**Resistance to dopamine agonists in prolactinoma is correlated with reduction of dopamine D₂ receptor long isoform mRNA levels**

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**Abstract**

**Objective:** Dopamine agonists normalize prolactin (PRL) levels and reduce tumour size in responsive prolactinoma. However, several cases have shown resistance to dopamine agonists upon initial treatment. Infrequently, prolactinoma initially responds, but then becomes refractory to prolonged treatment (secondary resistance). We investigated the possible mechanisms of resistance to dopamine agonists.

**Subjects and methods:** Twelve cases of prolactinoma were surgically resected and classified according to the responsiveness of PRL levels and tumour size to dopamine agonists: good responders (n = 5), poor responders (n = 5), or secondary resistance (n = 2). We examined the expression of dopamine D₂ receptor (D₂R) isoform (short: D₂S and long: D₂L) mRNA and protein. We investigated DNA methylation patterns in the promoter region of the DRD2 gene.

**Results:** The predominant D₂R isoform expressed in prolactinoma was D₂L. Levels of D₂L mRNA were significantly lower in secondary resistance and poor responders than in good responders. Expression of D₂R protein was variable among cases. Almost no CpG sites of the DRD2 gene promoter region were methylated.

**Conclusion:** Resistance of prolactinoma to dopamine agonists is correlated with a reduction in D₂L isoform mRNA levels. Silencing of the DRD2 gene by methylation in the promoter region is unlikely to play a role in dopamine agonist resistance in prolactinoma.

**European Journal of Endocrinology** 166 383–390

**Introduction**

Prolactin (PRL)-secreting pituitary adenomas (prolactinoma) are among the most common pituitary tumours. Dopamine agonists are the first-line treatment for prolactinoma, and treatment responses are highly variable. The majority of prolactinoma patients treated with dopamine agonists respond with a normalization of PRL levels and a reduction in tumour size. However, some patients do not exhibit a satisfactory response. Dopamine agonist resistance generally includes: i) a failure to achieve a normal PRL level on the maximally tolerated doses of a dopamine agonist; or ii) a failure to achieve a 50% reduction in tumour size (1, 2). However, there is no widely accepted definition of resistance to dopamine agonist with regard to the duration of therapy and the amount of tolerated doses used. There have been very few reported cases of prolactinoma exhibiting secondary resistance, i.e. cases that show an initial response to dopamine agonists, but then become refractory with prolonged treatment (3, 4, 5, 6, 7).

The molecular mechanism of dopamine agonist resistance is not fully understood, although dopamine D₂ receptor (D₂R) or post-receptor signaling in tumoral cells is thought to be involved in such resistance, as reduced D₂R expression and alterations in intracellular signal transduction have been reported (2, 8). The D₂R encoded by the DRD2 gene exists as one of the two alternatively spliced isoforms, short (D₂S) or long (D₂L), which structurally differ in a 29 amino acid fragment in the third cytoplasmic loop of the seven-transmembrane domain (9) and is also expected to function differently in each isoform (10). Previous reports have shown that the differential expression of D₂R isoforms might be related to treatment resistance in prolactinomas (11, 12). Very recently, we encountered rare cases of prolactinoma showing secondary resistance (13). In order to explore the possible molecular mechanism of secondary
resistance, we examined a total of 12 cases of prolactinoma that exhibited variable responses to dopamine agonists. In each case, the levels of expression of the D2R isoform were measured by quantitative RT-PCR; levels of membrane and cytosol D2R protein were determined by immunohistochemistry, and silencing of the DRD2 gene was investigated by methylation analysis of CpG dinucleotides in the promoter region using prolactinoma tissues.

Subjects and methods

Subjects

Twelve cases of prolactinoma were included in the present study. All the tissues were resected by one of the authors (S Y) at Toranomon Hospital between 2005 and 2010, an the diagnosis of all cases was verified as prolactinoma based on the histopathological findings. All tumour specimens were counterstained for anterior pituitary hormones and were shown to be negative except for PRL, confirming the absence of contaminated normal pituitary tissues. The 12 cases were provisionally classified according to their responsiveness to dopamine agonists. In each case, the levels of expression of the D2R isoform were measured by quantitative RT-PCR; levels of membrane and cytosol D2R protein were determined by immunohistochemistry, and silencing of the DRD2 gene was investigated by methylation analysis of CpG dinucleotides in the promoter region using prolactinoma tissues.

Quantitative RT-PCR to determine the levels of D2R isoform

The pituitary adenoma tissues were immediately frozen to −80 °C after surgery. Total DNA and RNA were extracted immediately from minced tissues using NucleoSpin RNA XS (Macherey-Nagel, Düren, Germany) and the NucleoSpin RNA/DNA Buffer Set as described in the manufacturer’s instructions. Pituitary cDNA was obtained using the SuperScript III Cells Direct cDNA Synthesis System (Invitrogen). A human embryonic kidney cell line (293T) and a human neuroblastoma cell line (SK-N-SH) were used as a negative and a positive control respectively.

Specific mRNA was measured by quantitative RT-PCR using a Fluorescent Quantitative Detection System with QuantiTect SYBR Green RT-PCR assay kits (Qiagen) according to the manufacturer’s protocol. Three cDNA plasmids were created using: Escherichia coli DH5α-competent cells; human glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a house-keeping gene; D2S; and D2L. A mixture of equal concentrations (ratio, 1:1:1) of each cDNA was used as the standard. The standard was quantified by real-time PCR, and the levels of G3PDH, D2S, and D2L were in the ratio of 2.58:1.08:1 respectively. We calculated the mRNA levels of each of the samples using this ratio of standard. All duplicated samples were measured using the same standard, and all experiments were performed in triplicate. The average of the data was taken in each case. The primers specific to D2S and D2L were designed according to NCBI database, NM_016574 and NM_000795 respectively. The forward primer for D2S was 5'-CCACCCCTGAGGCTCCACTAAAGGGG-3' and was located in exons 5 and 7 (exon 6 was spliced out) of the D2S mRNA. The forward primer for D2L was 5'-GGAGATTTCAGTAGAACAGCCGGAG-3', and was located in exon 6 of the D2L mRNA. The common reverse primer was 5'-ATGGTGCGGCGGTGCTGGAGAAGTC-3', and was located in exon 7 of the D2R mRNA. The expected
Table 1 Clinical data for 12 cases with prolactinoma.

<table>
<thead>
<tr>
<th>C.No.</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Tum. diam. (mm)</th>
<th>Before</th>
<th>Post</th>
<th>Max. treatment dosage, duration</th>
<th>Classification</th>
<th>Adverse event</th>
<th>MIB-1</th>
<th>p53</th>
<th>D2R</th>
<th>pRL (ng/ml)</th>
<th>D2S mRNA/G3PDH mRNA ratio</th>
<th>D2L mRNA/G3PDH mRNA ratio</th>
<th>Max. treatment dosage, duration</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>M</td>
<td>3000</td>
<td>54</td>
<td>24</td>
<td>15 (±20)</td>
<td>Secondary resistance</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>1+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 1 mg/week, 8 years</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>F</td>
<td>240</td>
<td>30</td>
<td>15</td>
<td>15 (±20)</td>
<td>Poor responder</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>2+</td>
<td>160</td>
<td>0.7</td>
<td>1.0</td>
<td>Cab 9 mg/week, 3 years</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>F</td>
<td>267</td>
<td>41</td>
<td>17</td>
<td>9 (±20)</td>
<td>Secondary resistance</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>1+</td>
<td>190</td>
<td>0.8</td>
<td>0.3</td>
<td>Cab 1 mg/week, 2 years</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>F</td>
<td>225</td>
<td>99</td>
<td>15</td>
<td>7 (±20)</td>
<td>Poor responder</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>2+</td>
<td>150</td>
<td>0.7</td>
<td>1.0</td>
<td>Cab 1 mg/week, 1 years</td>
</tr>
<tr>
<td>5</td>
<td>34</td>
<td>F</td>
<td>108</td>
<td>72</td>
<td>8</td>
<td>5 (±20)</td>
<td>Poor responder</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>0+</td>
<td>100</td>
<td>0.7</td>
<td>0.3</td>
<td>Cab 0.5 mg/week, 3 years</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>F</td>
<td>262</td>
<td>55</td>
<td>11</td>
<td>5 (±20)</td>
<td>Poor responder</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>0+</td>
<td>100</td>
<td>0.7</td>
<td>0.3</td>
<td>Cab 0.5 mg/week, 4 years</td>
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<tr>
<td>7</td>
<td>23</td>
<td>M</td>
<td>225</td>
<td>99</td>
<td>15</td>
<td>7 (±20)</td>
<td>Poor responder</td>
<td>Nausea</td>
<td>3%</td>
<td>3%</td>
<td>1+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 1 mg/week, 6 months</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>M</td>
<td>13</td>
<td>0.6</td>
<td>5</td>
<td>5 (±20)</td>
<td>Good responder</td>
<td>N/0</td>
<td>0%</td>
<td>0%</td>
<td>0+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 0.5 mg/week, 1 year</td>
</tr>
<tr>
<td>9</td>
<td>32</td>
<td>M</td>
<td>15</td>
<td>0.7</td>
<td>5</td>
<td>3 (±20)</td>
<td>Good responder</td>
<td>N/0</td>
<td>0%</td>
<td>0%</td>
<td>0+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 0.5 mg/week, 1 year</td>
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<tr>
<td>10</td>
<td>52</td>
<td>M</td>
<td>1700</td>
<td>36</td>
<td>9</td>
<td>15 (±20)</td>
<td>Good responder</td>
<td>N/0</td>
<td>0%</td>
<td>0%</td>
<td>0+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Ter 1.5 mg/day, 3 months</td>
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<tr>
<td>11</td>
<td>32</td>
<td>F</td>
<td>116</td>
<td>16</td>
<td>11</td>
<td>15 (±20)</td>
<td>Good responder</td>
<td>N/0</td>
<td>0%</td>
<td>0%</td>
<td>0+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 1 mg/week, 4 years</td>
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<tr>
<td>12</td>
<td>24</td>
<td>M</td>
<td>58</td>
<td>4</td>
<td>14</td>
<td>15 (±20)</td>
<td>Good responder</td>
<td>N/0</td>
<td>0%</td>
<td>0%</td>
<td>0+</td>
<td>150</td>
<td>0.6</td>
<td>2.0</td>
<td>Cab 2 mg/week, 6 months</td>
</tr>
</tbody>
</table>

PCR products were 150 and 159 bp in length for D2S and D2L, respectively. The primer sets for G3PDH were 5’-TGCACCACCAACTGTTAGC-3’ (forward) and 5’-AGTGATGGCGATGGACTGTGC-3’ (reverse). Real-time PCR was performed as follows: pre-denaturation at 95 °C for 30 s, 40–60 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A melting curve analysis was performed at the end of every run to ensure that a single PCR product of the expected melting temperature was produced in a given well. The D2S mRNA/G3PDH mRNA ratio and the D2L mRNA/G3PDH mRNA ratio were obtained, and the total D2R mRNA/G3PDH mRNA ratio was calculated by adding the D2S and D2L mRNA/G3PDH mRNA ratios, adjusted by the standards.

**Immunohistochemical analysis of D2R protein expression**

Immunohistochemistry was performed for 12 prolactinomas on the Ventana Benchmark XT Automated IHC Stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Histological sections were first incubated for 10 min in a microwave at 300 W, and then incubated with primary rabbit polyclonal antibody to human D2R (GTX-71745; Gene-Tex Inc., Irvine, CA, USA) diluted 1:200 and left for 30 min at room temperature. Antibody binding was detected using the Ventana iView DAB detection kit (Ventana Medical Systems, Inc.) and slides were counterstained with hematoxylin. Semi-quantitative analyses were performed with special attention paid to the pattern of immunostaining of D2R, i.e. membrane- or cytosol-associated staining. The intensity of the D2R signal was scored as 0 (negative), 1+ (cytoplasmic positive staining), 2+ (membranous positive staining <50% cells), and 3+ (membranous positive staining over 50% cells).

**Immunohistochemistry of MIB-1 and p53**

MIB-1 and p53 were stained using a commercially available anti-Ki-67 anti-body (Ki-67 Antigen, M7240; Dako, Glostrup, Denmark), and an anti-p53 antibody (p53 Protein, M7001; Dako) respectively. MIB-1 labeling index was counted as described previously (14). We adopted the threshold of labeling index of 3% for distinguishing pituitary adenomas, based on the observations by Thapar et al. (15) and on the WHO classification of atypical pituitary adenoma (16). Immunostaining of p53 was scored as positive or negative (16).

**Methylation analysis of the promoter region of the DRD2 gene**

The DNA methylation patterns in the promoter region of the DRD2 gene were investigated by a DNA bisulphate modification method using the MethylEasy Xceed
Rapid DNA Bisulphite Modification kit (Human Genetic Signatures Pty Ltd, North Ryde, Australia) according to the manufacturer’s instructions. The promoter region of the *DRD2* gene, which extends from $-239$ to $+146$ (the first nucleotide of exon 1 assigned as position $+1$), is a CG rich containing region ($+15$ to $+44$) that has been reported to be differentially methylated between striatum and lymphocytes (17). We set two forward primers from $-239$ to $-219$ (5’-TATTTTGGGTGGG-GTGGGAG-3’) and from $-124$ to $-104$ (5’-AGGAGGTATAGTTTTTGTTG-3’), and the reverse primer included two CpG sites extending from $+123$ to $+146$. Four patterns of reverse primers were selected (Rev1: 5’-CAACAACCTCAACCAACTCTAACC-3’; Rev2: 5’-CAACAACCTCAGACCAGCTCTAACC-3’; Rev3: 5’-CAACAACCTCAGACCGACTCTAACC-3’; and Rev4: 5’-CAACAACCTCAGACCAACTCTAACC-3’); mixtures of equal concentrations were prepared. Takara Taq Hot Start Version (Takara Biotechnology, Otsu, Japan) was used for the PCR analysis, which was carried out at 97 °C for 4 min, 60 °C for 3 min, and 72 °C for 2 min, followed by 35 cycles of amplification for the first PCR and 40 cycles for the second PCR at 95 °C for 1 min, using a gradient from 59 to 64 °C for 1 min, and 72 °C for 1 min. The PCR products were 270 bp in length and were run on 4% NuSieve 3:1 Agarose gel (Cambrex Bio Science, Rockland, ME, USA). The products were inserted into the pCR4-TOPO vector and transformed into competent cells using a TOPO-TA cloning kit for sequencing (Invitrogen) following the manufacturer’s instructions. Plasmid DNA was extracted from the cells using the Rapid DNA Bisulphite Modification kit and transformed into competent cells using a TOPO-TA cloning kit for sequencing (Invitrogen) following the manufacturer’s instructions. The promoter region of the *DRD2* gene was examined (Fig. 3). There were 36 CpG sites located in this promoter region that were mostly unmethylated. The DNA methylation patterns in the promoter region of the *DRD2* gene did not differ among prolactinomas.

**Expression of p53 and MIB-1 labeling index**

MIB-1 (Ki-67) labeling index was over 3% in all secondary resistance and poor responders. Only two cases in good responders were under 3%. The expression of p53 was positive in secondary resistance, and it was variable in poor and good responders (Table 1).

**Discussion**

In the present study, we investigated the expression of *D2R* isoforms in three categories of prolactinomas, i.e. good responders, poor responders, or secondary resistance. We found reduced expression of *D2L* isoform mRNA in poor responders and secondary resistance as compared with those in good responders; this reduction was more prominent in prolactinomas showing secondary resistance. This is the first demonstration that the reduction of *D2L* isoform mRNA is correlated with
resistant to dopamine agonist. Continued administration of dopamine agonist until TSS in poor responders and secondary resistant cases may affect the expression of D2R mRNA. However, chronic treatment with cabergoline has not been shown to alter D2R mRNA expression in the striatum of Parkinsonian monkeys (18) and no evidence has been reported so far in the pituitary adenoma.

The sensitivity of prolactinomas to dopamine agonist is highly variable and is considered as a spectrum, ranging from highly sensitive, responsive, partially resistant to complete resistance (19). It is difficult to define standard dose thresholds to assign the status of dopamine agonist resistance. However, a dose of 1.5, 2.0, or 3.5 mg/week of cabergoline was proposed to define resistance to treatment in macroprolactinoma (19, 20, 21). We have classified prolactinoma patients into good and poor responders according to the threshold dose of 1.0 mg/week of cabergoline, which was the same as the median dose able to normalise PRL levels in two previous retrospective studies (19, 20). Therefore, good responders and poor responders in the present study may correspond to the highly sensitive group and the combined group of responsive and partial resistance in the previous report (19) respectively.

The increasing doses of cabergoline would normalise PRL levels in our poor responders (22). The third category of prolactinoma patients was the secondary or acquired resistant cases, which initially responded to a dopamine agonist and subsequently became resistant or refractory to treatment.

In dopamine agonist-resistant prolactinomas, a reduction of D2R receptor levels has been demonstrated and accounts for the partial response to dopamine agonists (8, 10, 11, 23). In the present study, we confirmed at a quantitative level that mean D2R mRNA levels in poor responders were lower than those in good responders. However, a great variability in terms of D2R mRNA levels has been found among good responders and poor responders and a clear-cut threshold cannot be established between the two categories. Other studies examining D2R expression in dopamine agonist-resistant prolactinomas have yielded conflicting results (24, 25), which may have been related to tumour heterogeneity and particular techniques employed for analysis.

The D2L isoform was predominantly expressed in both the normal human pituitary and in the prolactinomas. This observation is in good agreement with those of most previous studies using RT-PCR and in situ hybridisation assays (9, 10, 26, 27). However, Neto et al. (28) reported that the D2S isoform was the dominant isoform in the normal pituitary. It should be noted that in that study, D2S-specific primer pairs for quantitative RT-PCR were not set, and therefore, the quantity of D2S mRNA could only be estimated by calculating the ratio of D2L mRNA to the total D2R mRNA. Here, we were able to select specific primer pairs for D2S and D2L using similar GC-content percentages and similar amplified product lengths, which yielded equivalent amplification efficiencies for both D2S and D2L. An investigation by Caccavelli et al. (11) focused on differences in the proportion of D2S and D2L isoforms. The two molecular isoforms of the D2R display comparable binding characteristics, but they are regulated differently (10, 29), and they may exhibit differential coupling to selective G-proteins (30, 31). The D2S receptor appears to be more efficient than the D2L receptor at coupling to adenylate cyclase (32, 33).

The proportion of D2S mRNA was reported to be lower in cases of resistance than in responsive prolactinomas (11, 12). Our present study could not demonstrate clear correlation between the proportion of D2S mRNA and the responsiveness to dopamine agonists, failing to confirm the findings in previous studies (11, 12). Changes in the ratio of the receptor isoforms alone are unlikely to determine the spectrum of dopamine agonist responsiveness observed among prolactinomas (34). Nonetheless, the pathophysiological significance of D2R isoforms in prolactinomas requires further investigation (35).

The presence of D2R protein in prolactinoma tissues was examined by immunohistochemical analysis. The expression of D2R protein was found to be highly...
variable in prolactinomas, and was not clearly correlated with the state of resistance to dopamine agonists. In contrast, one prolactinoma case showing secondary resistance did not express any D2R protein, in agreement with the very low levels of mRNA expression. The subcellular localisation of D2R protein appeared to be diverse among prolactinomas. Incomplete membrane-bound immunoreactivity as well as cytoplasmic and nucleic immunoreactivities was noted in the present study. Previous immunohistochemical studies of normal lactotrophs and prolactinomas have detected D2R immunoreactivity, primarily in the cytoplasm and the nuclei (36, 37), but occasional immunostaining of the cell membrane has been observed (38). It remains to be determined whether cytoplasmic or membrane-bound D2R is closely correlated with responses to dopamine agonists and receptor internalisation.

DNA methylation induces the silencing of DNA transcription. In humans, CpG dinucleotides are the preferential target of methylation. The methylation of the promoter region is important because there are certain transcription factors that have differential affinity for methylated CpG and unmethylated CpG. Al-Azzawi et al. (39) examined the promoter region of the rodent Drd2 gene (−538 to + 7 bp) in GH3 and MMQ cells and the normal rat pituitary, and they found that methylation patterns were closely correlated with D2 receptor reduction. We investigated DNA methylation in the compatible region (−103 to +122 bp) of the human DRD2 gene. We could not find any differences in methylation status among prolactinomas in this promoter region. These findings suggest that silencing of transcription is not related to responses to dopamine agonists in human prolactinoma. Additional molecular alterations may contribute to the sensitivity to inhibitory dopaminergic influence (40).

We report here the two prolactinoma cases of secondary resistance to dopamine agonist. Only six such patients have been reported in the literature (excluding malignant transformation) (3, 4, 5, 6, 7). The possible explanations for acquired non-responsive-ness include non-compliance, onset of gonadal steroid replacement that causes dopamine resistance in the lactotrophs, and, rarely, transformation to carcinoma (37, 41). However, none of these reasons applied in our cases, which showed very low levels of D2R mRNA expression and loss of D2R protein, indicating de-differentiation of the tumour. In the present study, 11 cases had a high MIB-1 labeling index of more than 3%, and six cases expressed positive p53 immunostaining, which suggested the possible atypical adenomas according to the WHO classification (16). However, there appeared to be no correlation between MIB-1 labeling index and the responsiveness to dopamine agonists. The difference between atypical adenoma and pituitary carcinoma lies in whether or not there is evident metastasis. Therefore, careful surveillance of these patients with possible atypical adenoma is mandatory.

The limitations of this study must be considered. The number of prolactinoma patients was relatively small. Since the first-line therapy of prolactinoma is medical treatment with dopamine agonists, few cases having intolerance and resistance to medications, cerebrospinal rhinorrhea, and pituitary apoplexy were selected for surgery. The duration of dopamine agonist therapy would be very short in cases of sensitive prolactinoma, which might affect the biochemical and functional changes in tumoral cells.

In conclusion, the resistance of prolactinoma to dopamine agonists is correlated with a reduction in D2L mRNA levels. D2L mRNA levels were reduced in cases showing secondary resistance and in poor responders compared with those in good responders. The silencing of DRD2 gene expression by methylation in the promoter region is unlikely to play a role in dopamine agonist resistance.

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 study was supported in part by Grants-in-Aid from the Ministry of Health, Labor, and Welfare for the 3rd-term Comprehensive 10-Year Strategy for Cancer Control and National Cancer Center Research and Development Fund (grant numbers 21-8-6, 23-A-11 and 20) and from the MHLW for the Intractable Diseases (Hypothalamo-pituitary Dysfunction). SS was a recipient of the research resident fellowship from the Foundation for Promotion of Cancer Research, Japan.

Acknowledgements

The authors are indebted to Drs R Y Osamura (Center for Diagnostic Pathology, International University of Health and Welfare) and T Sano (Edogawa Hospital) for their advice on the immunohistochemical analyses.
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Received 26 July 2011
Revised version received 27 November 2011
Accepted 29 November 2011