CHARTER IV

RESULTS

1. Sequence analysis in the TRβ gene

From the six unrelated individuals, only one patient had a mutation in exon 9 of the *TRβ* gene. Analysis of the *TRβ1* gene by PCR-sequencing revealed that the proband harbored a heterozygous missense mutation (c.1223T>C) resulting in a methionine (ATG) to threonine (ACG) substitution at codon 313 (p.M313T). This was a *de novo* mutation as her unaffected parents did not carry this mutation (figure 18).

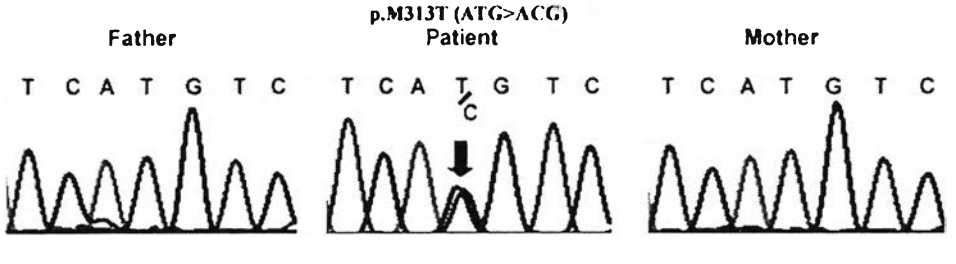

Figure 18 Mutation analysis. An electropherogram of the proband showing a heterozygous missense mutation (an arrow) resulting in a methionine (ATG) to threonine (ACG) substitution at codon 313 (p.M313T) (the middle panel). The identified mutation was not detected in her parents (the left and right panels).

This mutation has been previously reported in other populations but never been investigated for its functional significance. A previous report identified one Japanese patient with goiter[48]. Thyroid function tests were shown in table 14.

Table 14 Thyroid function tests of a Thai patient and a Japanese patient with a similar mutation in the *TRβ1* gene.

	Thai patient	Japanese patient	Normal range
FT_3 (pg/ml)	12.03	6.10	1.6- 4.0
FT_4 (ng/dl)	4.52	4.97	0.8-1.8
TSH (mU/ml)	1.41	1.20	0.3-4.1

From the structure of TRβ, the p.M313T mutation is located at the T_3-binding domain (figure 19) [11]. The uncharacterized p.M313T mutation could be pathogenic by impairing T_3-mediated transcriptional activity and interfering with the function of the wild type TRβ.

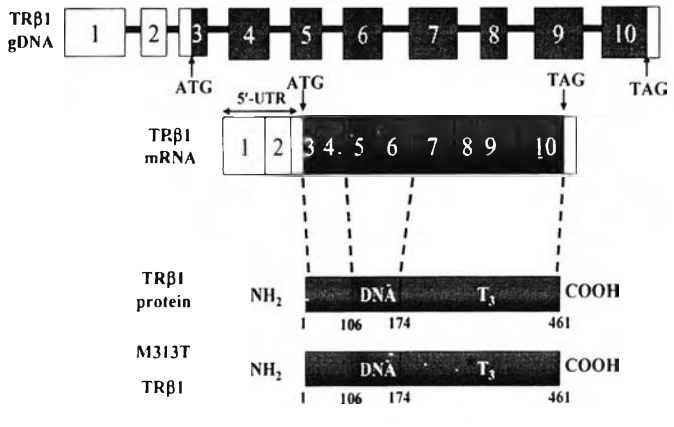

Figure 19 Structure of the *TRβ*1 isoform[11]. Star (*) indicates the position of the p.M313T.

2. Functional analysis of mutant TR*β*1s.

2.1 T_3-dependent transactivation activity

The objective of this experiment is to determine the effect of the mutant TRβ1s on T_3-transcriptional activity using the luciferase reporter system. The Palx3-Luc is a positively regulated reporter containing a binding site for TRs. In the presence of T_3, the TRβ1 can effectively activate the Luc gene expression. In transient co-transfection, the wild-type or mutant TRβ1s were cotransfected with the Palx3-Luc into COS-7 cells. For efficient activation, 10^{-7} M of T_3 were added to the culture media. This concentration of T_3 was previously used and shown to have a significant effect. The transactivation activity was represented as relative luciferase activity (firefly luciferase activity was normalized with renilla luciferase activity). All experiments were performed in triplicate and repeated two times. Cotransfection with the renilla luciferase reporter was used as the internal control to minimize the variability of the obtained results caused by differences in the transfection efficiency between different samples of the transfected cells (figure 20).

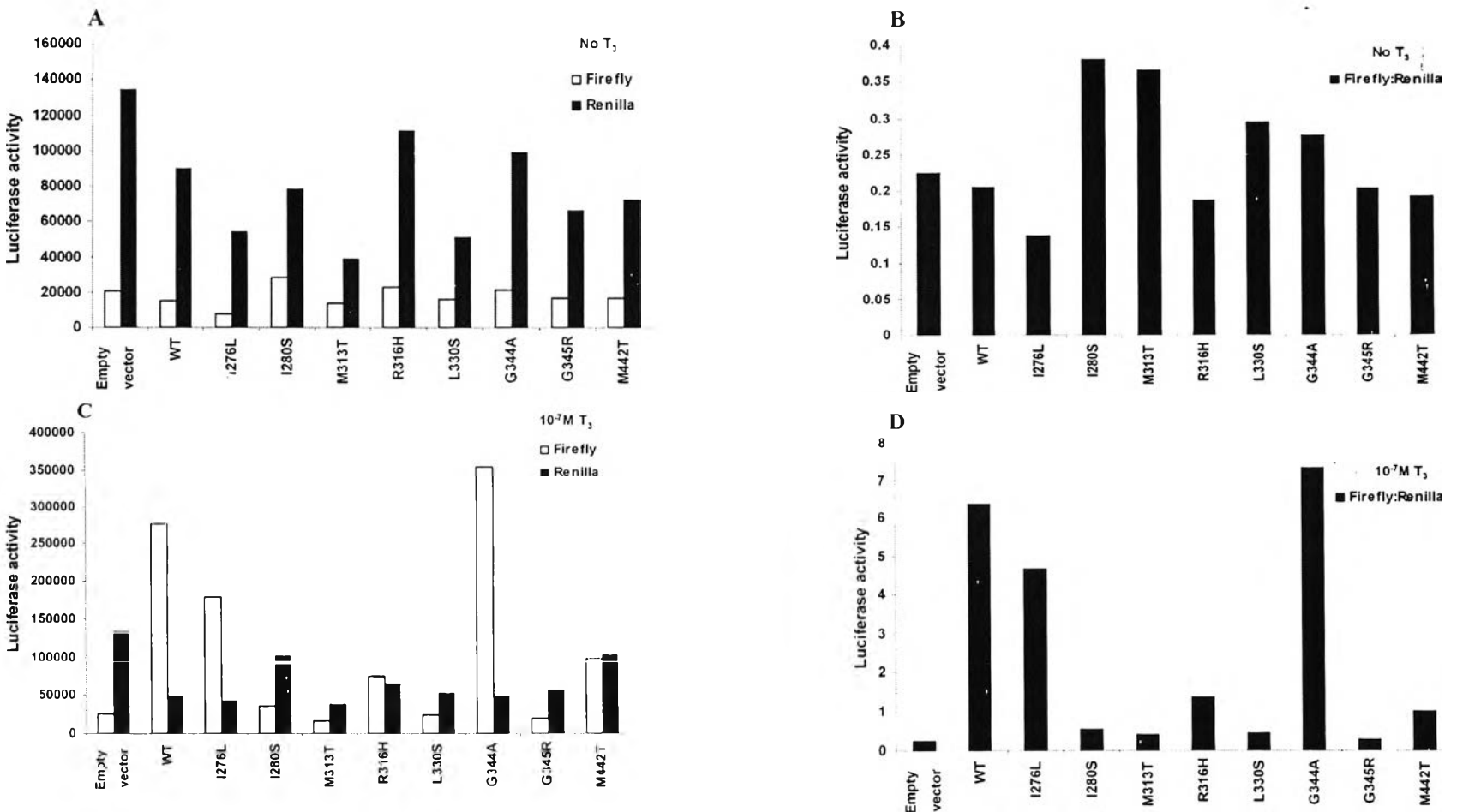

Figure 20 Firefly and Renilla luciferase activities in testing of T_3-dependent transactivation.

Panel A shows activities of Firefly and Renilla luciferases in each culture without T_3.
Panel B shows normalization of luciferase in each culture without T_3.
Panel C shows activities of Firefly and Renilla luciferases in each culture with 10^{-7}M T_3.
Panel D shows normalization of luciferase in each culture with 10^{-7}M T_3.

Statistical analysis

All data of the luciferase activity were calculated as relative luciferase activity and shown as fold induction (relative to the luciferase activity of the control vector pcDNA^{TM} 3.1/myc-His B). All experiments were performed in triplicate and repeated two times. The results were reported as fold induction ± SD. Statistical analyses were performed using ANOVA (figure 4).

At 10^{-7} M of T_3, the T_3-dependent transactivation of the Palx3-Luc reporter observed in the COS-7 cells transfected with the wild-type TRβ, was significantly abolished when cells were transfected with each of the mutant TRβs except for the I276L and G344A mutants. The mutant I276L had transcriptionally activity close to the wild-type response (I276L = 20-fold; wild-type = 28-fold), and the mutant G344A showed normal transactivation activity at 10^{-7} M of T_3 (G344A = 32-fold; wild-type = 28-fold). This suggested that the I276L and G344A mutants retained their transactivation properties at 10^{-7} M of T_3 (figure 21).

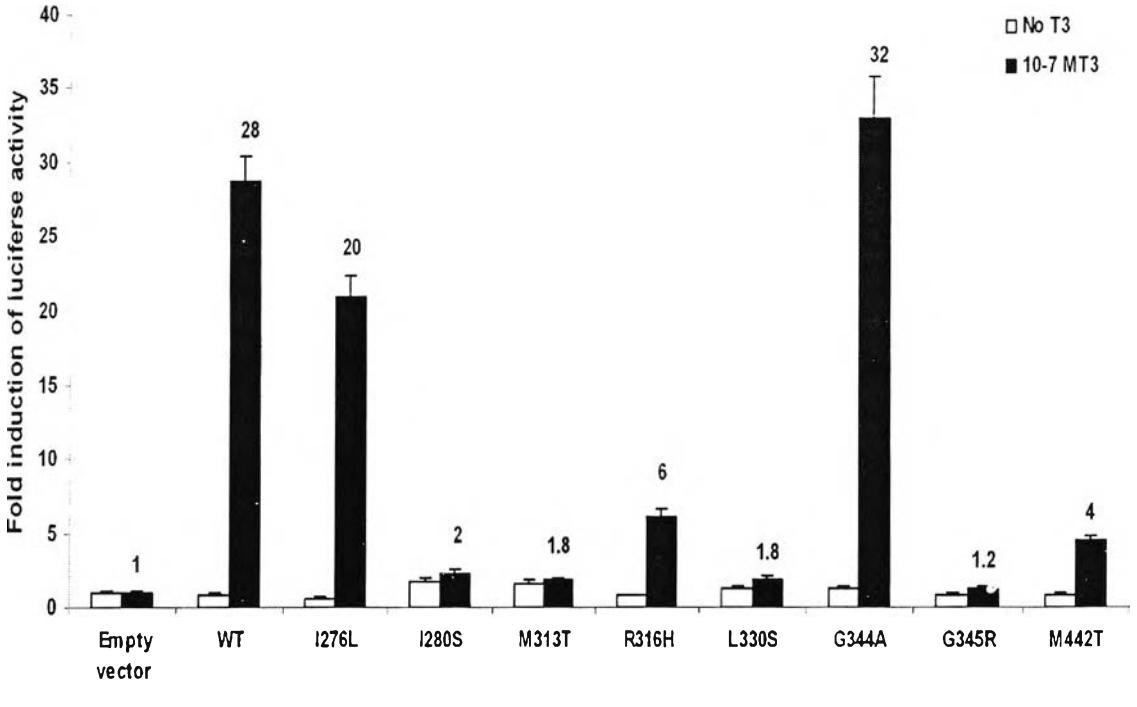

Figure 21 T_3-dependent transactivation of the Palx3-Luc reporter in COS-7 cells transfected with different constructs. All except the I276L and G344A mutants showed a significant reduction of the T_3-dependent transactivation activity compared with that of the WT TRβ1. Data were represented in fold induction relative to the luciferase activity of the vector control. Relative stimulation in the presence of T_3 treatment is indicated above the bars.

2.2 Dominant negative effect (DNE)

Dominant negative effect was tested by transfection of the all mutant TRβ1s exhibited a dominant negative effect in the presence of 10^{-7}M of T_3 together with equal and quadruple amounts of each mutant TRβ1 to COS-7 cells in the presence of 10^{-7} M T_3. The firefly luciferase activity was normalized with renilla luciferase activity. Experiments were performed twice with triplicate per experiment.

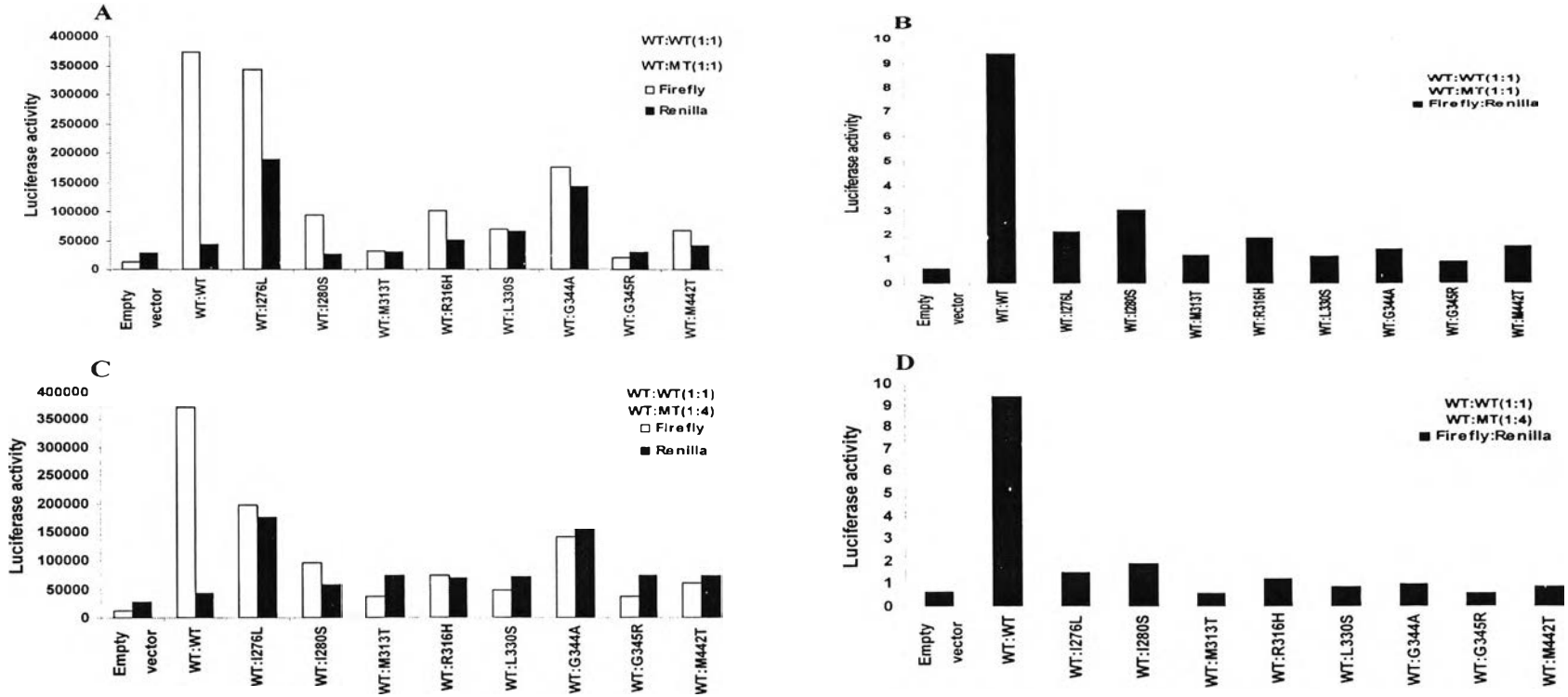

Figure 22 Firefly and Renilla luciferase activities in testing of dominant negative effect in each culture with 10^{-7}M T_3.

Panel A shows activities of Firefly and Renilla luciferase at ratio of WT:MT=1:1.
Panel B shows normalization of luciferase at ratio of WT:MT=1:1.
Panel C shows activities of Firefly and Renilla luciferase at ratio of WT:MT=1:4.
Panel D shows normalization of luciferase at ratio of WT:MT=1:4.

Statistical analysis

In cotransfection experiments to test for dominant negative effect, all mutant TRβ1s exhibited a dominant negative effect in the presence of 10^{-7}M of T_3 providing strong evidence supporting the pathogenic mechanism of these mutant TRβ1s identified in patients with RTH (figure 23).

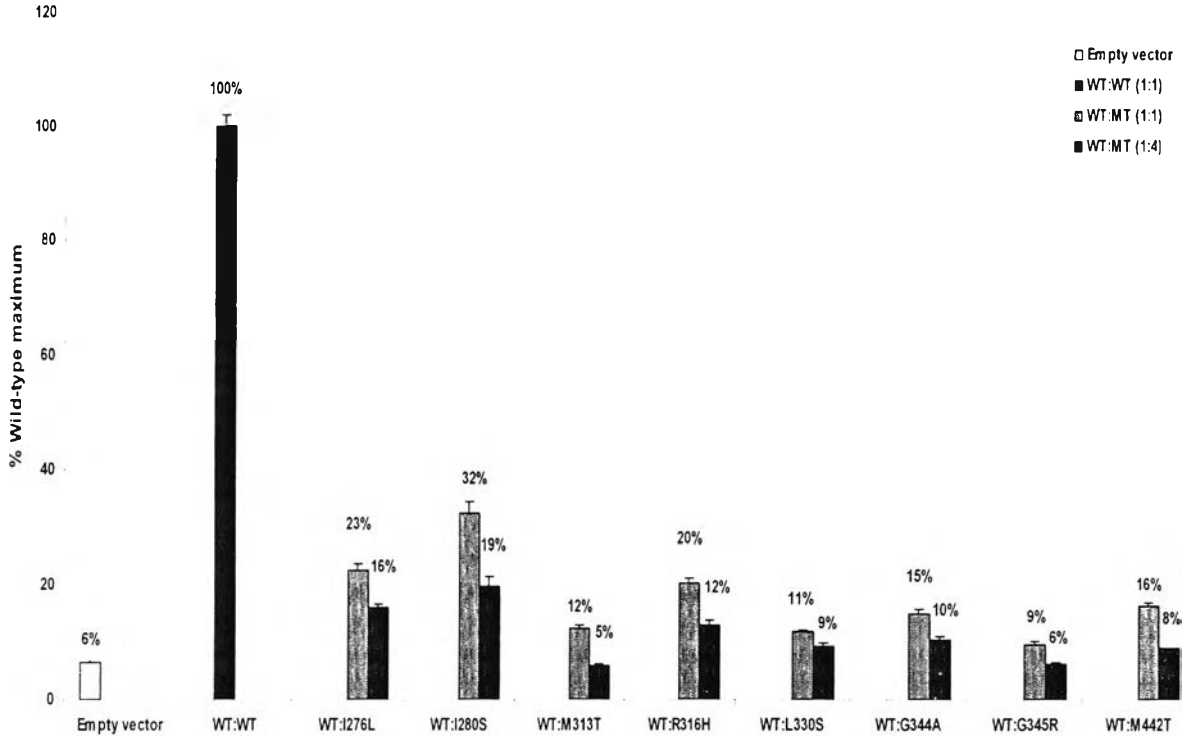

Figure 23 Dominant negative effect of the mutant TRβ1s. At both ratios of all wild type to mutant TRβ1s, all mutant TRβ1s exhibited a dominant negative effect in the presence of 10^{-7}M of T_3. Data were presented as percent activity of the WT receptor (100%).