# Germline ESR2 Mutation Predisposes to Medullary Thyroid Carcinoma and Causes Up-Regulation of RET Expression

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## Abstract

Familial medullary thyroid cancer (MTC) and its precursor, C cell hyperplasia (CCH), is associated with germline *RET* mutations causing multiple endocrine neoplasia type 2. However, some rare families with apparent MTC/CCH predisposition do not have a detectable *RET* mutation. To identify novel MTC/CCH predisposition genes we undertook exome resequencing studies in a family with apparent predisposition to MTC/CCH and no identifiable *RET* mutation. We identified a novel *ESR2* frameshift mutation, c.948delT, which segregated with histological diagnosis following thyroid surgery in family members and demonstrated loss of *ESR2* encoded ERβ expression in the MTC tumour. ERα and ERβ form heterodimers binding DNA at specific estrogen response elements (ERE) to regulate gene transcription. ERβ represses ERα mediated activation of the ERE and the *RET* promoter contains three ERE. *In vitro*, we showed that *ESR2* c.948delT results in unopposed ERα mediated increased cellular proliferation, activation of the ERE and increased RET expression. *In vivo*, immunostaining of CCH and MTC using an anti-RET antibody demonstrated increased RET expression. Together these findings identify germline *ESR2* mutation as a novel cause of familial MTC/CCH and provide important insights into a novel mechanism causing increased RET expression in tumourigenesis.

## Introduction

Thyroid cancer is the most common endocrine malignancy. Although medullary thyroid cancer (MTC), arising from the calcitonin secreting para-follicular cells, only accounts for ~5% of thyroid cancers (1), nearly 50% of patients present with stage III or IV disease and only 21% of patients presenting with stage IV disease survive 10 years (2).

In ~25% of MTC there is a germline *RET* mutation (3) predisposing to multiple endocrine neoplasia type 2 (MEN2), a dominantly inherited endocrine tumour predisposition syndrome characterised by predisposition to the development of young onset MTC and often primary C cell hyperplasia (CCH), a precursor to MTC (4). Other features may also occur such as phaeochromocytoma, primary hyperparathyroidism (HPT) and, rarely, developmental abnormalities, for example, a marfanoid habitus and ganglioneuromatosis of the mouth and gut (4).

The majority of *RET* mutations predisposing to MEN2 result in single amino acid substitutions affecting key residues in the extra-cellular and kinase domains of the RET receptor (5,6) causing inappropriate constitutive RET activation. Detection of a germline *RET* mutation, enables pre-symptomatic interventions such as prophylactic thyroidectomy to be offered to at-risk gene carriers (6). Thus all individuals presenting with MTC or primary CCH should be offered germline *RET* testing (7); however, some families with an apparent predisposition to MTC/CCH do not harbour a germline *RET* gene alteration (8,9) suggesting that further predisposing gene alterations remain to be identified. We investigated a kindred with non-*RET* MTC/CCH and detected a novel constitutional frameshift mutation in *ESR2* encoding the beta subunit of the oestrogen receptor, ERβ (10).

## Results

### Case Report

The index case presented age 22 years with MTC (Supplementary Material, Fig. S1). Although no constitutional mutation of exons 10, 11, or 13-16 of *RET* was detected, in view of the young age of diagnosis his monozygotic twin brother was offered follow-up and underwent thyroidectomy age 33 years because of abnormal pentagastrin stimulation testing; histological review showed the presence of CCH. Prophylactic thyroid surgery was then undertaken in the offspring of both brothers (individuals III:1-5); individuals III:2 and III:3 were found to have CCH and individuals III:1, III:4 and III:5 to have normal thyroid tissue. Pentagastrin stimulation testing was subsequently offered to the wider family and individual I:1 noted to have mildly abnormal results. In the index case, further constitutional molecular genetic testing did not detect any mutations in the remaining exons of *RET*, karyotype analysis was normal and array CGH analysis did not demonstrate any clinically significant CNVs (data not shown). We therefore hypothesised a novel gene alteration was predisposing to MTC/CCH in the family and undertook exome sequencing in two affected members (II:3 and III:3).

### Identification of Germline *ESR2* mutation in Familial MTC/CCH

We filtered the variants identified using the dbSNP (11), NHLBI Exome Variant Server (EVS) (12) and 1000 genomes (13) datasets looking for potentially disrupting variants present in both samples, prioritising frameshift and nonsense changes. We did not identify any deleterious alterations in the genes known to be mutated in hereditary cancer predisposition (14, Supplementary Material, Table S1) but did identify a novel frameshift alteration of *ESR2* (c.948delT; p.Gly318Alafs*22) present in both affected individuals but not in the NHLBI EVS nor in 2577 individuals from the 1000 genome project (12,13).

Constitutional *ESR2* sequencing in other family members demonstrated the c.948delT variant to be present in the two family members found to have CCH (individuals II:1 and III:2) but not in the

three (individuals III:1, III:4, III:5) with normal thyroid histology (Fig. 1A, Supplementary Material, Fig. S1).

**Loss of ERβ staining in *ESR2* c.948delT associated MTC**

To investigate the role of ESR2 in MTC tumourigenesis we initially undertook immunohistochemistry using an anti-ERβ antibody and demonstrated complete loss of nuclear staining in the c.948delT associated MTC (Fig. 1B) with retained staining in the corresponding normal thyroid tissue. However, despite direct sequencing of the complete *ESR2* coding region and LOH studies using closely linked microsatellite markers in archived tumour material, we did not detect a somatic alteration of *ESR2* (data not shown). Whole genome copy number analysis by molecular inversion probe technology did not reveal any pathological CNVs affecting either chromosomes 14 or 10 (harbouring the *ESR2* and *RET* loci respectively) (Supplementary Material, Fig. S2).

**No additional constitutional *ESR2* mutations in other cases of apparent MTC predisposition**

To ascertain whether constitutional alterations of *ESR2* might be involved in other cases of apparent MTC predisposition, we undertook sequencing of the coding region of *ESR2* in 19 individuals with apparently isolated MTC, eight of whom were diagnosed <40 years of age (mean age at diagnosis 47.5y, range 33-71y, SEM 2.96). The entire coding region of *RET* had been previously sequenced and no alterations detected other than known polymorphisms. We identified a novel germline missense alteration, c.382G>C; p.V128L, in a female who developed an apparently isolated MTC age 36 years (Fig. 2A). This variant was also detected in a pair of siblings, one of whom had a papillary thyroid cancer age 60 years and the other a medullary thyroid cancer age 57 years. We did not detect any constitutional *ESR2* alterations, other than known polymorphisms, in a further three families with familial non-*RET* MTC (data not shown).

Further investigation using archived tumour material from the individual with the apparently isolated MTC and *ESR2* c.382G>C (no blocks were available from the sibling pair) revealed preserved ERβ staining (Fig. 2B) and no somatic *ESR2* sequence alterations, LOH or pathological

CNVs of chromosomes 14 or 10 were detected. Although this variant was not present in the NHLBI EVS (12) or 1000 genome project (13) datasets, further analysis using SIFT (15) predicted it to be tolerated, and PolyPhen-2 (16) to be benign; we were therefore unable to exclude that *ESR2* c.382G>C may represent a rare polymorphism and did not evaluate further.

**No somatic mutations of *ESR2* detected in sporadic MTC**

As hereditary cancer predisposing genes are often implicated in sporadic tumours (17), we investigated whether somatic mutations of *ESR2* also occurred in sporadic MTC. However, direct sequencing of *ESR2* in 15 fresh frozen sporadic MTC did not reveal any alterations other than known polymorphisms (data not shown). Whilst the cosmic (18) and TCGA data sets (19), containing data for over 21,000 tumours, did not have any data regarding *ESR2* in MTC, we did note the presence of somatic *ESR2* mutations in other tumours of neuroectodermal origin, for example glioma and melanoma.

**ESR2 c.948delT is associated with unrestrained ERα driven cell proliferation**

We then sought to understand how this rare novel *ESR2* frameshift alteration might cause tumourigenesis. Initially we determined stability of the *ESR2* c.948delT mutant compared to wild-type (wt) *ESR2*. Transient transfection of HCT116 cells, followed by the addition of the transcription inhibitor, actinomycin, resulted in significantly lower *ESR2* c.948delT mRNA levels over a 24 hour time period compared with wt*ESR2* indicating reduced stability of the mutant mRNA (Supplementary Material, Fig. S3).

*ESR2*-encoded ERβ forms either homo- or, preferentially, hetero-dimers with *ESR1*-encoded ERα to bind DNA at specific estrogen-responsive elements (EREs) within the promoters of target genes to regulate transcription (20). ERα is the more potent activator and ERβ can repress ERα; thus transcriptional activity is determined by the relative ERα/ERβ proportion (21-23).

We next investigated whether ESR2 c.948delT altered cellular growth. In MCF-7 cells, *ESR2* c.948delT failed to restrain the ERα-driven proliferation of MCF-7 cells in response to either 17β-

estradiol (E2) or the ERα selective agonist, PPT. In contrast, wt*ESR2* was capable of inhibiting cell proliferation by up to 30% in both E2 and PPT treated MCF7 cells over-expressing ERα (Fig. 3).

**Loss of ERβ function causes increased estrogen-responsive element (ERE) activity and RET expression**

In both HCT116 and MCF-7 cells, in response to either E2 or PPT agonist, wt*ESR1* activated the ERE, as indicated by increased luciferase expression, and this was significantly restrained in the presence of wt*ESR2*. However, co-transfection of wt*ESR1* with *ESR2* c.948delT restored expression to a level similar to that seen with wt*ESR1* alone indicating that the *ESR2* c.948delT mutant has lost the ability to restrain ERα-mediated activation of the ERE (Fig. 4).

We then investigated how inappropriate activation of the ERE by c.948delT might cause MTC tumourigenesis. Inappropriate up-regulation of RET activity causing MTC is well established (4) and we noted that the *RET* promoter (-34 to -314) contains three ERE (24). Furthermore, in *in vitro* studies, *RET* has been established as an ER target gene (25-27). We therefore investigated whether loss of ERβ function might lead to up-regulation of RET expression.

As MCF7 cells have intrinsic ER activity, we used HCT116 cells to investigate the effect of *ESR2* c.948delT on RET expression. Co-transfection of wt*ESR1* with wt*ESR2* in HCT116 cells, in the presence of either E2 or PPT, resulted in decreased RET expression as compared with wtESR1 alone; however, over-expression of wt*ESR1* and *ESR2* c.948delT, restored RET expression to a level similar to that seen with wt*ESR1* alone (Fig. 5A, B).

*In vivo*, RET immunostaining using archived sections from the ESR2 c.948delT associated MTC (individual II:3) and analysis of archived stained slides showing CCH (individual II:1) demonstrated increased RET staining (Fig. 5C). In particular, the intensity of RET expression in the MTC was stronger than in corresponding normal thyroid tissue and similar to that in an MEN2-associated MTC with a known constitutional *RET* mutation (Fig. 5C). Analysis of *RET* exons 10, 11 and 13-16 in the *ESR2* c.948delT associated MTC did not detect a somatic mutation to account for the increased expression (data not shown).

## Discussion

By targeted capture array-based exome sequencing we detected a novel loss of function *ESR2* mutation in a family with predisposition to MTC/CCH. As the interpretation of novel rare variants identified through next generation sequencing approaches in isolated families is challenging, we sought functional evidence to establish pathogenicity.

Initially we demonstrated tumour specific loss of ERβ expression in the c.948delT associated MTC which we considered indicative of loss of function as ERβ is known to be expressed by human para-follicular C cells (28). Whilst *ESR2* knockout mice display a phenotype more in keeping with a reduced fertility phenotype in females rather than one associated with tumourigenesis (29), *ESR2* has been shown to inhibit cell proliferation in vitro (30) and tumour formation in nude mice (31). Although we did not identify a somatic *ESR2* alteration in this tumour and were unable to investigate ESR2 promoter hypermethylation, we note that *ESR2* promoter hypermethylation associated with ERβ down-regulation has been detected in a variety of tumour types (32-34). As with other cancer predisposition syndromes (35), it is also probable that additional somatic mutations are required in the transition from normal thyroid to MTC and we did detect low level loss of chromosomes 3 and 13.

It is likely that ERβ loss of function in MTC is rare as we did not detect loss of ERβ expression in 12 apparently sporadic MTC tumours nor an intragenic *ESR2* mutation in 15 sporadic MTCs. Interestingly, studies of MTC have indicated that *RAS* and *RET* are the dominant driver pathways in tumourigenesis, with few mutations being detected in other genes and none in *ESR2* (36). Our findings are also in keeping with large scale studies of sporadic tumours where no CNVs, and only one putative mutation of *ESR2* (missense alteration in a single papillary thyroid cancer), have been detected in thyroid tumours (18,19). Such relative infrequency of somatic mutation in sporadic tumours in genes predisposing to hereditary cancers is well recognised, for example the low rate of somatic *BRCA1* and *BRCA2* mutations in sporadic breast cancer (37,38). We did note that somatic *ESR2* mutations have been detected in other neuroectodermal derived tumours such as glioma and melanoma and also breast cancer samples indicating that inappropriate up-regulated ERα activity may also be involved in their tumourigenesis (18,19).

We did not detect a constitutional *ESR2* mutation in a further three families with an apparent genetic predisposition to MTC and CCH and it is likely that, akin to other familial cancer predisposition syndromes (39), there is also heterogeneity in the genetic predisposition to MTC/CCH.

The association of increased RET activity with MTC tumourigenesis is well established (4) and here we demonstrate a novel mechanism of MTC tumourigenesis whereby loss of ERβ function results in ERα-driven RET expression, likely mediated through the ERE on the *RET* promoter. Whilst there may be an unidentified constitutional *RET* mutation, or another mechanism of *RET* up-regulation, we have also shown *ESR2* c.948delT to cause increased cellular proliferation indicating a likely role in tumourigenesis; however, we cannot exclude that other mechanisms of ERβ action may also be involved, for example through loss of ERβ mediated inhibition of HIF-1α causing inappropriate HIF pathway activation (40).

Although we detected a mutation in just one family, our results suggest that constitutional *ESR2* mutation is of high penetrance for CCH/MTC. However, as individual I:1 has not overtly presented with MTC, we cannot exclude that there may be variable penetrance, as occurs for germline *RET* mutations and in other familial cancer predisposition syndromes such as paraganglioma-phaeochromocytoma predisposition (4, 41).

It is interesting to speculate whether constitutional *ESR2* mutations are also associated with predisposition to HPT and phaeochromocytoma. These tumours have not occurred to date in the family (relevant clinical and laboratory investigation has been undertaken) and whole exome sequencing of both constitutional DNA from individuals with apparent genetic predisposition to phaeochromocytoma (12 individuals) and sporadic phaeochromocytoma (seven tumours) has not detected any deleterious *ESR2* mutations (ERM unpublished observations). Nonetheless, given the known tumour predisposition associated with dysregulated *RET* activity, it would seem prudent to offer clinical surveillance for phaeochromocytoma and HPT, and prophylactic thyroidectomy, where a loss of function constitutional *ESR2* mutation is detected.

The investigation of rare familial cancer predisposition often provides important insights into cell biology with the potential for novel therapeutic approaches (42) and our findings also provide further evidence of the interaction between ERα and RET in tumourigenesis. Many invasive breast

cancers expressing ERα also express RET (4) and in recent pre-clinical models of breast cancer, targeting both RET and ERα decreases the growth and metastatic potential of breast cancer cells (43). Thus tumours associated with loss of ERβ function might be suitable candidates for therapeutic agents targeting the RET pathway (44), RET tyrosine kinase active site (45) or ERα (46).

In summary, our data is the first to identify a novel genetic cause for familial MTC since the identification of the *RET* gene. We demonstrate a novel mechanism promoting up-regulation of *RET* activity through constitutional *ESR2* mutation which provides opportunities for pre-symptomatic testing and potential novel therapeutic strategies.

## Materials and Methods

### Whole Exome Sequencing Analysis

Exome sequencing was performed at the Biomedical Research Centre at Kings College London as previously described (47).

### Sanger Sequencing

Genomic DNA was extracted from formalin-fixed paraffin embedded (FFPE) tissue using the QIAamp DNA FFPE kit (Qiagen) and from peripheral leucocytes using the Nucleon BACC2 kit (Amersham Biosciences) (sequences and PCR conditions available upon request).

### Immunohistochemistry

Slides were de-paraffinized using Benchmark Special Stains Deparaffinization Solution. Heat-induced antigen retrieval was performed using EDTA buffer as previously described (48). Immunostaining was performed using the Ventana Benchmark Automated Slide Stainer (Ventana Medical Systems) with antibodies against ERβ (Leica Biosystems, 1:50), calcitonin (Dako IR515) and RET (Abcam EPR2871, 1:500).

### Loss of Heterozygosity (LOH) analyses

Microsatellite markers were used to determine LOH at the *ESR2* locus on chromosome 14q23.2: one, previously described (49) within intron six (chr14:64,720,279-64,720,323) and one 5' upstream (chr14:64,802,692-64,802,742) (sequences available upon request). PCR amplification was performed on genomic DNA templates using fluorescently labelled primers. PCR products were electrophoresed on an ABI Genetic Analyzer and analysed by GeneMapper fragment analysis software (Applied Biosystems).

### OncoScan® FFPE Assay Kit

The OncoScan® FFPE assay kit platform was utilized to identify whole-genome copy number variations (CNV) as previously described (50).

**Cell Culture**

In the absence of a suitable MTC cell line we used HCT116 (Health Protection Agency Culture Collections, UK) and MCF-7 cells (European Collection of Animal Cell Cultures) cultured in McCoy's 5A (Life Technologies) with 5% fetal bovine serum (Invitrogen), penicillin ($10^4$ U/ml) and streptomycin ($10^4$ mcg/ml) (Invitrogen) for HCT116 cells and RPMI 1640 (Life Technologies) with 5% fetal bovine serum (Invitrogen), penicillin ($10^5$ U/L) and streptomycin (100 mg/L) for MCF-7 cells. Cells were treated with 17β-oestradiol (E2, Sigma) and 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl(trisphenol) (PPT, Tocris) at final concentrations of 10 nM and 100 nM, in phenol red-free medium (Life Technologies) with 5% charcoal-stripped serum.

**Transfection**

The ESR1/2 ORFS were cloned into Sgf1 and Mlu1 restriction sites of pCMV6-AN-Myc (Origene). QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) was used to delete the thymine at position 948 of ESR2, generating ESR2, c.948delT. Plasmid DNA transfections were performed with TransIT LT1 (Mirus Bio LLC). In some experiments, plasmid DNA was introduced into cells by reverse transfection (lipid: DNA complexes plated prior to addition of cells).

**RNA extraction, Real-time PCR and mRNA Stability Assay**

Total RNA was extracted and reverse transcribed using RNeasy Micro Kit (Qiagen) and Reverse Transcription System (Promega) with expression determined by the 7500 Real-time PCR system (Applied Biosystems). qRT-PCR was carried out on 96 well plates using FAM-labelled Taqman probes (Life Technologies) for ESR2 (Hs01100359_m1), RET (Hs01120030_m1) and PPIA (Hs04194521_s1). In mRNA stability experiments, HCT116 cells were treated with actinomycin D at a final concentration of 10 μg/ml for 4h, 8h and 24h prior to total RNA extraction.

**Western Blotting**

Western blots, performed as described previously (51), were probed with antibodies against RET (C31B4, Cell Signaling Technology), 1:1000; ERβ (H150, Santa Cruz Biotechnology), 1:1000; c-Myc, 1:1000 and β actin; 1:10,000 (Sigma-Aldrich). Antigen-antibody complexes were detected using ECL Plus (Amersham Biosciences).

**Luciferase Reporter Assays**

Luciferase reporter assays were performed using the Cignal ERE Reporter Kit (Qiagen). HCT116 (7,500 cells/ well) and MCF-7 (15,000 cells/ well) cells were reverse transfected with 50ng Cignal ERE reporter along with 100ng ER expression vectors with TransIT LT1. After 24h hormone incubation, cells were harvested with lysis buffer (Promega) and the firefly and renilla luciferase activities determined with a dual luciferase assay kit (Promega), measuring luminescence with a Centro LB960 microplate luminometer (Berthold Technologies).

**Cell Proliferation**

Proliferation of MCF-7 cells was determined using the Celltiter-Glo® Luminescent Cell Viability Assay (Promega). In brief, 12,500 MCF-7 cells were reverse transfected with ER expression vectors and TransIT LT1 in 96 wells and then treated with hormones as indicated for 72h prior to analysis of the luminescent signal using the Centro LB960 microplate luminometer (Berthold Technologies).

**Statistical Analyses**

Data are displayed as mean ± SE. Normally distributed data were analysed using a two-tailed Student's t-test, unless otherwise indicated. A $P$-value < 0.05 was considered to be statistically significant.

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## Conflict of Interest

The authors have no conflicts of interest to declare.

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## Figure legends

Figure 1. Germline *ESR2* mutation detected in family members with MTC/CCH and loss of ERβ staining in associated MTC tumour. (A) Sanger sequencing traces showing presence of *ESR2* c.948delT (upper panel) and corresponding normal trace (lower panel). Affected nucleotide is indicated by a red arrow. (B) Representative histological stains in sections following thyroid surgery in representative family members II:3 (MTC), III:2 (CCH), III:4 (normal thyroid). Haematoxylin and eosin stain showing MTC (upper panel) and apparently normal thyroid tissue (middle and lower panels). Calcitonin staining demonstrating MTC (upper panel), areas of CCH within apparently normal thyroid tissue (black arrowhead, middle panel), and normal thyroid tissue (lower panel). ERβ immunohistochemistry showing loss of nuclear expression in the MTC (upper panel, white arrowhead). Middle and lower panels – preserved nuclear staining of ERβ in follicular epithelial cells. It was not possible to determine if there was loss of ERβ staining associated with areas of CCH (middle panel).

Figure 2. Germline *ESR2* alteration detected in an individual with early onset MTC and preserved ERβ staining in associated MTC tumour. (A) Sanger sequencing traces showing constitutional *ESR2* alteration c.382G>C (upper panel) and corresponding normal trace (lower panel) detected in an individual with apparently isolated MTC age 36 years. Affected nucleotide is indicated by a red arrow. (B) Representative histological stains in sections following thyroid surgery. Left, haematoxylin and eosin stain showing MTC (white arrowhead) and corresponding normal thyroid tissue (black arrowhead). Middle, calcitonin stain demonstrating MTC (white arrowhead) with corresponding unstained normal thyroid tissue (black arrowhead). Right, ERβ immunohistochemistry showing preserved nuclear expression in both MTC (white arrowhead and high power insert) and corresponding normal thyroid tissue (black arrowhead and high power insert).

Figure 3. *ESR2* c.948delT is associated with unrestrained ERα-driven cellular proliferation. MCF-7 cells were reverse transfected with wtESR1 (ESR1) alone or in combination with either wt*ESR2* (ESR2) or *ESR2* c.948delT (948ΔT) for 24h and then treated with 10nM E2, 100nM E2, or 10nM PPT

for 72h, compared with vehicle (ethanol) treated controls. Cell proliferation status was quantified by luciferase assay using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and expressed relative to ethanol treated controls. Data presented as mean values ± SE (n = 4). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS - not significant.

Figure 4. Diminished ability of *ESR2* c.948delT to inhibit wt*ESR1*. Luciferase assays evaluating transcriptional activity of a transfected ERE by vector only (VO), wt*ESR1* (ESR1) alone, or in combination with either wt*ESR2* (ESR2) or *ESR2* c.948delT (948ΔT) mutant at a ratio of 1:3 in (A) HCT116 and (B) MCF-7 cell lines. Cells were treated with 10nM E2, 10nM PPT or ethanol control as indicated for 24h prior to cell lysis and luciferase activity measurement. Relative fold change presents mean values ± SE from at least two independent experiments (n = 4 wells per experiment). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS - not significant.

Figure 5. *ESR2* c.948delT is associated with elevated RET expression. (A,B) real-time PCR (upper panel) and Western blot (lower panel) analyses of RET expression levels in HCT116 cells transfected with wtESR1 (ESR1) alone or in combination with either wt*ESR2* (ESR2) or *ESR2* c.948delT (948ΔT) and then treated with either (A) 10nM E2 (+) or (B) 10nM PPT (+) compared to ethanol control (-). Real-time data presented as mean *RET* mRNA levels ± SE (n = 4). In control experiments cells were transfected with vector only (VO) as indicated. (C) Representative histological sections showing immunohistochemical staining for RET (upper panels) with corresponding calcitonin stains (lower panels) to confirm presence of CCH/MTC. Left - archived slides from individual II:1 showing areas of intense RET staining (white arrow, upper panel) and area of marked calcitonin staining indicating presence of CCH (white arrowhead, lower panel) within normal thyroid tissue. Middle - MTC tumour from individual II:3 showing intense RET expression (white arrowhead, upper panel) as compared with corresponding normal thyroid tissue (black arrowhead, upper panel). Calcitonin stain (lower panel) confirms presence of MTC (same panel as in Fig.1B). Right - MEN2 associated MTC (white arrowhead, upper panel) also showing intense RET expression as compared with corresponding normal thyroid tissue (black arrowhead, upper panel), for comparison with middle

panel. Calcitonin stain (lower panel) confirms presence of MTC (white arrowhead, lower panel) with corresponding normal thyroid tissue also shown (black arrowhead, lower panel).

## Abbreviations

| | |
|---|---|
| MTC | medullary thyroid cancer |
| CCH | C cell hyperplasia |
| ERβ | oestrogen receptor beta |
| ERE | estrogen response element |
| MEN2 | multiple endocrine neoplasia type 2 |
| HPT | hyperparathyroidism |
| SNP | single nucleotide polymorphism |
| NHLBI | National Heart, Lung and Blood Institute |
| EVS | exome variant server |
| CNV | copy number variant |
| SIFT | Sorts Intolerant From Tolerant |
| TCGA | The Cancer Genome Atlas |
| ERα | oestrogen receptor alpha |
| E2 | 17β-estradiol |
| PPT | 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl(trisphenol) |
| FFPE | formalin-fixed paraffin embedded |
| PCR | polymerase chain reaction |
| LOH | loss of heterozygosity |

| | | |
|---|---|---|
| 602 | COSMIC | catalogue of somatic mutations in cancer |

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## SUPPLEMENTARY FIGURES

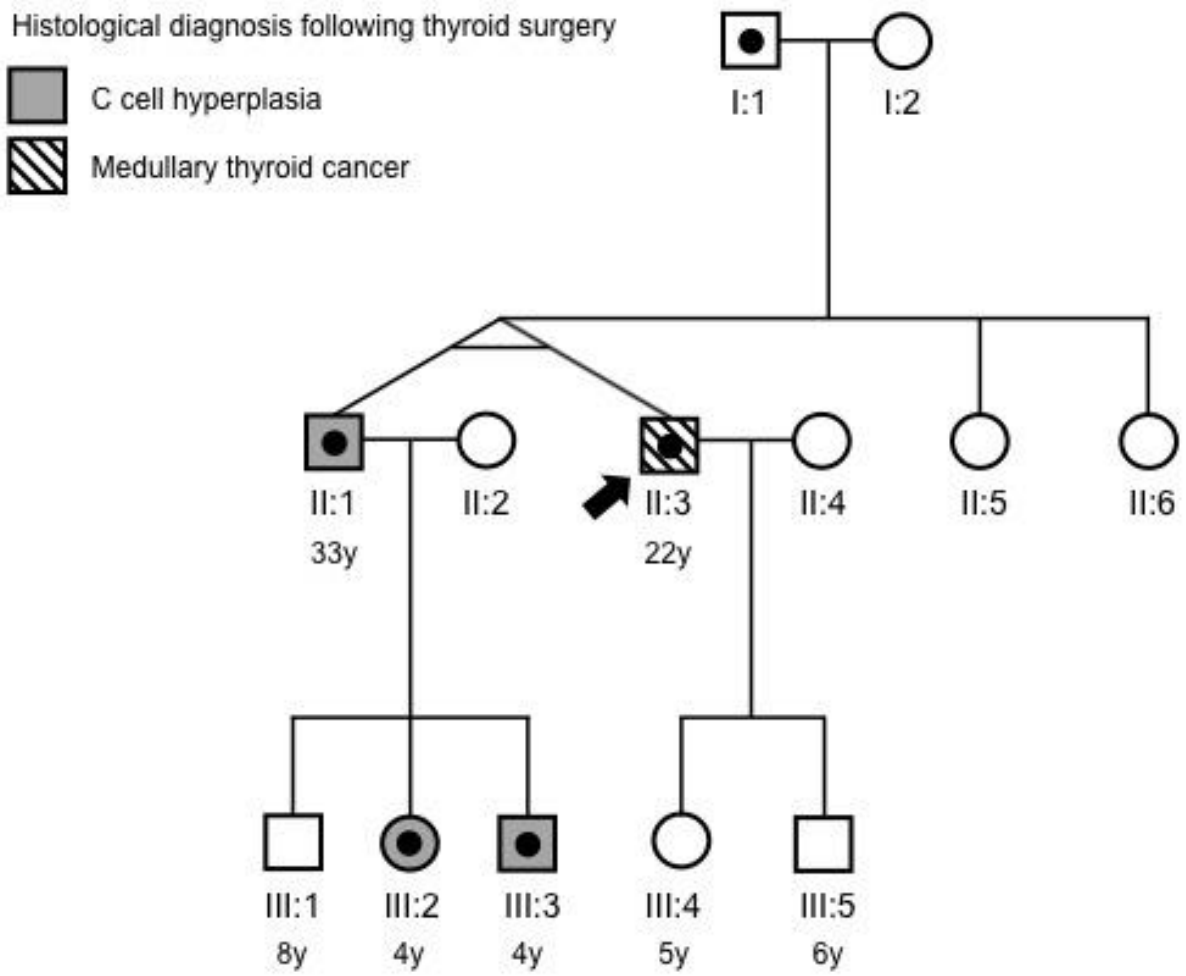


**Figure S1. Pedigree of family with MTC/CCH.** Diagnoses marked are from histological review following thyroid surgery. The index case (arrowed) presented with MTC age 22 years. ESR2 c.948delT carriers are marked and the ages (y) at which thyroidectomy was undertaken are indicated.

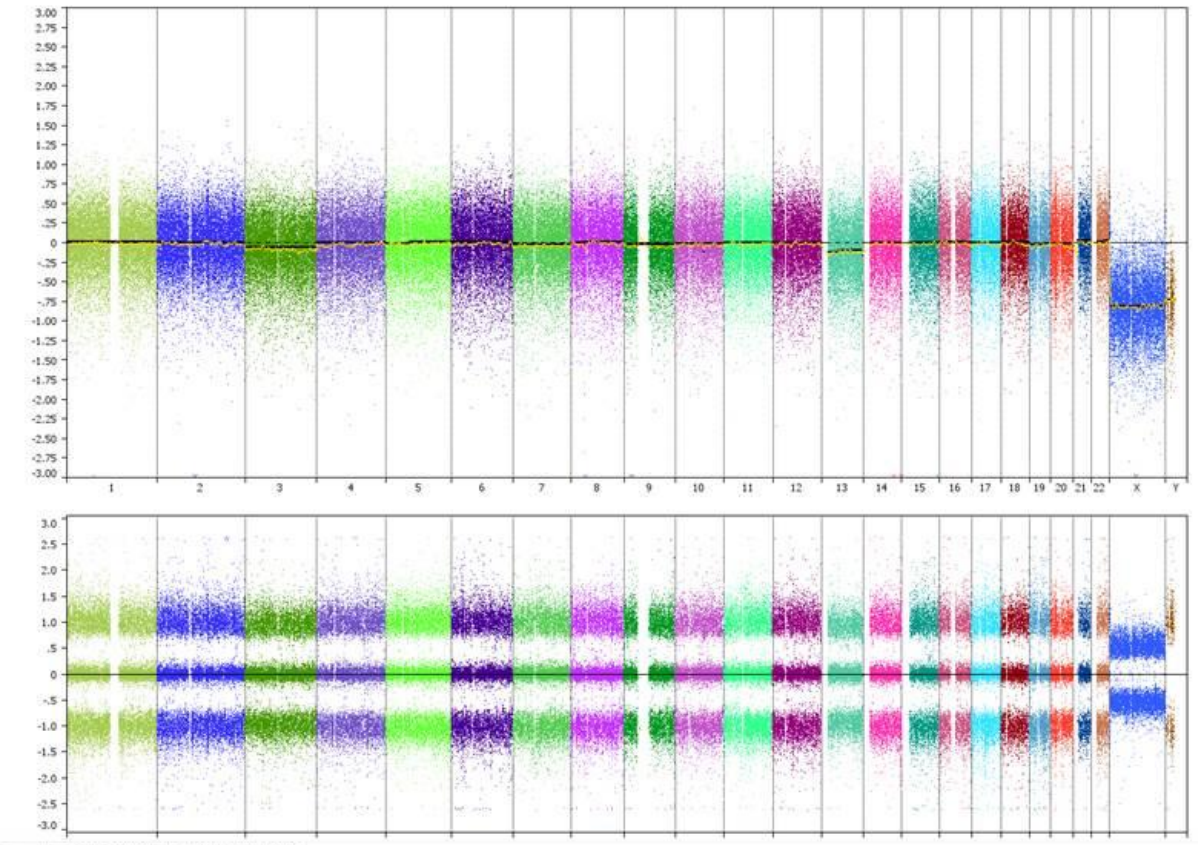

**Figure S2. OncoScan® FFPE assay of MTC tumour.** Log2 ratio (upper panel) and BAF plots (lower panel) showing results from the OncoScan® FFPE Assay Kit for MTC tumour from individual II:3. Data shows no copy number abnormality of either chromosome 10 or 14. There is some evidence of low level loss of chromosomes 3 and 13.

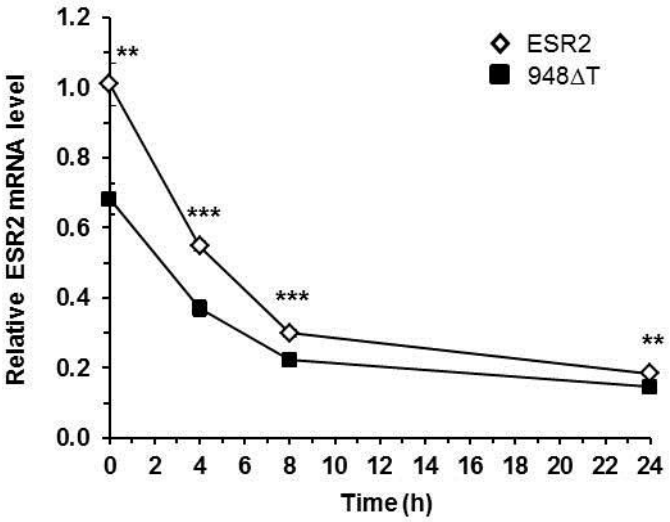


**Figure S3. ESR2 c.948delT results in reduced mRNA levels.** At 24 hours after transfection with either wt ESR2 (ESR2, white diamonds) or ESR2 c.948delT (948ΔT, black squares), time 0 hours (h) on x-axis, HCT116 cells were treated with actinomycin D (10 mg/ml) for 0, 4, 8 and 24 hours. The relative wt ESR2 and c.948delT mRNA levels were monitored by RT-qPCR. All values are relative to wt ESR2 mRNA levels at 0 hours (set as 1). Values represent means ± SE (n = 4) from at least two independent experiments. ESR2 mRNA expression levels were normalized against the 18S rRNA housekeeping gene. **, $P < 0.01$; ***, $P < 0.001$. NS - not significant.

| | | | | |
|---|---|---|---|---|
| *AIP* | *DDB2* | *FANCL* | *PALB2* | *SLX4* |
| *ALK* | *DICER1* | *FANCM* | *PHOX2B* | *SMAD4* |
| *APC* | *DIS3L2* | *FH* | *PMS1* | *SMARCB1* |
| *ATM* | *EGFR* | *FLCN* | *PMS2* | *STK11* |
| *BAP1* | *EPCAM* | *GATA2* | *PRF1* | *SUFU* |
| *BLM* | *ERCC2* | *GPC3* | *PRKAR1A* | *TMEM127* |
| *BMPR1A* | *ERCC3* | *HNF1A* | *PTCH1* | *TP53* |
| *BRCA1* | *ERCC4* | *HRAS* | *PTEN* | *TSC1* |
| *BRCA2* | *ERCC5* | *KIT* | *RAD51C* | *TSC2* |
| *BRIP1* | *EXT1* | *MAX* | *RAD51D* | *VHL* |
| *BUB1B* | *EXT2* | *MEN1* | *RB1* | *WRN* |
| *CDC73* | *EZH2* | *MET* | *RECQL4* | *WT1* |
| *CDH1* | *FANCA* | *MLH1* | *RET* | *XPA* |
| *CDK4* | *FANCB* | *MSH2* | *RHBDF2* | *XPC* |
| *CDKN1C* | *FANCC* | *MSH6* | *RUNX1* | |
| *CDKN2A* | *FANCD2* | *MUTYH* | *SBDS* | |
| *CEBPA* | *FANCE* | *NBN* | *SDHAF2* | |
| *CEP57* | *FANCF* | *NF1* | *SDHB* | |
| *CHEK2* | *FANCG* | *NF2* | *SDHC* | |
| *CYLD* | *FANCI* | *NSD1* | *SDHD* | |

**Table S1. List of genes on TruSight Cancer Panel.**

(http://www.illumina.com/products/trusight_cancer.html)