Polymorphisms in \textit{RET} and Its Coreceptors and Ligands as Genetic Modifiers of Multiple Endocrine Neoplasia Type 2A

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Abstract

Germ line missense mutations in the \textit{RET} proto-oncogene are responsible for the inherited cancer syndrome multiple endocrine neoplasia type 2A (MEN2A). The clinical presentation of the disease and the age at onset varies even within families, where patients carry the same mutation. These variations in phenotypes suggest a role for genetic modifiers, and recently, it has been reported that polymorphisms within \textit{RET} (G691S/S904S) may have such a modifier effect on the age at onset. Here, we investigate whether this observed association could be confirmed in a larger set of 384 individuals from MEN2 families from four different European populations. In addition, we tested as modifiers four other single nucleotide polymorphisms (SNPs), which we have found in a previous association study of \textit{RET}, its coreceptors, and ligands to be associated with the risk of developing sporadic medullary thyroid carcinoma. We could not replicate the association between G691S/S904S and modifier effects in MEN2A families in any of the four European families analyzed. Of the other SNPs tested, only RET A432A showed a positive weak effect on tumor spectrum within MEN2A, which requires replication in a larger series. (Cancer Res 2006; 66(2): 1177-80)

Introduction

The inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN2) can be divided in three clinically distinct forms: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC; ref. 1). All forms are transmitted as an autosomal dominant trait with a high degree of penetrance and variable clinical expression and have in common MTC. In patients with FMTC, only the thyroid gland is affected, whereas patients with MEN2A may also develop phaeochromocytoma and primary hyperparathyroidism. Between 93% and 98% of MEN2A families have mutations of one of the five conserved cysteine residues in exon 10 (codons 609, 611, 618, and 620) or exon 11 (codon 634) in the extracellular domain of the \textit{RET} proto-oncogene (2). Mutations in Cys\textsuperscript{634} are the most commonly found in MEN2A families.

The different specific activating mutations in \textit{RET} are associated with different clinical types of MEN2A. The explanations for the genotype-phenotype correlations are not yet certain. Several lines of evidence suggest that they may result either from different levels of \textit{RET} activation induced by different mutations or, in one particular class of mutation, from altered substrate specificity of the \textit{RET} tyrosine kinase.

The range of phenotypic expression seen between families with different mutations and the variation within families with the same mutation provide a potentially interesting and tractable system for analysing both the relationship between phenotype and \textit{RET} genotype and the effects of modifier genes. We have found six single nucleotide polymorphisms (SNPs) of the \textit{RET} pathway to be associated with the risk of developing sporadic MTC (sMTC): \textit{GHRz1 STOP+946bp} (rs1061413), \textit{RET A45A} in exon 2 (rs1800858), \textit{RET A432A} in exon 7 (rs1800860), \textit{RET G691S} in exon 11 (rs1799939), \textit{RET S904S} (rs1800863), and \textit{ARTN START-797bp} (rs3762422; ref. 3). In a recent report, Robledo et al. have shown that two of these \textit{RET} SNPs (G691S and S904S) may modify the age at onset of MTC tumor in family members (4). We have analyzed all six of the variants in four sets of Caucasian MEN2A families of different geographic origin to assess their influence on age at onset and on MEN2 phenotype. Because the modifier effect of a variant could depend on the type of mutation, the analyses were done in two different groups; the first considering all \textit{RET} mutations and the second including only mutations located at codon 634, which were the commonest in all four populations.

Materials and Methods

Subjects. Details of the study subjects are shown in Table 1. The U.K. patients were identified through the register of MEN2 families established by the Cancer Research Campaign (now Cancer Research UK) since 1982. The Spanish patients were those used by Robledo et al. (4) with the addition of 10 new MEN2 families. The French patients were recruited through the GETC database (5). One large Swedish family, referred to the U.K. register of MEN2, was also included in the study.

In all populations, data on family history, mode of presentation, treatment, pathology, and screening of family members were collected. For all patients, informed consent was obtained for all diagnostic procedures and for taking a family history. All selected cases were carriers for a known mutation in \textit{RET}. They were classified by the following phenotypic features: (a) asymptomatic at the time of the study, (b) symptomatic patients who had developed only MTC, or (c) symptomatic with MTC and another syndrome tumor (hyperparathyroidism and/or phaeochromocytoma). They were also classified by age of onset for the first clinical symptom in symptomatic patients and by age of last clinical screening (basal calcitonin determination, calcium and PTH determinations, and urinary 

Note: F. Lesueur and A. Cebrian contributed equally to this work. B.A.J. Ponder is a Gibb fellow of Cancer Research UK.

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©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-05-2995

www.aacrjournals.org 1177 Cancer Res 2006; 66: (2). January 15, 2006

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measurements of catecholamines and derivatives) that was negative in asymptomatic cases (<20, 20-34, >35 years).

**Genotyping.** We genotyped all samples for selected polymorphisms using the ABI PRISM 7900 sequence detection system or Taqman (Applied Biosystems, Foster City, CA). We carried out PCR on DNA (20 ng) using Taqman universal PCR master mix, forward and reverse primers (900 nmol/L), and FAM- and VIC-labeled probes (200 nmol/L) in a 5-μL reaction. Amplification conditions on an MJ Tetrad thermal cycler (Genetic Research Instrumentation, MJ Research, Cambridge, MA) were as follows: one cycle of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. We read the completed PCRs on an ABI PRISM 7900 Sequence Detector and analyzed them using the Allelic Discrimination Sequence Detector Software (Applied Biosystems). For the software to recognize the genotypes, we included four nontemplate controls in each 384-well plate. We designed Taqman primers and probes using the Primer Express Oligo Design Software v2.0 (Applied Biosystems). All sequences are available upon request.

**Statistical methods.** Unconditional logistic regression was used to test for association between the phenotypes of interest and genotype, while controlling for study center. We have previously observed that each of the SNPs that was significantly associated with predisposition to sporadic MTC had a dominant mode of action (3). We therefore compared individuals who were homozygous for the common allele with those who were carriers of the rare allele (heterozygotes and rare homozygotes).

Permutation testing was used to allow for the effect of multiple hypothesis testing. Case-control status was randomly permuted within study strata and the number of times a P was obtained that was as small as that observed provides an indication of its true significance.

**Results and Discussion**

Genotype frequencies by age at onset of clinically apparent disease and tumor subtype are shown in Tables 2 and 3. Because...
G691S and S904S are in perfect linkage disequilibrium (6, 7), we analyzed only the nonsynonymous variant G691S, likely to affect the structure or the function of the tyrosine kinase receptor (3, 4). We could not confirm in our combined set of four different populations the association between G691S and age at onset previously reported by Robledo et al. (P = 0.29). This highlights the importance of replication in different populations of any significant association. Apparently, significant results in a small sample set may not be replicated when larger sets are analyzed.

There was a nominally significant association for GFRA1 STOP-946bp genotype and age at onset (P = 0.01). However, the proportion of carriers of the rare allele was higher in both the cases diagnosed ages <20 years and those diagnosed ages ≥35 years compared with cases diagnosed ages 20 to 34 years, and the median age of diagnosis was similar for both genotypes. This suggests that this result is a chance finding (type I statistical error). None of the other SNPs were significantly associated with age at onset.

We compared putative modifier genotype frequencies in patients who developed MTC alone to patients who additionally developed clinical symptoms of other characteristic tumors of MEN2A. We found a weak positive association between the SNP A432A (c.1296 g>a) in RET and phenotypic expression (Table 3). The rare allele for this variant (frequency 31% in the normal population) was overrepresented in patients carrying a mutation at codon 634 compared with those with MTC only (P = 0.03). This result is consistent with our previous study in sMTC (3) in which the c.1296-A allele seems to protect the general population from developing sMTC, independently of the age at onset. However, this result should be treated with some caution because it may be a chance finding in the context of multiple testing. To examine this, we carried out permutation testing, in which a P ≤ 0.036 was achieved 141 times out of 10,000, indicating that a larger series of cases will be needed to assess whether the association is real or not. Subgroup analysis would in principle be useful; however, there are many different codon 634 mutations, and the most common comprises just 42%. Therefore, we do not have the power to show differences in the effect of different mutations.

Some support for a real association would come from a clear functional effect of the c.1296 g>a substitution. Codon 432 is located in cadherin-like domain 4 (8). Although this is not a conserved residue, an effect of synonymous codon usage on gene expression has been supported by the detection of epistatic interactions between nucleotides that are important in maintaining pre-mRNA/RNA secondary structures (9). The presence of the rare allele of SNP A432A might modify the mRNA folding, stability, and translation, leading to decreased amount of mutant 634 protein and consequently leading to less oncogenic activity. Despite this being a weak association, the fact that a similar association has been observed in the four different populations warrants the investigation of the effect of the A432A polymorphism on phenotypic expression in RET mutation carriers at residue 634 in other populations to determine if it is a common real association.

In conclusion, we have used 384 gene carriers from four different European populations; this is a large study for a rare disease, and we could not confirm the previously reported association between G691S and age at onset of the MEN2A syndrome. Our analysis of four other SNPs in RET and its coreceptors and ligands, which we had found to be associated with susceptibility to MTC in a large study of apparently sporadic cases, showed an inconclusive weak effect of the variant c1296 g>a in RET on the probability of multiple organs involvement in RET codon 634.

### Table 3. Effect of genotypes on the phenotypic expression of the disease for all mutation carriers and 634 mutation carriers only

<table>
<thead>
<tr>
<th>SNP</th>
<th>Tumors*</th>
<th>All mutation in RET</th>
<th>Mutation at codon 634</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Common 1</td>
<td>Rare</td>
<td>LR, χ²</td>
</tr>
<tr>
<td>GFRα1 3UTR (c&gt;g)</td>
<td>MTC</td>
<td>85</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>MTC + others</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>RET A45A (g&gt;a)</td>
<td>MTC</td>
<td>120</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>MTC + others</td>
<td>74</td>
<td>35</td>
</tr>
<tr>
<td>RET A432A (g&gt;a)</td>
<td>MTC</td>
<td>75</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>MTC + others</td>
<td>49</td>
<td>57</td>
</tr>
<tr>
<td>RET G691S (g&gt;a)</td>
<td>MTC</td>
<td>113</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>MTC + others</td>
<td>74</td>
<td>29</td>
</tr>
<tr>
<td>ARTN 5UTR (a&gt;t)</td>
<td>MTC</td>
<td>129</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>MTC + others</td>
<td>77</td>
<td>28</td>
</tr>
</tbody>
</table>

Abbreviations: UTR, untranslated region; MTC, medullary thyroid carcinoma.
*To test an association between the presence of specific polymorphism and the phenotypic expression of the disease, the population was divided in two groups: MTC, all patients who had developed only MTC at the time of the study; MTC + others, all patients who developed clinical symptoms of pheochromocytoma and/or hyperparathyroidism in addition to MTC.
1 Common, number of common allele carriers; rare, number of rare allele carriers (we analyzed together both heterozygous and rare homozygous because these SNPs had a dominant manner of action).
mutation carriers, consistent with the effect of this variant on susceptibility to sporadic MTC.

Each of these results illustrates the necessity, when association studies are used to search for small effects in rare diseases, for very large data sets and for replication of positive results in independent samples.

Acknowledgments

Received 8/22/2005; revised 10/20/2005; accepted 11/8/2005.

Grant support: Cancer Research UK.

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We thank all the subjects who participated in this study.

References


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