

Thyroid cancer and co-occurring *RET* mutations in Hirschsprung disease

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Abstract

The objective of this study was to assess the occurrence of thyroid cancer and co-occurring *RET* mutations in a population-based cohort of adult Hirschsprung disease (HD) patients. All 156 patients operated for HD in a tertiary center during 1950–1986 were followed for thyroid malignancies up to 2010 through the nationwide Finnish Cancer Registry. Ninety-one individuals participated in clinical and genetic screening, which included serum calcitonin and thyroid ultrasound (US) with cytology. Exons 10, 11, 13, and 16 were sequenced in all, and all exons of *RET* in 43 of the subjects, including those with thyroid cancer, *RET* mutations, suspicious clinical findings, and familial or long-segment disease. Through the cancer registry, two cases (aged 35 and 37 years) of medullary thyroid cancer (MTC) were observed; the incidence for MTC was 340-fold (95% CI 52–1600) compared with average population. These individuals had C611R and C620R mutations in exon 10. One papillary thyroid cancer without *RET* mutations was detected by clinical screening. Four subjects (aged 31–50 years) with co-occurring *RET* mutations in exons 10 (C609R; $n=1$) and 13 (Y791F, $n=3$) had sporadic short-segment HD with normal thyroid US and serum calcitonin. Three novel mutations and five single-nucleotide polymorphisms were found outside exons 10 and 13 without associated signs of thyroid cancer. MTC-associated *RET* mutations were restricted to exons 10 and 13 affecting ~5% of unselected adults with HD. Clinical thyroid assessment did not improve accuracy of genetic screening, which should not be limited to patients with familial or long-segment disease.

Key Words

- ▶ hirschsprung disease
- ▶ oncology
- ▶ thyroid cancer
- ▶ multiple endocrine neoplasia

Endocrine-Related Cancer
(2013) 20, 595–602

Introduction

Hirschsprung disease (HD) is a congenital malformation characterized by absence of ganglion cells in the myenteric and submucosal plexuses of the distal intestine

(Martucciello *et al.* 2000). It is thought to be caused by disordered migration and/or differentiation of neural crest cells during embryonic period and is thereby regarded as a

neurocristopathy. Neural crest also gives rise to other neuronal, endocrine, and paraendocrine tissues, explaining increased incidence of other neurocristopathies in HD, including familial medullary thyroid carcinoma (MTC), multiple endocrine neoplasia (MEN) syndromes, and neuroblastoma (Le Douarin & Kalcheim 1999).

Currently, at least ten genes are known to be involved in pathogenesis of HD. The *RET* proto-oncogene is the major gene associated with HD and MEN2 syndromes (Donis-Keller *et al.* 1993, Mulligan *et al.* 1993, Carlson *et al.* 1994, Hofstra *et al.* 1994, Attié *et al.* 1995). *RET* mutations are found in up to 50% of the familial and in 15–30% of the sporadic HD cases (Amiel *et al.* 2008). Loss-of-function mutations of the *RET* gene result in HD whereas gain-of-function mutations lead to MEN2 syndromes (Arighi *et al.* 2004). In contrast to these cancer syndromes, which are caused by a germline mutation and associate with MTC, somatic gene rearrangements of the *RET* gene are associated with papillary thyroid cancer (Grieco *et al.* 1990). The incidence of MTC has been reported to vary between 2.5 and 5% among HD patients (Decker *et al.* 1998, Sijmons *et al.* 1998, Pakarinen *et al.* 2005).

The majority of mutations causing MEN2A are located in exons 10 and 11 of the *RET* gene, including mainly germline missense mutations in one of the six cysteines (codons 609, 611, 618, and 620 in exon 10 and codons 630 and 634 in exon 11; Donis-Keller *et al.* 1993, Mulligan *et al.* 1993, 1994a,b, Hansford & Mulligan 2000). Only mutations in codons 609, 611, 618, and 620 of exon 10 are known to co-segregate with HD and MEN syndromes (Mulligan *et al.* 1994a,b, Romeo *et al.* 1998, Sijmons *et al.* 1998, Nishikawa *et al.* 2003). Mutations in codon 791 of the *RET* gene are suggested to be weak or nonpathogenic mutations for MEN2A and familial medullary thyroid carcinoma (FMTC) (Frank-Raue *et al.* 2008, Erlic *et al.* 2010).

Previous studies have focused on genetic screening of certain germline *RET* mutations in non-population-based cohorts of selected HD and/or MTC patients or their families (Nishikawa *et al.* 2003, De Groot *et al.* 2005, Bütter *et al.* 2007, Fialkowski *et al.* 2008). Taking into account that genetics of HD is still incompletely understood, this approach may lead to biased conclusions regarding the risk of thyroid malignancies or co-occurring *RET* mutations among HD patients in general. In the present study, we combined extensive genetic screening with clinical thyroid assessment after reliable registry-based patient identification. This study design enabled accurate evaluation of genotype–phenotype correlation between *RET* mutations and thyroid cancer in an unselected population-based cohort of adult HD patients.

Subjects and methods

Patients and study design

All patients operated for HD at our tertiary center between years 1950 and 1986 were identified from the hospital records and those alive after January 1st 1967 were enrolled. Since January 1st 1967, all residents of Finland have had a personal identity (ID) code. The individuals in the study cohort were compared with those listed under the Population Register Center of Finland and the correctness of each ID code was checked. Vital status was verified through the Cause-of-death of Statistic Finland and cancer history of the study population was reviewed from the Finnish Cancer Registry until December 31st 2010.

Of the 156 patients, five had emigrated and 11 had died from causes unrelated to thyroid cancer. The remaining 140 eligible patients were traced from the database of the Population Register Centre and contacted by mail during years 2007–2009. Of them, 91 (65%) subjects volunteered to participate, and they were studied cross-sectionally during their outpatient visit. Participants underwent a series of examinations including the measurement of serum calcitonin concentration and ultrasound (US) examination of the thyroid gland combined with a fine-needle biopsy (FNB) when indicated. They were interviewed and asked to complete a questionnaire assessing history of thyroid disorders and family history of HD, and whole blood samples were withdrawn for genetic analysis.

Data abstracted from the completely reviewed patient records included indication to the surgery, type of the operation, operative details, and the length of the aganglionic bowel segment. The diagnosis of HD was based on disease history, operative findings, and histology of the resected colonic specimen featuring absence of ganglion cells together with an increased acetylcholinesterase staining.

Cancer registry

Follow-up of the 156 HD patients for cancer was performed automatically based on the ID codes, in linkage with the population-based countrywide Finnish Cancer Registry. The follow-up started at the date of the subject's birth (which is very close to the date of operation of HD) or January 1st 1967, whichever was later, and ended upon the subject's death or December 31st 2010, whichever occurred first. In addition, medical records were reviewed for 1950–1967 to ensure that no MTC cases were missed

because MTC can be an early-onset disease. The Finnish Cancer Registry maintains records of all the cancer patients from every medical facility in Finland and has practically a complete coverage of the population and a high accuracy (Teppo *et al.* 1994, Pukkala 2011).

Thyroid US

An experienced radiologist performed all US examinations. The whole neck area was examined with special attention to morphology of the thyroid gland and the nearby lymph nodes. A FNB was obtained from any solid or cystic solid hypoechoic or isoechoic solitary lesions in the thyroid gland or from extra-glandular focal lesions with a low threshold. The FNB was not obtained from simple cysts or typical multiple goiter nodules with a sonolucent halo sign. US examination was repeated with or without FNB if initial US and cytology were not entirely suggestive of a benign lesion.

Serum calcitonin

Serum calcitonin level was measured immunoluminometrically after an overnight fast using standard hospital laboratory methods. The upper normal limit for fasting serum calcitonin was 1.7 pmol/l for women and 3.8 pmol/l for men.

Genetic analyses

DNA was extracted from 30 ml whole blood samples. In all participants, exons 10, 11, 13, and 16 of the *RET* cDNA were PCR amplified and nucleotide sequences were resolved with the ABI3730xl DNA Analyzer capillary electrophoresis instrument (Applied Biosystems) using standard methods. All exons of the *RET* gene were additionally sequenced in 43 of the subjects including those with thyroid cancer, *RET* mutation in exons 10, 11, 13, or 16, suspicious clinical findings, familial or long-segment disease, and a group of randomly selected subjects.

Statistical analyses

The numbers of observed cases of thyroid cancer among the HD patients and numbers of person-years at risk were counted by 5-year age groups, separately for both sexes and four calendar periods (1967–1977, 1978–1988, 1989–1999, and 2000–2010). The expected number of cancer cases was calculated by multiplying the number of

person-years in each stratum by the corresponding cancer incidence in all of Finland. The standardized incidence ratio (SIR) was calculated by dividing the observed number of cases with the expected number. Exact 95% CIs were defined on the assumption that the data followed a Poisson distribution.

Ethics

The study protocol was approved by the Ethical Committee of the Hospital for Children and Adolescents, University of Helsinki.

Results

Occurrence of thyroid cancer

In the entire cohort of 156 subjects, two MTCs and one papillary thyroid cancer were observed. The SIR for all types of thyroid cancer combined was 20 (95% CI 4.1–58) and for MTC 340 (95% CI 41–1200). Both MTC cases were in age category 30–44 years; SIR in this category was 700 (95% CI 85–2500).

Subject characteristics

A total of 91 subjects participated. Age, gender, and the level of aganglionosis were similarly distributed among participants and non-participants, making significant selection bias unlikely (Table 1). There were a total of 15 familial and 76 sporadic HD cases.

Previously diagnosed benign thyroid or parathyroid diseases included hyperthyroidism, hypothyroidism, and hyperparathyroidism one each. One subject had Down syndrome, one cartilage hair hypoplasia, one was paraplegic and deaf, and one was blind and had Marfan

Table 1 Subject characteristics

	Participants (n=91)	Non-participants (n=49)
Age (years) ^a	44 (19–68)	44 (19–68)
Females	18 (20%)	5 (10%)
BMI ^a	25.6 (18.0–36.2)	Unknown
Family history of HD	15 (16%)	Unknown
Level of aganglionosis		
Rectosigmoid	83 (91%)	42 (86%)
Long-segment	5 (5%)	4 (8%)
Unclear	3 (3%)	3 (6%)

HD, Hirschsprung disease.

^aData are shown as mean and range.

syndrome. Cardiovascular diseases ($n=9$), asthma ($n=5$), type 2 diabetes ($n=3$), psychiatric conditions ($n=3$), and lactose intolerance represented the most common other chronic medical conditions. Two sporadic short-segment HD patients had a family history of thyroid cancer. Down syndrome ($n=2$) was the only associated disorder, which could be traced among the non-participants (Table 1).

Clinical screening

Thyroid US examination and cytology A total of 110 thyroid US examinations were performed at least once to all participants excluding one patient with previous thyroidectomy (Fig. 1). The first examination was normal in 81% of cases. US was repeated up to four times in 13 participants with an average interval of 10 months (range 1–38 months) between the first and the last examinations. Overall, FNB of a thyroid lesion was obtained in 11 patients. Cytological evaluation revealed one papillary thyroid carcinoma, benign sialoblastoma, three goiters, one cyst, and two cases of thyroiditis. Rest of the samples were regarded as normal ($n=1$) or insufficient ($n=2$). The patients with insufficient specimens had undetectable serum calcitonin levels and they underwent two to three repeated US examinations confirming benign and unprogressive nature of the lesions. The patient with papillary carcinoma underwent uncomplicated thyroid lobectomy at the age of 46 years, and the sialoblastoma was surgically removed. Histology of the resected specimen confirmed the cytological diagnosis in both cases.

Serum calcitonin A total of 104 measurements of serum calcitonin concentration were performed at least once to all participants. Calcitonin concentration was undetectable in 62 (68%) and detectable (1.9, range 1.1–3.4 pmol/l) within normal limits in 26 (29%) participants. Only three patients (3%) had elevated serum calcitonin concentration (4.5, range 2.9–7.2 pmol/l). Of them, one underwent pentagastrin-stimulated measurement of serum calcitonin with a normal finding, and serum calcitonin concentration had normalized in repeated measurement in one. The third patient underwent four follow-up calcitonin measurements and US examinations within 31 months. Calcitonin concentration remained slightly increased (3.8, range 3.5–4.2 pmol/l) and US findings were equivocal. Serum calcitonin measurement was repeated in additional eight patients with detectable calcitonin concentration in the first measurement. The repeated measurement was within the normal range in every case.

RET mutations and their clinical correlations

Primary sequencing of exons 10, 11, 13, and 16 Four different mutations and three different single-nucleotide polymorphisms (SNP) of the *RET* were found by sequencing exons 10, 11, 13, and 16. Three of the mutations included the same TGC to CGC mutation changing cysteine to arginine in codons 609, 611, and 620 in exon 10. The fourth mutation was a TAT to TTT mutation changing tyrosine to phenylalanine in codon 791 in exon 13

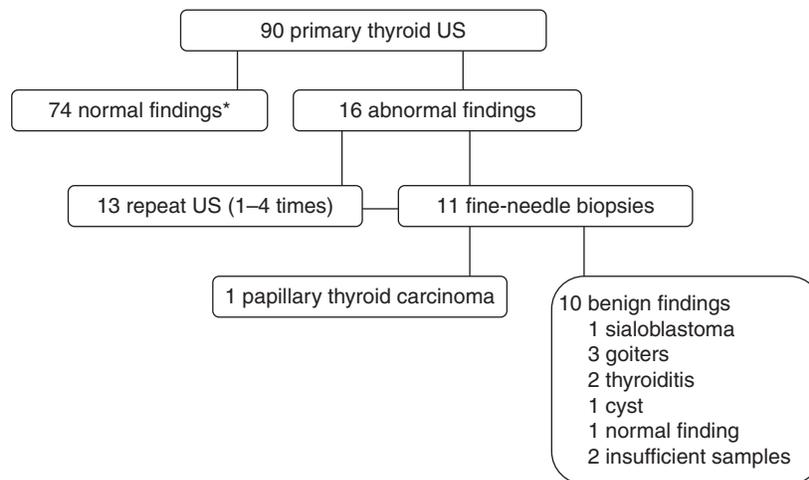


Figure 1

Results of thyroid ultrasound examinations and fine-needle biopsies.
*Normal findings included ten goiters.

Table 2 Observed mutations and rare SNPs in all exons of RET with their clinical correlations

Mutation/SNP	Exon	Thyroid cancer	Age (years)	Familial HD	Level of aganglionosis	Serum calcitonin	Thyroid ultrasound
C611R (<i>n</i> =1)	10	MTC	35	Yes	RS	NA	NA
C620R (<i>n</i> =1)	10	MTC	37	No	RS	NA	NA
C609R (<i>n</i> =1)	10	No	31	No	RS	Normal	Normal
Y791F (<i>n</i> =3)	13	No	38, 45, 50	All no	All RS	All normal	All normal
W37* (<i>n</i> =1)	2	No	46	Yes	RS	Normal	Normal
F147S (<i>n</i> =1) ^a	3	No	33	Yes	RS	Normal	Normal
V125V (<i>n</i> =1)	3	No	34	Yes	RS	Normal	Normal
47665insT (<i>n</i> =1)	17	No	46	Yes	LS	Normal	Normal
R982C (<i>n</i> =1) ^a	18	No	33	Yes	RS	Normal	Normal

SNP, single-nucleotide polymorphism; MTC, medullary thyroid cancer; HD, Hirschsprung disease; RS, rectosigmoid; LS, long-segment; NA, not applicable.
^aSame patient having both mutations.

(Table 2). The patient with C620R *RET* mutation had familial MEN2A with previously treated pheochromocytoma and MTC at the age of 37 years, and the patient with C611R *RET* mutation had previously treated MTC at the age of 35 years. None of the four sporadic short-segment HD patients with missense mutations in codons 609 (*n*=1) and 791 (*n*=3) had any clinical evidence suggestive of thyroid cancer (Table 2). The mean age of these four patients was 41 years. The C609R *RET* mutation carrier was 31 years old and the carriers of Y791F *RET* mutation were 38, 45, and 50 years old. Observed SNPs were rs1799939 in exon 11, rs1800861 in exon 13, and rs3026773 in the intronic region. Their carriers had normal thyroid US and serum calcitonin.

Additional sequencing of all exons of RET All exons of RET were additionally sequenced in 43 of the subjects. No additional *RET* mutations were observed among the patients with either medullary (*n*=2) or papillary thyroid cancer (*n*=1), *RET* mutations in primary sequencing of exons 10, 11, 13, or 16 (*n*=4), or suspicious clinical findings (*n*=2), including one patient with a history of hyperparathyroidism and the patient with chronically elevated calcitonin level described earlier. The remaining 34 patients, who underwent sequencing of all exons of RET, included all subjects with familial HD (*n*=15) or long-segment aganglionosis (*n*=5) and a group of randomly chosen subjects with sporadic HD (*n*=19). Among them, three novel mutations, two rare SNPs, and three commonly shared SNPs, which were not associated with clinical signs of MTC, were found (Table 2). The novel mutations were W37* in exon 2, F147S in exon 3, and a frameshift-introducing insertion 47665insT (CM000672.1:g.43619181_43619182insT) in exon 17. The rare SNPs were rs1800859 (V125V) in exon 3 and rs17158558 (R982C) in exon 18. The three commonly

shared SNPs included rs1800858 in exon 2, rs1800860 in exon 7, and rs1800863 in exon 15.

Co-segregating *RET* mutations

In total, we found six patients with *RET* mutations co-occurring in HD and MTC. Two of the patients were diagnosed and operated for MTC before this study. The remaining four were identified through the clinical screening of 91 subjects. Assuming corresponding frequency among the 49 non-participants would mean two additional mutation carriers among them and an overall approximate point prevalence of 5% (8/156).

Discussion

Here, we have combined clinical, genetic, and registry screening of co-occurring *RET* mutations and thyroid cancer in an unselected population-based cohort of adults with HD. The cancer history of the whole cohort was reviewed from the Finnish Cancer Registry and participation to clinical screening was offered to all eligible subjects. Participants (65%) were representative for the entire study population and they underwent combined clinical and genetic cancer screening. Age, gender, and level of the aganglionosis were comparable between the participants and non-participants making a significant selection bias unlikely. The major findings of this survey suggest that MTC-associated *RET* mutations are restricted to exons 10 and 13 affecting 5% of unselected adults with HD. Accuracy of genetic screening was not improved by clinical thyroid assessment. Genetic screening should not be limited to patients with familial or long-segment disease. Based on our findings, mutational screening of *RET* exons 10 and 13 is warranted in HD.

Some earlier studies have recommended mutation analysis of the *RET* gene in HD (De Groot *et al.* 2005, Skába *et al.* 2006, Fialkowski *et al.* 2008). MEN2A/FMTC has been found to co-occur with HD in few patients worldwide (Borst *et al.* 1995, Borrego *et al.* 1999, Eng 1999, Fernández *et al.* 2003, Nishikawa *et al.* 2003, De Groot *et al.* 2005, Dvoráková *et al.* 2005, Bütter *et al.* 2007, Fialkowski *et al.* 2008). The main focus of these studies has been in the genetic screening of certain *RET* germline mutations (Pasini *et al.* 2002, Nishikawa *et al.* 2003, Dvoráková *et al.* 2005). In some studies, HD patients or their relatives with MTC were genetically screened after the diagnosis of thyroid cancer (Fernández *et al.* 2003). In other studies, smaller and younger selected patient cohorts have undergone genetic screening after the diagnosis of HD followed by prophylactic thyroidectomy in patients with high-risk *RET* mutations (Dvoráková *et al.* 2005, Fialkowski *et al.* 2008). Genetic screening of MTC patients has also revealed *RET* mutations co-occurring with HD in some cases (Bütter *et al.* 2007). To our best knowledge, there are no previous studies on unselected population-based cohort of adult HD patients screened for thyroid malignancies both genetically and clinically. Screening of thyroid malignancies in HD could potentially benefit patients by enabling an early diagnosis and treatment of cancer resulting in decreased cancer-related morbidity and mortality.

Previous studies suggest that MTC co-occurs with familial and sporadic HD, with short- and long-segment as well as with syndromic and non-syndromic forms of HD, suggesting that all patients regardless of family history or disease characteristics should be screened (Le Douarin & Kalcheim 1999, Fernández *et al.* 2003, Amiel *et al.* 2008). In general, patients with non-syndromic and long-segment HD and those with familial history of endocrine tumors appear to have the highest risk for MEN2A (Garipey 2003). Here, we combined extensive genetic screening with clinical thyroid cancer screening after reliable registry-based patient identification. This study design enabled accurate evaluation of genotype–phenotype correlation between *RET* mutations and thyroid cancer. In accordance with previous studies, we found that the majority of co-segregating *RET* mutations were found among patients with sporadic and short-segment HD (Table 2).

All thus far identified HD-associated thyroid cancer mutations are located in exon 10 of the *RET* gene (Mulligan *et al.* 1994a,b, Romeo *et al.* 1998, Sijmons *et al.* 1998, Nishikawa *et al.* 2003). Mutations in codons 609, 611, and 620 carry markedly increased risk for developing MTC (Brandi *et al.* 2001, Frank-Raue *et al.* 2006). Mutations

in codon 791 of the *RET* gene appear to be weak or nonpathogenic mutations for MEN2A and FMTC (Frank-Raue *et al.* 2008, Erlic *et al.* 2010). Our screening identified four patients aged between 31 and 50 years with mutations of the *RET* gene associated with development of MTC without any clinical evidence of thyroid cancer at screening (Mulligan *et al.* 1994a,b, Romeo *et al.* 1998, Sijmons *et al.* 1998, Nishikawa *et al.* 2003). These mutations included a C609R of exon 10 and Y791F ($n=3$) of exon 13. Further follow-up including prophylactic thyroidectomy in the case of C609R aged 31 years seems warranted. This is further supported by linear age-dependent increase in MTC incidence after the age of 20 years (Frank-Raue *et al.* 2011). Previously, Y791F *RET* mutations were thought to cause less aggressive form of thyroid cancer, and a life-long calcitonin screening was suggested instead of prophylactic thyroidectomy (Frank-Raue *et al.* 2008). However, more recent findings question pathogenicity of an isolated Y791F *RET* mutation in MTC (Erlic *et al.* 2010). More likely, Y791F *RET* is a modifying mutation acting together with some other synchronous mutations (Toledo *et al.* 2010).

Records of all cancers from every medical institute in Finland are maintained by the Finnish Cancer Registry, which also provides information about the incidence of each cancer type in the entire Finnish population with a complete coverage and high accuracy since 1953 (Teppo *et al.* 1994). The computerized record linkage procedure is very precise (Pukkala 2011). In this study, two cases of MTC were identified through the Finnish Cancer Registry, which is over 300 times more than would be expected in such a small cohort on the basis of national MTC incidence. Clinical screening revealed only one papillary thyroid cancer case corresponding the incidence in all of Finland. In total, we found six patients with co-occurring *RET* mutations. Assuming similar frequency among participants and non-participants, the estimated overall prevalence of MTC-associated *RET* mutations was 5% in the whole cohort.

All exons of *RET* gene were not sequenced in every subject leaving a theoretical possibility that some co-occurring mutations were missed. However, in addition to sequencing exons 10, 11, 13, and 16 in all, we sequenced all exons of *RET* gene in 47% of the subjects, including the ones with thyroid cancer, *RET* mutation, any clinical findings suggestive of thyroid cancer at screening, or predisposing disease characteristics. Three novel mutations and five SNPs were found outside exons 10 and 13, but none of their carriers showed any signs suggestive of thyroid cancer in clinical assessment (Table 2).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was financially supported by the Helsinki University Central Hospital Grant, the Sigrid-Juselius Foundation, and the Finnish Pediatric Research Foundation.

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Received in final form 25 May 2013

Accepted 5 June 2013

Made available online as an Accepted Preprint

6 June 2013