NJ 1997 Allele-sharing models: LOD scores and accurate linkage tests. Am J Hum Genet 61:1179-1188.

27. Wang K, Li M, Hadley D, Liu R, Glessner J, Grant SF, Hakonarson H, Bucan M 2007 PennCNV: an integrated hidden Markov model designed for high-resolution copy number variation detection in whole-genome SNP genotyping data. Genome Res 17:1665-1674.

28. Volzke H, Ludemann J, Robinson DM, Spieker KW, Schwahn C, Kramer A, John U, Meng W 2003 The prevalence of undiagnosed thyroid disorders in a previously iodine-deficient area. Thyroid 13:803-810.

29. Teumer A, Rawal R, Homuth G, Ernst F, Heier M, Evert M, Dombrowski F, Volker U, Nauck M, Radke D, Ittermann T, Biffar R, Doring A, Gieger C, Klopp N, Wichmann HE, Wallaschofski H, Meisinger C, Volzke H 2011 Genome-wide association study identifies four genetic loci associated with thyroid volume and goiter risk. Am J Hum Genet 88:664-673.

30. Iafrate AJ, Feuk T, Van Puymbroeck L, Rivera MN, Listewnik ML, Ying QP, Scherer SW, Lee C 2004 Detection of large-scale variation in the human genome. J Mol Diagn 6:411-411.

31. Conrad DF, Pinto D, Redon R, Feuk L, Gokcumen O, Zhang Y, Aerts J, Andrews TD, Barnes C, Campbell P, Fitzgerald T, Hu M, Ihm CH, Kristiansson K, MacArthur DG, MacDonald JR, Onyiah I, Pang AWC, Robson S, Stirrups K, Valsesia A, Walter K, Wei J, Tyler-Smith C, Carter NP, Lee C, Scherer SW, Hurles ME, Wellcome Trust Case C 2010 Origins and functional impact of copy number variation in the human genome. Nature 464:704-712.

32. Kadamur G, Ross EM 2013 Mammalian Phospholipase C. In: Julius D, (ed) Annu Rev Physiol 75:127-154.

33. Grubb DR, Vasilevski O, Huynh H, Woodcock EA 2008 The extreme C-terminal region of phospholipase C beta 1 determines subcellular localization and function; the "b" splice variant mediates alpha(1)-adrenergic receptor responses in cardiomyocytes. Faseb Journal 22:2768-2774.

34. Giordano J, Ge Y, Gelfand Y, Abrusan G, Benson G, Warburton PE 2007 Evolutionary history of mammalian transposons determined by genome-wide defragmentation. PLOS Comput Biol 3:1321-1334.

35. Ott J, Wang J, Leal SM 2015 Genetic linkage analysis in the age of whole-genome sequencing. Nature Rev Genet 16:275-284.

36. Dunham I, Kundaje A, Aldred SF, Collins PJ, Davis C, Doyle F, Epstein CB, Frietze S, Harrow J, Kaul R, Khatun J, Lajoie BR, Landt SG, Lee B-K, Pauli F, Rosenbloom KR, Sabo P, Safi A, Sanyal A, Shoresh N, Simon JM, Song L, Trinklein ND, Altshuler RC, Birney E, Brown JB, Cheng C, Djebali S, Dong X, Dunham I, Ernst J, Furey TS, Gerstein M, Giardine B, Greven M, Hardison RC, Harris RS, Herrero J, Hoffman MM, Iyer S, Kellis M, Khatun J, Kheradpour P, Kundaje A, Lassmann T, Li Q, Lin X, Marinov GK, Merkel A, Mortazavi A, Parker SCJ, Reddy TE, Rozowsky J, Schlesinger F, Thurman RE, Wang J, Ward LD, Whitfield TW, Wilder SP, Wu W, Xi HS, Yip KY, Zhuang J, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, Snyder M, Pazin MJ, Lowdon RF, Dillon LAL, Adams LB, Kelly CJ, Zhang J, Wexler JR, Green ED, Good PJ, Feingold EA, Bernstein BE, Birney E, Crawford GE, Dekker J, Elnitski L, Farnham PJ, Gerstein M, Giddings MC, Gingeras TR, Green ED, Guigo R, Hardison RC, Hubbard TJ, Kellis M, Kent WJ, Lieb JD, Margulies EH, Myers RM, Snyder M, Stamatoyannopoulos JA, Tenenbaum SA, Weng Z, White KP, Wold B, Khatun J, Yu Y, Wrobel J, Risk BA, Gunawardena HP, Kuiper HC, Maier CW, Xie L, Chen X, Giddings MC, Bernstein BE, Epstein CB, Shoresh N, Ernst J, Kheradpour P, Mikkelsen TS, Gillespie S, Goren A, Ram O, Zhang X, Wang L, Issner R, Coyne MJ, Durham T, Ku M, Truong T, Ward LD, Altshuler RC, Eaton ML, Kellis M, Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, Xue C, Marinov GK, Khatun J, Williams BA, Zaleski C, Rozowsky J, Roeder M, Kokocinski F, Abdelhamid RF, Alioto T, Antoshechkin I, Baer MT, Batut P, Bell I, Bell K, Chakrabortty S, Chen X, Chrast J, Curado J, Derrien T, Drenkow J, Dumais E, Dumais J, Duttagupta R, Fastuca M, Fejes-Toth K, Ferreira P, Foissac S, Fullwood MJ, Gao H, Gonzalez D, Gordon A, Gunawardena HP, Howald C, Jha S, Johnson R, Kapranov P, King B, Kingswood C, Li G, Luo OJ, Park E, Preall JB, Presaud K, Ribeca P, Risk BA, Robyr D, Ruan X, Sammeth M, Sandhu KS, Schaeffer L, See L-H, Shahab A, Skancke J, Suzuki AM, Takahashi H, Tilgner H, Trout D, Walters N, Wang H, Wrobel J, Yu Y, Hayashizaki Y, Harrow J, Gerstein M, Hubbard TJ, Reymond A, Antonarakis SE, Hannon GJ, Giddings MC, Ruan Y, Wold B, Carninci P, Guigo R, Gingeras TR, Rosenbloom KR, Sloan CA, Learned K, Malladi VS, Wong MC, Barber G, Cline MS, Dreszer TR, Heitner SG, Karolchik D, Kent WJ, Kirkup VM, Meyer LR, Long JC, Maddren M, Raney BJ, Furey TS, Song L, Grasfeder LL, Giresi PG, Lee B-K, Battenhouse A, Sheffield NC, Simon JM, Showers KA, Safi A, London D, Bhinge AA, Shestak C, Schaner MR, Kim SK, Zhang ZZ, Mieczkowski PA, Mieczkowska JO, Liu Z, McDaniell RM, Ni Y, Rashid NU, Kim MJ, Adar S, Zhang Z, Wang T, Winter D, Keefe D, Birney E, Iyer VR, Lieb JD, Crawford GE, Li G, Sandhu KS, Zheng M, Wang P, Luo OJ, Shahab A, Fullwood MJ, Ruan X, Ruan Y, Myers RM, Pauli F, Williams BA, Gertz J, Marinov GK, Reddy TE, Vielmetter J, Partridge EC, Trout D, Varley KE, Gasper C, Bansal A, Pepke S, Jain P, Amrhein H, Bowling KM, Anaya M, Cross MK, King B, Muratet MA, Antoshechkin I, Newberry KM, McCue K, Nesmith AS, Fisher-Aylor KI, Pusey B, DeSalvo G, Parker SL, Balasubramanian S, Davis NS, Meadows SK, Eggleston T, Gunter C, Newberry JS, Levy SE, Absher DM, Mortazavi A, Wong WH, Wold B, Blow MJ, Visel A, Pennachio LA, Elnitski L, Margulies EH, Parker SCJ, Petrykowska HM, Abyzov A, Aken B, Barrell D, Barson G, Berry A, Bignell A, Boychenko V, Bussotti G, Chrast J, Davidson C, Derrien T, Despacio-Reyes G, Diekhans M, Ezkurdia I, Frankish A, Gilbert J, Gonzalez JM, Griffiths E, Harte R, Hendrix DA, Howald C, Hunt T, Jungreis I, Kay M, Khurana E, Kokocinski F, Leng J, Lin MF, Loveland J, Lu Z, Manthravadi D, Mariotti M, Mudge J, Mukherjee G, Notredame C, Pei B, Rodriguez JM, Saunders G, Sboner A, Searle S, Sisu C, Snow C, Steward C, Tanzer A, Tapanari E, Tress ML, van Baren MJ, Walters N, Washietl S, Wilming L, Zadissa A, Zhang Z, Brent M, Haussler D, Kellis M, Valencia A, Gerstein M, Reymond A, Guigo R, Harrow J, Hubbard TJ, Landt SG, Frietze S, Abyzov A, Addleman N, Alexander RP, Auerbach RK, Balasubramanian S, Bettinger K, Bhardwaj N, Boyle AP, Cao AR, Cayting P, Charos A, Cheng Y, Cheng C, Eastman C, Euskirchen G, Fleming JD, Grubert F, Habegger L, Hariharan M, Harmanci A, Iyengar S, Jin VX, Karczewski KJ, Kasowski M, Lacroute P, Lam H, Lamarre-Vincent N, Leng J, Lian J, Lindahl-Allen M, Min R, Miotto B, Monahan H, Moqtaderi Z, Mu XJ, O'Geen H, Ouyang Z, Patacsil D, Pei B, Raha D, Ramirez L, Reed B, Rozowsky J, Sboner A, Shi M, Sisu C, Slifer T, Witt H, Wu L, Xu X, Yan K-K, Yang X, Yip KY, Zhang Z, Struhl K, Weissman SM, Gerstein M, Farnham PJ, Snyder M, Tenenbaum SA, Penalva LO, Doyle F, Karmakar S, Landt SG, Bhanvadia RR, Choudhury A, Domanus M, Ma L, Moran J, Patacsil D, Slifer T, Victorsen A, Yang X, Snyder M, White KP, Auer T, Centanin L, Eichenlaub M, Gruhl F, Heermann S, Hoeckendorf B, Inoue D, Kellner T, Kirchmaier S, Mueller C, Reinhardt R, Schertel L, Schneider S, Sinn R, Wittbrodt B, Wittbrodt J, Weng Z, Whitfield TW, Wang J, Collins PJ, Aldred SF, Trinklein ND, Partridge EC, Myers RM, Dekker J, Jain G, Lajoie BR, Sanyal A, Balasundaram G, Bates DL, Byron R, Canfield TK, Diegel MJ, Dunn D, Ebersol AK, Frum T, Garg K, Gist E, Hansen RS, Boatman L, Haugen E, Humbert R, Jain G, Johnson AK, Johnson EM, Kutyavin TV, Lajoie BR, Lee K, Lotakis D, Maurano MT, Neph SJ, Neri FV, Nguyen ED, Qu H, Reynolds AP, Roach V, Rynes E, Sabo P, Sanchez ME, Sandstrom RS, Sanyal A, Shafer AO, Stergachis AB, Thomas S, Thurman RE, Vernot B, Vierstra J, Vong S, Wang H, Weaver MA, Yan Y, Zhang M, Akey JM, Bender M, Dorschner MO, Groudine M, MacCoss MJ, Navas P, Stamatoyannopoulos G, Kaul R, Dekker J, Stamatoyannopoulos JA, Dunham I, Beal K, Brazma A, Flicek P, Herrero J, Johnson N, Keefe D, Lukk M, Luscombe NM, Sobral D, Vaquerizas JM, Wilder SP, Batzoglou S, Sidow A, Hussami N, Kyriazopoulou-Panagiotopoulou S, Libbrecht MW, Schaub MA, Kundaje A, Hardison RC, Miller W, Giardine B, Harris RS, Wu W, Bickel PJ, Banfai B, Boley NP, Brown JB, Huang H, Li Q, Li JJ, Noble WS, Bilmes JA, Buske OJ, Hoffman MM, Sahu AD, Kharchenko PV, Park PJ, Baker D, Taylor J, Weng Z, Iyer S, Dong X, Greven M, Lin X, Wang J, Xi HS, Zhuang J, Gerstein M, Alexander RP, Balasubramanian S, Cheng C, Harmanci A, Lochovsky L, Min R, Mu XJ, Rozowsky J, Yan K-K, Yip KY, Birney E, Consortium EP 2012 An integrated encyclopedia of DNA elements in the human genome. Nature 489:57-74.

37. Manole D, Schildknecht B, Gosnell B, Adams E, Derwahl M 2001 Estrogen promotes growth of human thyroid tumor cells by different molecular mechanisms. J Clin Endocrinol Metab 86:1072-1077.

38. Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, Barnett DH, Stossi F, Yeo A, George J, Kuznetsov VA, Lee YK, Charn TH, Palanisamy N, Miller LD, Cheung E, Katzenellenbogen BS, Ruan Y, Bourque G, Wei CL, Liu ET 2007 Whole-genome cartography of estrogen receptor alpha binding sites. PLOS Genet 3:867-885.

39. Kobayashi K, Shaver JK, Liang W, Siperstein AE, Duh QY, Clark OH 1993 Increased Phospholipase-C activity in neoplastic thyroid membrane. Thyroid 3:25-29.

40. Strassheim D, Shafer SH, Phelps SH, Williams CL 2000 Small cell lung carcinoma exhibits greater phospholipase C-beta 1 expression and edelfosine resistance compared with non-small cell lung carcinoma. Cancer Res 60:2730-2736.

41. Eszlinger M, Krohn K, Berger K, Lauter J, Kropf S, Beck M, Fuhrer D, Paschke R 2005 Gene expression analysis reveals evidence for increased expression of cell cycle-associated genes and G(q)-protein-protein kinase C signaling in cold thyroid nodules. J Clin Endocrinol Metab 90:1163-1170.

42. Kero J, Ahmed K, Wettschureck N, Tunaru S, Wintermantel T, Greiner E, Schuetz G, Offermanns S 2007 Thyrocyte-specific G(q)/G(11) deficiency impairs thyroid function and prevents goiter development. J Clin Investig 117:2399-2407.