No evidence of RET germline mutations in familial pituitary adenoma

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Abstract

Pituitary adenomas are common in the general population. Although most of them are sporadic, some occur in a familial setting. In familial pituitary adenoma patients it is common that no germline defects are found after screening of aryl hydrocarbon receptor interacting protein (AIP) and other genes known to underlie the condition, suggesting the existence of yet unknown predisposition genes. Recently, the RET proto-oncogene was found to be a novel in vivo interaction partner of AIP in the pituitary gland. Here, we have screened patients from 16 AIP mutation negative (AIPmut−) pituitary adenoma families for RET germline mutations to assess whether RET could play a role in pituitary adenoma predisposition, similar to AIP. We found five novel germline RET changes: one in RET Exon 4 and the rest in noncoding regions of RET. Two changes, c.1560G>A and c.1285G>A, were segregated in affected family members. We also analyzed the RET region with enhancer element locator (EEL) to identify RET regulatory elements, and to see whether the changes resided in these. None of the variants mapped to the regions predicted by EEL. Expression of RET was examined in ten AIPmut− and seven AIP mutation positive (AIPmut+) somatotropinomas by immunohistochemistry, with a trend showing reduced expression in the latter (P=0.05). We conclude that the RET variants are presumably not related to pituitary adenoma predisposition, although reduced RET expression may play a role in AIP-related genesis of somatotropinomas.

Journal of Molecular Endocrinology (2011) 46, 1–8

Introduction

Pituitary adenomas are common benign neoplasms, which account for ~15% of all intracranial tumors. They can be clinically nonfunctioning or hormone secreting. Among the latter, prolactin (PRL) and GH-secreting adenomas are the most common (Heaney & Melmed 2004). The majority of pituitary adenomas arise sporadically, although a subset occurs as component tumors of well-characterized familial cancer syndromes, such as multiple endocrine neoplasia type I (MEN1), Carney complex, and an MEN1-like syndrome (MEN4; Chandrasekharappa et al. 1997, Stratakis et al. 2001, Pellegrata et al. 2006, Georgiatis et al. 2007a, Tichomirowa et al. 2009). A fourth pituitary adenoma susceptibility gene, aryl hydrocarbon receptor interacting protein (AIP) (11q13), was recently identified (Vierimaa et al. 2006).

Germline mutations in AIP cause a pituitary adenoma predisposition characterized by young age at disease onset and occurrence of somatotropinomas (Daly et al. 2007). Germline AIP mutations, such as nonsense and missense mutations and large genomic deletions, have been reported in different populations, in patients with and without a positive family history of pituitary adenoma (Vierimaa et al. 2006, Georgiatis et al. 2007b, 2008, Cazabat et al. 2009, Tichomirowa et al. 2009). Loss of heterozygosity at the AIP locus in tumors and recent...

The RET proto-oncogene (10q11) is a tyrosine kinase transmembrane receptor for glial cell line-derived neurotropic factor ligands (Trupp et al. 1996, 1998). Gain-of-function mutations of RET are associated with the tumor syndromes MEN2A and MEN2B and with familial medullary thyroid carcinoma. Loss-of-function mutations of RET cause the neurodevelopmental disorder, Hirschsprung’s disease, characterized by congenital intestinal agangliosis (Mulligan et al. 1993, Romeo et al. 1994, Arighi et al. 2005). A common noncoding RET variant (rs2435357) within a conserved enhancer-like sequence in Intron 1 is also associated with the risk of Hirschsprung’s disease (Emison et al. 2005). Haplotypes of the human RET proto-oncogene, which are associated with Hirschsprung’s disease, have been shown to derive from a single ancestral combination of alleles in the Italian population. The variant rs2435357 is part of a large haplotype that extends from the RET promoter up to Intron 19 (Lantieri et al. 2006).

In the normal pituitary, RET is expressed in somatotrophs where it is associated with apoptosis and differentiation (Urbano et al. 2000, Japón et al. 2002, Cánibano et al. 2007). In pituitary adenomas, it is present in somatotropinomas and a subset of corticotropinomas (Japón et al. 2002). Recently, RET was found to interact with AIP in mammalian cell lines and in vivo in the rat pituitary gland. AIP interacts with the proapoptotic domain of RET, and clinically pathogenic RET or AIP mutations that were introduced to cell constructs did not impair this interaction. In the same study, no somatic RET mutations were found in the 28 screened somatotropinomas (whole gene; 28 cases) (Vargioli et al. 2009). RET mutations have also previously been searched for in human pituitary adenomas in studies by Komminoth et al. (1996) (Exons 10, 11, 13, 15, and 16; 8 cases), Yoshimoto et al. (1999) (Exons 10, 11, 13, and 16; 172 cases), and Vieira Neto et al. (2007) (whole gene; 1 case) but no relevant mutations have been found.

A significant subset of familial pituitary adenoma patients does not display mutations in the known predisposition genes. These patients are often diagnosed with familial isolated pituitary adenoma, which is characterized by a heterogeneous phenotype of pituitary adenomas with unknown underlying genetic defects (Tichomirowa et al. 2009). Although previous studies have found no somatic pathogenic RET mutations in pituitary adenoma, to our knowledge, RET germline mutations have not been previously assessed in familial pituitary adenoma. Perceiving that specific RET mutations have a clear connection with various human disease phenotypes including endocrine tumors, and that AIP and RET are cellular interaction partners, we aimed to determine whether RET mutations could cause a rare familial pituitary adenoma phenotype, similar to AIP mutations.

We screened 16 familial AIP mutation negative (AIPmut−) pituitary adenoma patients for RET mutations. Enhancer element locator (EEL) was applied to map RET regulatory elements, to create new data on regulation of this key oncogene and to assist in evaluation of the noncoding variants identified in the study. In addition, RET immunohistochemistry was performed to assess the expression of RET in AIP mutation positive (AIPmut+) and AIPmut− pituitary somatotropinomas.

Materials and methods

Patients and control samples

Sixteen patients from families with pituitary adenomas were included in this study. All the patients had previously been sequenced negative for AIP mutations, and AIP multiplex ligation-dependent probe amplification assay had also been performed in 12 out of 16 patients (Vierimaa et al. 2006, Georgitsi et al. 2007a, 2008; Table 1). The patients were from the United Kingdom (n=6), Italy (n=5), Finland (n=3), Turkey (n=1), and New Zealand (n=1) (Table 1). Age at diagnosis or in some cases age at operation ranged from 25 to 67 years with a mean of 46 years. The tumors secreted GH (n=8), PRL (n=3), and ACTH (n=1). Four adenomas were nonfunctioning. DNA samples from available relatives were analyzed in cases where segregation of RET variants was evaluated (Table 1). DNA samples from healthy population controls were used as control samples: 279 Caucasians from the United Kingdom (Human Random Control DNA panels, Sigma–Aldrich) and 41 samples from Italy. Appropriate informed consent for sample and patient information usage were obtained from all the patients. The study was approved by the appropriate ethical committees.

RET mutation analysis

Mutation screening was performed from blood-isolated genomic DNA. PCR protocols and primer sequences are available on request. PCR products were purified using Exo-SAP-IT PCR purification kit (USB Corporation, Cleveland, OH, USA). DNA sequencing was performed by Big Dye 3.1 termination chemistry on an ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The whole coding region of RET (Ensembl version 55 gene ENSG00000165731, transcript ENST00000340058) and flanking intronic sequences of exons were sequenced, as well as
Table 1 Patient information and the detected RET changes

<table>
<thead>
<tr>
<th>Family</th>
<th>Sex</th>
<th>Age at Dg</th>
<th>Tumor secretes</th>
<th>Negative screening in family</th>
<th>Familial background (relation)</th>
<th>Patient’s other clinical data</th>
<th>RET change</th>
<th>Segregation</th>
<th>Controls</th>
<th>rs2435357</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK</td>
<td>M</td>
<td>67</td>
<td>NFPA</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>Pituitary adenomas (brother, sister)</td>
<td>GH (mother, brother)</td>
<td></td>
<td>T/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>60</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td></td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>50</td>
<td>NFPA</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>NFPA (daughter) PRL (sister), uterine fibroids</td>
<td>c.1560(^{*})G &gt; A in 3’UTR IVS17 + 105delG</td>
<td>No</td>
<td>T/T</td>
<td>0/279</td>
<td>C/C</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>47</td>
<td>PRL</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>NA</td>
<td>29</td>
<td>NFPA</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>NFPA (mother)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>F</td>
<td>54</td>
<td>NFPA</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>NFPA (mother)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>44 (op)</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>NFPA (niece)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td>42</td>
<td>GH</td>
<td>AIP(^a), AIP-MLPA(^b)</td>
<td>GH (niece)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>F</td>
<td>36 (op)</td>
<td>PRL</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>GH (aunt)</td>
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<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>67 (op)</td>
<td>ACTH</td>
<td>AIP(^a), CDKN1B(^a)</td>
<td>GH (son)</td>
<td>V262A c.785T &gt; C Exon 4</td>
<td>No</td>
<td>0/41</td>
<td>C/C</td>
<td></td>
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<tr>
<td></td>
<td>F</td>
<td>49 (op)</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a)</td>
<td>PRL (daughter)</td>
<td></td>
<td></td>
<td>T/C</td>
<td></td>
<td></td>
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<tr>
<td>Finland</td>
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<td>40</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>PRL (maternal cousin) TC (mother)</td>
<td>PTC, MB, SCH</td>
<td></td>
<td>T/C</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>M</td>
<td>37 (op)</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a), AIP-MLPA(^b)</td>
<td>GH (paternal brother)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>35</td>
<td>GH</td>
<td>AIP(^a), CDKN1B(^a)</td>
<td>GH (nephew’s daughter)</td>
<td></td>
<td></td>
<td>T/T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td>M</td>
<td>25</td>
<td>PRL</td>
<td>AIP</td>
<td>GH (father)</td>
<td></td>
<td></td>
<td>T/T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turkey</td>
<td>M</td>
<td>54</td>
<td>GH</td>
<td>AIP(^c), CDKN1B(^a), MEN1(^a), AIP-MLPA(^b)</td>
<td>GH (mother)</td>
<td></td>
<td></td>
<td>C/C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dg, diagnosis; NFPA, nonfunctioning pituitary adenoma; NA, not available; op, age at operation; TC, thyroid cancer; PTC, papillary thyroid cancer; MB, medulloblastoma; SCH, Schwannoma; MLPA, multiplex ligation-dependent probe amplification. Family 5 is of Vietnamese origin. \(^a\)Georgitsi et al. (2007a). \(^b\)Georgitsi et al. (2008). \(^c\)Vierimaa et al. (2006). The rest of the negative screenings are unpublished data.
5' and 3' UTRs, promoter regions, and a noncoding RET variant (rs2435357). Evaluation of sequences was done with Mutation Surveyor software V3.24 (Soft-Genetics, State College, PA, USA).

**Prediction of enhancer elements around RET**

EEL is a freely available computational tool that predicts enhancer elements based on, for example, analysis of transcription factor binding affinity (Hallikas et al. 2006). Genomic human and mouse sequences 50 kb up- and downstream from RET and Ret (GRCh37:10:43522517:43675799:1 and NCBIM37:6:118051766:118197762:1 respectively) were aligned and the EEL computer algorithms were applied as previously described to detect possible enhancer elements in and around RET (Palin et al. 2006). We used 149 previously described transcription factor binding site matrices (from Jaspar2, Hallikas et al. 2006, Badis et al. 2009).

**RET immunohistochemistry**

RET was stained on slides from paraffin-embedded pituitary tumors from seven AIPmut+ somatotropinoma patients and ten AIPmut– somatotropinoma patients. PoverVision Poly-HRP IHC Detection System kit (PV6104; Leica Biosystems Newcastle Ltd, Newcastle, UK) was used for detection of RET (ab51122; 1:75 dilution; Abcam, Cambridge, UK). Diaminobenzidine was used as a chromogen and hematoxylin as a counterstain. RET was scored as negative (−) or positive (+). In cases where the tumor stained partly negative and partly positive, the tumor was screened as (+/−). A Leica DM LB was used in microscopy (×40 objective; Meyer Instruments, Houston, TX, USA). Imaging was performed with an Olympus DP50 camera and Studio Lite Imaging Software.

**Results**

**RET mutation analysis**

Twenty-eight RET fragments were sequenced in 16 patient samples. In total, 99-3% of fragments (445 of 448) were successfully sequenced and analyzed. Five novel heterozygous RET variants were found. A review of these genetic findings in the RET region is provided in Fig. 1.

A heterozygous V262A missense change c.785T>C was found in RET Exon 4 in an Italian prolactinoma patient. However, this variant was absent in the patient’s aunt with acromegaly. The change was not found in 41 Italian controls (Table 1).

**Figure 1** Schematic RET region illustrating the five genetic findings from familial pituitary adenoma patients.

A heterozygous 3'UTR change c.1560G>A was detected in a British prolactinoma patient and her sister with prolactinoma. The change was not found in an unaffected sister with uterine fibroids and endometriosis or in 279 British Caucasian controls. In the same patient, we also found an unreported heterozygous IVS17+105delG. However, the affected sister did not share the deletion and it was also found in the unaffected sister and 10 of 91 analyzed British Caucasian controls (Table 1).

A heterozygous –1285G>A change upstream of RET was found in a British nonsecreting adenoma patient of Vietnamese origin. It was also present in the patient’s mother with nonfunctioning pituitary adenoma. No Asian controls were available. The mother also had a heterozygous –1491C>T change that was not present in her child (Table 1).

**EEL analysis of the RET region**

The EEL tool was used to predict whether the two new segregating RET variants, c.1560G>A and –1285G>A, could reside in putative enhancer elements of the RET region. The 100 highest scored alignments were mapped in the RET region and the 6 highest scored alignments were taken into closer examination (Fig. 2). No overlap between the variants and the predicted enhancer elements was found. The highest scored element (score 258.42) is a 295 bp long fragment beginning 5624 bp upstream of RET and comprising 5 putative transcription factor binding sites (Fig. 2). Detailed RET EEL results on the remaining 94 alignments are available on request.

We also screened a previously described noncoding RET variant (rs2435357 C>T) associated with the risk of Hirschsprung’s disease and situated within a conserved enhancer-like sequence in RET Intron 1. The disease-associated allele is T and the wild-type allele is C (Emison et al. 2005). The T/C genotype was detected in three samples. T/C alleles were present in three samples and C/C in ten samples (Table 1). Near this variant, EEL predicted a putative enhancer element (score 184.23) of 152 bp and comprising 9 transcription factor binding sites (Fig. 2).
RET immunohistochemistry

RET expression was positive (+) in nine out of ten AIPmut− somatotropinomas. One AIPmut− somatotropinoma stained partly negative and partly positive (+/−). AIPmut+ somatotropinoma results were as follows: negative (−) in three out of seven samples; (+/−) in two out of seven samples; and (+) in two out of seven samples (Fig. 3 and Table 2). When considering (−) staining versus (+) and (+/−) staining with Fisher’s exact test, the two-sided P value is 0.05 (Table 2).

Discussion

Germline mutations in AIP cause pituitary adenoma predisposition (Vierimaa et al. 2006). Recently, AIP was shown to interact in vivo in the pituitary gland with the RET proto-oncogene, whose mutations have a well-characterized role in a variety of endocrine tumors (Mulligan et al. 1993, Arighi et al. 2005, Vargiolu et al. 2009). We screened 16 AIPmut− familial pituitary adenoma patients for RET germline mutations to assess...
whether RET could have an impact on pituitary tumorigenesis. We found five previously unreported RET variants of which two segregated with the phenotype. Three heterozygous variants (V262A missense change c.785T>C in RET Exon 4, IVS17+105delG, and -1491C>T) did not segregate with pituitary adenoma phenotype indicating that they are likely to be rare polymorphisms with little clinical relevance (Table 1).

In a British prolactinoma patient, we found a heterozygous c.1560G>A change in the 3'UTR, which was present in the affected sister. The change was absent in 279 control samples and an unaffected sister (Table 1). A heterozygous -1285G>A change was found in a Vietnamese patient and her affected child. No Asian control samples were available. The variant occurs quite far from the coding region of RET and is not located on any known promoter or regulatory region of RET (Guo et al. 2007). We applied the EEL tool to detect whether it would predict enhancer elements overlapping the two segregating RET changes, but no such elements were found. Thus, it seems unlikely that these variants were related to pituitary tumorigenesis.

We also analyzed the noncoding region harboring rs2435357 C>T, a low-penetrance risk allele for Hirschsprung’s disease. The T allele reduces in vitro RET enhancer activity and decreases transcription (Emison et al. 2005). The disease-associated allele T was present in three samples in homozygous form. Three specimens were heterozygous, and the remaining ten were homozygous for the ‘wild-type’ allele C (Table 1). Pituitary tumors of the patients having the T allele (n=6) were histologically heterogeneous and the age of the patients ranged from 25 to 67 years. The allele frequencies we found are similar to the previously described population frequencies of C and T alleles (Emison et al. 2005). Thus, it seems unlikely that rs2435357 would have an impact on pituitary tumorigenesis.

Table 2  RET expression in AIPmut+ and AIPmut− pituitary somatotropinomas

<table>
<thead>
<tr>
<th>RET expression in tumor</th>
<th>AIPmut+ samples</th>
<th>AIPmut− samples</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (−)</td>
<td>3</td>
<td>0</td>
<td>0.05147</td>
</tr>
<tr>
<td>Positive/negative (+/−)</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Positive (+)</td>
<td>2</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

*Two-sided P value with Fisher’s exact test, − versus +/− and + expression.
the amount of transcription factor binding matrices could enlarge the predicted element and increase the number of putative transcription factor binding sites. All in all, finding overlapping sequence elements with different techniques increases their reliability. Based on these data, the biological impact of rs2435357 on Hirschsprung’s disease predisposition remains unclear.

Recent studies on the RET regulatory landscape and enhancers have enlightened the regulation of RET (Emison et al. 2005, Grice et al. 2005, Fisher et al. 2006). Our predicted RET enhancer with the highest EEL score (258±42) was a 295 bp long element beginning 5624 bp upstream of RET and comprising 9 transcription factor binding sites (Fig. 2). Grice et al. (2005) showed that MCS −5−2, an MCS comprising our predicted enhancer, enhanced luciferase expression in neuronal cells. Located in the vicinity of the RET promoter area, this predicted element could be significant in the control of RET expression, although further functional and in vivo studies are needed to verify its role.

RET immunohistochemistry results on AIPmut+ and AIPmut− somatotropinomas might indicate RET underexpression in AIPmut+ pituitary adenomas (P=0.05; Fig. 3 and Table 2). This interesting finding could suggest a putative role for RET in AIP-mediated pituitary tumorigenesis. However, with such a small set of samples this is an issue that must be unraveled in further studies.

The present study presents five novel genomic heterozygous variants of RET in AIPmut− familial pituitary adenoma patients with currently unknown pathogenic mechanisms underlying adenoma formation. While definite exclusion of a pathogenic role is always challenging it seems that none of these variants are likely to be causative, and the study thus found no evidence for a role for RET as a pituitary adenoma susceptibility gene. However, this study cannot fully exclude the possibility of RET mutations as an exceedingly rare cause of familial pituitary tumorigenesis. Thus, additional studies would be needed to verify this issue. Although EEL did not reveal putative enhancer elements overlapping the identified sequence variants, the EEL results provide valuable data on transcriptional regulation of RET for further validation. Finally, although RET immunohistochemistry indicated underexpression of RET in AIPmut+ pituitary somatotropinomas (P=0.05), the validation of this observation requires further studies.

Funding
This study was supported by the Helsinki Biomedical Graduate School, the Academy of Finland (the Center of Excellence in Translational Genome-Scale Biology) (grant number 639232), the Sigrid Juselius Foundation (grant number 4701169), and the Cancer Society of Finland (grant number 4700325).

Author contribution statement
E H performed RET mutational analysis. E H and S T aligned RET sequences with EEL and validated the results. S H, E M, O K, L I, R J M G, S G, and A L provided the patient samples. A T and M M provided tumor slides for RET immunohistochemistry, M A and E H performed immunohistochemistry and J A scored the stainings. A K and L A A contributed to the research design and supervised the project. E H, S T, A K, and L A A wrote the manuscript.

Acknowledgements
We are grateful to Sini Marttinen, Inga-Lill Svedberg, Ilna Vuoristo, and Yilong Li for excellent technical assistance.

References

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.


Received in final form 4 October 2010

Accepted 15 October 2010

Made available online as an Accepted Preprint 18 October 2010