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CLINICAL STUDY

Resistance to dopamine agonists in prolactinoma is correlated with reduction of dopamine D_2 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 D_2 receptor (D_2R) isoform (short: D_2S and long: D_2L) mRNA and protein. We investigated DNA methylation patterns in the promoter region of the *DRD2* gene.

Results: The predominant D_2R isoform expressed in prolactinoma was D_2L. Levels of D_2L mRNA were significantly lower in secondary resistance and poor responders than in good responders. Expression of D_2R 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 D_2L 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 D_2 receptor (D_2R) or post-receptor signaling in tumoral cells is thought to be involved in such resistance, as reduced D_2R expression and alterations in intracellular signal transduction have been reported (2, 8). The D_2R encoded by the *DRD2* gene exists as one of the two alternatively spliced isoforms, short (D_2S) or long (D_2L), 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 D_2R 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

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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 D_2R isoform were measured by quantitative RT-PCR; levels of membrane and cytosol D_2R 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 into the following three categories: good responders, poor responders, or secondary resistance. Good responders were sensitive prolactinomas achieving normalisation or 95% reduction of basal PRL levels, or reducing tumour maximal diameter more than 50% of the initial diameter by treating with <1 mg/week of cabergoline or terguride of <1.5 mg/day ($n=5$). Poor responders were defined as cases neither achieving 95% reduction of basal PRL levels, nor reducing tumour maximal diameter more than 50% of the initial diameter in spite of the treatment of 1 mg/week or more of cabergoline for more than 2 years ($n=5$). Secondary resistance was defined as cases showing good responses to a dopamine agonist for more than 2 years and a subsequent increase in tumour size with elevated PRL levels in spite of continuing treatment ($n=2$) (5).

The various indications for surgical treatment of these 12 prolactinoma patients included pregnancy, intolerable adverse events induced by dopamine agonists, cerebrospinal fluid rhinorrhea, and the patients' desire for surgery. Two cases (#8 and #9) were not treated with a dopamine agonist after transsphenoidal surgery (TSS) because of the failure to normalise PRL levels. Cases #1 and #2 were secondary resistance which showed good responses to cabergoline 1 mg/week or bromocriptine 10–15 mg/day for 7 and 4 years respectively, with more than 50% reduction in tumour size, but showed rapid expansion of tumour size with elevation of PRL levels. Dopamine agonist therapy was continued until TSS in the cases #1–#3 and #5–#7, whereas it was stopped for over a month in cases #4 and #8–#12. The relevant demographic and clinical data for these patients are shown in Table 1.

The present clinical study was approved by the ethical committees of both the National Cancer Center and Toranomon Hospital in Tokyo. Informed consent was obtained from all patients.

Assay of PRL

Serum PRL levels were measured by the automated immunoenzymometric assay (Lumipulse Presto Prolactin Assay; Fujirebio, Inc., Tokyo, Japan). The minimum detectable concentration was 0.02 ng/ml. The inter-assay coefficients of variation were 3.2% at 8.2 ng/ml, 1.1% at 58.6 ng/ml, and 4.7% at 215 ng/ml respectively, while the intra-assay coefficients of variation were 2.3% at 8.2 ng/ml, 1.3% at 57.5 ng/ml, and 1.4% at 192 ng/ml respectively. The PRL standards were calibrated with the World Health Organization (WHO) 3rd International Reference Preparation (IRP) 84/500. The normal values for PRL are as follows: male: 3–12 ng/ml and non-pregnant female: 6–30 ng/ml.

Quantitative RT-PCR to determine the levels of D_2R 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 quantiative 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; D_2S; and D_2L. 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, D_2S, and D_2L 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 D_2S and D_2L were designed according to NCBI database, NM_016574 and NM_000795 respectively. The forward primer for D_2S was 5′-CCACCTGAGGGCTCCACTAAAGGAGG-3′, and was located in exons 5 and 7 (exon 6 was spliced out) of the D_2S mRNA. The forward primer for D_2L was 5′-GGGAGTTTCCCAGTGAACAGGCGGAG-3′, and was located in exon 6 of the D_2L mRNA. The common reverse primer was 5′-ATGGTGGGACGGGTCGGGGAGAGTC-3′, and was located in exon 7 of the D_2R mRNA. The expected

Table 1 Clinical data for 12 cases with prolactinoma.

C.No.	Age/ gender	PRL (ng/ml)[a]			Tum. diameter (mm)[b]		Max. treatment dosage, duration	Adverse event	MIB-1[c]	p53[d]	D_2R[e]	Classification
		Pre	Post	Before TSS	Pre	Post (size red. %)						
1	45/M	3000	54	1519[f,g]	25	10 (−60) 20[g]	Cab 1 mg/week, 9 years	–	> 3%	+	0[h]	Secondary resistance
2	58/F	240	29	909[f,g]	20	7 (−65) 17[g]	Cab 9 mg/week, 6 years	–	> 3%	+	1+	Secondary resistance
3	20/F	267	41	75[f]	7	7 (0)	Cab 1 mg/week, 2 years	–	> 3%	–	1+	Poor responder
4	23/F	225	99	260[f]	15	15 (0)	Cab 1 mg/week, 3 years	Nausea	> 3%	–	3+	Poor responder
5	34/F	108	72	72[f]	13	13 (0)	Cab 1 mg/week, 3 years	–	> 3%	+	2+	Poor responder
6	23/F	262	55	55[f]	11	13 (+18)	Cab 1 mg/week, 2.5 years	–	> 3%	–	2+	Poor responder
7	59/M	2577	475	2253[f]	25	25 (0)	Cab 9 mg/week, 15 years	–	> 3%	+	1+	Poor responder
8	26/M	13[i]	0.6	312	ND[j]	ND[j]	Cab 0.5 mg/week, 1 year	–	> 3%	–	2+	Good responder
9	32/M	15[i]	0.7	198	ND[j]	ND[j]	Cab 0.5 mg/week, 1 year	–	> 3%	+	1+	Good responder
10	52/M	1700	36	962	20	ND[j] (> −90)	Cab 0.5 mg/week, 4 years	Liquorrhea	< 3%	–	1+	Good responder
11	32/F	116	16	115	5	ND[j] (> −60)	Ter 1.5 mg/day, 3 months	Nausea	< 3%	–	2+	Good responder
12	24/M	58	4	56	18	14 (−22)	Cab 0.25 mg/week, 6 months	Palpitation	> 3%	+	1+	Good responder

C.No, case number; Tum., tumour; red., reduction.
[a]Serum PRL levels measured before and after dopamine agonist therapy and before transsphenoidal surgery (TSS).
[b]Maximal tumour diameter evaluated on MRI.
[c]MIB-1 labeling index: percentage of immunopositive nuclei.
[d]p53 immunostaining: + (positive), over 1% of positive cells; – (negative), <1% of positive cells.
[e]Dopamine D_2 receptor immunohistochemistry score: 0, negative; 1+, cytoplasmic positive staining; 2+, membranous positive staining <50% cells; 3+, membranous positive staining over 50% cells.
[f]Dopamine agonist therapy was continued until TSS.
[g]Elevation after 4 years' response to dopamine agonists.
[h]The tissue before dopamine agonist therapy. ND, not detectable; Cab, cabergoline; Ter, terguride.
[i]After TSS.
[j]Minimal detectable diameter was estimated as 2 mm.

PCR products were 150 and 159 bp in length for D_2S and D_2L, respectively. The primer sets for G3PDH were 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-AGTGATGGCATGGACTGTGG-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 D_2S mRNA/G3PDH mRNA ratio and the D_2L mRNA/G3PDH mRNA ratio were obtained, and the total D_2R mRNA/G3PDH mRNA ratio was calculated by adding the D_2S and D_2L mRNA/G3PDH mRNA ratios, adjusted by the standards.

Immunohistochemical analysis of D_2R 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 D_2R (GTX-71745; Gene-Tex Inc., Irvine, CA, USA) diluted to 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. Semiquantitative analyses were performed with special attention paid to the pattern of immunostaining of D_2R, i.e. membrane- or cytosol-associated staining. The intensity of the D_2R 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′-TATTTTGGGTGTGG-GTGGGAG-3′) and from −124 to −104 (5′-AGGAGG-TATAGTTTTTTTGGT-3′), and the reverse primer included two CpG sites extending from +123 to +146. Four patterns of reverse primers were selected (Rev1: 5′-CAACAACTCAACCAACTCTAACCC-3′; Rev2: 5′-CAACAACTCGACCGACTCTAACCC-3′; Rev3: 5′-CA-ACAACTCAACCGACTCTAACCC-3′; and Rev4: 5′-CAA-CAACTCGACCAACTCTAACCC-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. Transformed cells were plated and incubated overnight at 37 °C, and cells were isolated independently over ten clones for each of the samples. The insert containing plasmid DNA was extracted from the cells using the NucleoSpin Plasmid QuickPure (Macherey-Nagel). Then 36 CpG methylation sites were sequenced in the promoter region of the *DRD2* gene.

Statistical analysis

The amounts of D_2R mRNA normalised by G3PDH mRNA of each sample were expressed as mean ± S.D. of three determinations. One-way ANOVA followed by Tukey's *post hoc* test was used to compare D_2R mRNA among the three groups. The expression of D_2R protein and p53 was analysed by Kruskal–Wallis test.

Results

Quantitative RT-PCR of the short and long isoforms of the D_2R

D_2R mRNA was expressed in all prolactinoma samples and in the normal pituitary control samples (Fig. 1). The average total D_2R mRNA level in poor responders was half that of good responders, and it was ninefold lower in secondary resistance than in good responders. The D_2L isoform was predominantly expressed in the pituitary. D_2L mRNA levels were, on average, four times higher in secondary resistance and poor responders and 12 times higher in good responders than D_2S mRNA levels. Therefore, the differences in total D_2R mRNA levels were largely accounted for by D_2L mRNA levels. The expression of D_2L mRNA was significantly higher in good responders than in secondary resistance ($P<0.01$) and in poor responders ($P<0.05$). The expression of D_2L mRNA was very low in secondary resistance. There appeared to be no correlation between D_2S mRNA and responsiveness to dopamine agonists.

D_2R protein expression determined by immunohistochemical analysis

Most immunostaining of the D_2R protein was observed in the cytoplasm of prolactinoma cells, and punctuate immunostaining was occasionally observed along the cell surface membrane (Fig. 2). The intensity of D_2R immunoreactivity varied among prolactinomas, and was not significantly different between poor responders and good responders (Table 1). D_2R immunostaining was completely absent in one of the secondary resistant cases (Fig. 2A).

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

The region spanning 103 bp upstream to 122 bp downstream from the transcription initiation site 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 D_2R isoforms in three categories of prolactinomas, i.e. good responders, poor responders, or secondary resistance. We found reduced expression of D_2L 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 D_2L isoform mRNA is correlated with

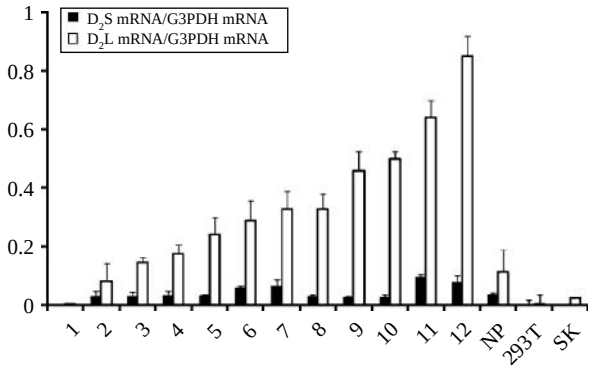

Figure 1 Expression of D_2R isoforms (short: D_2S filled square and long: D_2L open square) in prolactinomas (patient #1 to #12) and normal pituitary (NP) gland, and 293T, a human embryonic kidney cell line, and SK-N-SH (SK), a human neuroblastoma cell line as determined by quantitative RT-PCR. Cases #1 and #2 showed secondary resistance, cases #3 through #7 were poor responders, and cases #8 through #12 were good responders.

resistant to dopamine agonist. Continued administration of dopamine agonist until TSS in poor responders and secondary resistant cases may affect the expression of D_2R mRNA. However, chronic treatment with cabergoline has not been shown to alter D_2R 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 D_2R 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 D_2R mRNA levels in poor responders were lower than those in good responders. However, a great variability in terms of D_2R 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 D_2R 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 D_2L 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 D_2S isoform was the dominant isoform in the normal pituitary. It should be noted that in that study, D_2S-specific primer pairs for quantitative RT-PCR were not set, and therefore, the quantity of D_2S mRNA could only be estimated by calculating the ratio of D_2L mRNA to the total D_2R mRNA. Here, we were able to select specific primer pairs for D_2S and D_2L using similar GC-content percentages and similar amplified product lengths, which yielded equivalent amplification efficiencies for both D_2S and D_2L. An investigation by Caccavelli *et al.* (11) focused on differences in the proportion of D_2S and D_2L isoforms. The two molecular isoforms of the D_2R display comparable binding characteristics, but they are regulated differently (10, 29), and they may exhibit differential coupling to selective G-proteins (30, 31). The D_2S receptor appears to be more efficient than the D_2L receptor at coupling to adenylate cyclase (32, 33). The proportion of D_2S 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 D_2S 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 D_2R isoforms in prolactinomas requires further investigation (35).

The presence of D_2R protein in prolactinoma tissues was examined by immunohistochemical analysis. The expression of D_2R protein was found to be highly

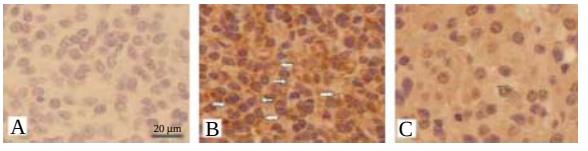

Figure 2 Immunohistochemical analysis of D_2R protein in prolactinomas. Representative cases: (A) complete absence in case #1, (B) membrane-associated staining in case #4, and (C) cytoplasmic staining in case #12. Membrane-associated D_2R is indicated by arrows.

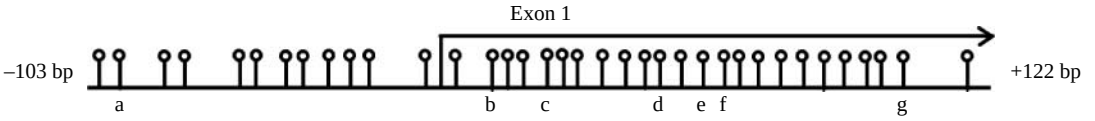

Figure 3 Methylation analysis of 36 CpG sites located in the promoter region of the *DRD2* gene in 12 prolactinoma samples. Unmethylated CpG sites are represented by open circles. Cases #1, #6–#8, #10, and #11 have no methylated CpG sites in over ten clones. Cases #2 and #4 show methylated CpG sites at (a) in two of 12 clones and one of 11 clones respectively. Case #3 shows a methylated CpG site at (b) in one of 11 clones. Case #9 shows methylated CpG sites at (c–e) in one, one, two of ten clones respectively. Case #5 shows a methylated CpG site at (f) in one of 11 clones. Case #12 shows a methylated CpG site at (g) in one of 11 clones.

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 D_2R protein, in agreement with the very low levels of mRNA expression. The subcellular localisation of D_2R 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 D_2R 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 D_2R 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 D_2 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-responsiveness 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 D_2R mRNA expression and loss of D_2R 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 D_2L mRNA levels. D_2L 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.

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