Prognostic Factors in Prolactin Pituitary Tumors: Clinical, Histological, and Molecular Data from a Series of 94 Patients with a Long Postoperative Follow-Up

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Context and Objective: Predicting pituitary tumor behavior remains a challenge. This multiparameter investigation aimed to identify markers for recurrence and progression in prolactin tumors.

Design: From a cohort of patients treated for prolactin tumors by surgery, we retrospectively studied clinical data, tumor characteristics, clinical outcome, and the expression of nine genes by quantitative RT-PCR.

Results: This study included 94 patients (62 females and 32 men), with long postoperative follow-up periods (mean, 138 $\pm$ 46 months); 54.3% of patients had a macro or giant adenoma. Tumors were classified into three pathological groups based on their radiological and histological characteristics (noninvasive, 61; invasive, 22; and aggressive-invasive, 11). Immediately after surgery, 60 patients (63.8%) went into remission (prolactin level normalization). Persistently elevated prolactin levels (36.2%) were associated with increasing age, male sex, high preoperative prolactin levels, large tumor size on univariate analysis, and invasion and pathological classification on univariate and multivariate ($P = 8 \times 10^{-10}$ and 3×10^{-8}) analysis. During follow-up, 19 patients (20%) had tumors that recurred or progressed under dopamine agonist treatment. Invasion and pathological classification were associated with recurrence or progression on univariate analysis. Seven genes (*ADAMTS6*, *CRMP1*, *PTTG*, *ASK*, *CCNB1*, *AURKB*, and *CENPE*) were associated with tumor recurrence or progression and five of these (*ADAMTS6*, *CRMP1*, *ASK*, *CCNB1*, and *CENPE*) were associated with the pathological classification.

Conclusion: This study identifies both the clinical and histological factors that relate to prolactin tumor recurrence or progression. Molecular markers give additional information for prognosis of such tumors. Altogether, our results could influence the management of patients with pituitary tumors. **(*J Clin Endocrinol Metab* 95: 1708–1716, 2010)**

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Abbreviations: MRI, Magnetic resonance imaging; PTTG, pituitary transforming tumor gene; q-RT-PCR, quantitative RT-PCR; RPL4, ribosomal protein L4.

Pituitary tumors are generally considered benign. However, many of them (45 to 55%) invade the sphenoid, cavernous sinus, or the dura mater (1), and some are aggressive, with a high proliferation rate and short postoperative time before recurrence (2). Only metastatic tumors are considered as malignant, and these are rare (0.2%) (3). Predicting pituitary tumor behavior remains a challenge (4–6). In pathological studies, increased levels of mitotic, Ki-67, proliferation cell nuclear antigen, and P53 indexes (7–10) have been found in invasive tumors, as well as expression of polysialic acid neural cell adhesion molecule (11, 12) and overexpression of pituitary transforming tumor gene (*PTTG*) (13). However, results conflict from one series to another, and these markers have not yet been correlated with postoperative results and recurrence in clinical studies. In two recent surgical series of prolactin pituitary tumors (14, 15), preoperative prolactin levels, tumor size, and invasion were related to cure rates. Our group demonstrated that prolactin tumors are more often invasive macroadenomas with worse outcomes in men than women (16, 17), which was confirmed by Schaller (18). In these earlier clinical studies, follow-up was limited (mean <50 months), and the histological data were poor. Recently, we identified a set of nine genes (*ADAMTS6, CRMP1, DCAMKL3, PTTG, ASK, CCNB1, AURKB, CENPE,* and *PITX1*) expressed differentially in human and rat prolactin tumors (19).

The need for specific markers of recurrence led us to initiate this study. We examined clinical and histological prognostic markers in a series of 94 patients with prolactin tumors, including three carcinomas. This series derived from a collaborative multicenter study on prognostic factors in pituitary tumors in France (HYPOPRONOS). Patients' characteristics (age, sex, preoperative plasma prolactin levels), tumor characteristics (size, invasion, pathological classification), and clinical outcome (early postoperative results and progression-free survival) were assessed from retrospective data with long-term postsurgical follow-up (more than 10 yr). In this study, we evaluated the association of this set of genes with pathological classification and tumor recurrence or progression in 30 patients.

Patients and Methods

Patients

We selected 94 patients with a prolactin-secreting tumor, without clinical or biological signs of acromegaly, from the HYPOPRONOS (Programme Hospitalier de Recherche Clinique National 27-43) study records. This French multicenter retrospective study followed a cohort of 350 patients surgically treated for pituitary tumors. The patients were operated on by transsphenoidal route between 1987 and 2004 by five experienced neurosurgeons (G. Perrin and E. Jouanneau at Hospices Civils de Lyon, F. Grisoli and H. Dufour at Hôpital La Timone Marseille, and M. Jan at Hôpital Bretonneau Tours). The majority of patients were referred directly to a surgeon without undergoing preoperative medical treatment (n = 54). Forty patients received preoperative dopamine agonist treatment, either because they expressed a personal preference for surgery instead of chronic medical treatment (n = 16) or because they experienced drug intolerance (n = 10) or resistance (n = 14), as previously defined (*i.e.* no normalization of prolactin plasma levels and no significant tumor shrinkage). Dopamine treatment stopped for 1 month before surgery in all except four cases. Recorded data included: age at surgery, sex, hormonal data, and postoperative events. All patients underwent magnetic resonance imaging (MRI) at the time of diagnosis and again before surgery. Tumor size was determined by MRI before surgery. Tumors were classified as microadenomas (diameter, <1 cm), macroadenomas (>1 cm and <4 cm), and giant adenomas (>4 cm). In 86 patients, the tumor volume was estimated (height × length × width/2). Invasion was evaluated on MRI (20) and/or histology. Long-term information for patients followed up outside our departments was obtained by contacting the patient or their physician by telephone or mail. Plasma prolactin levels were measured before surgery, 1 to 2 wk after surgery, and every year for at least 10 yr after surgery. Patients without clinical symptoms, with normal plasma prolactin level without visible radiological tumor remnants, and not requiring dopamine agonist therapy were considered in remission. Persistent increased plasma prolactin levels, with or without radiological evidence, defined persistence. Tumor progression was defined as evidence on MRI of regrowth and/or an increase of prolactin plasma levels during treatment. Recurrence was defined as an increase in plasma prolactin levels, with or without radiological evidence of any tumor mass, after a previous remission.

The studies were approved by the ethics committee of Lyon, and informed consent was obtained for each patient according to French law.

Pituitary tumors

For each tumor, fragments were fixed in Bouin fixative and embedded in paraffin for pathological diagnosis, including immunocytochemistry. Other fragments were frozen immediately and stored at −80 C (Neurobiotec Bank, Lyon, France). Pituitary hormone detection was performed using the indirect immunoperoxidase method. Only tumors with prolactin immunostaining were considered. The following antibodies were used: antiprolactin (1/400; Immunotech, Marseille, France), Ki-67 (Mib1, 1/50; Dako, Glostrup, Denmark), and anti-p53 (clone DO-7, 1/200; Novocastra Laboratories, Newcastle upon Tyne, UK). For the last two antibodies, we performed immunostaining both manually with microwave pretreatment (19) and automatically with Benchmark XT (Ventana Medical System, Tucson, AZ). To determine the Ki-67 labeling and mitotic indexes, we counted cells for 10 representative fields per tumor at 400X magnification, with an average count of 5000 nuclei. Ki-67 labeling was expressed as a maximum percentage and the mitoses by their absolute number.

Pathological classification

The same pathologist (J.T.) studied all the prolactin tumors at the time of the first surgery, classifying them into three pathological groups (19) based on radiological and histological data: noninvasive, invasive, and aggressive-invasive. The aggressive-invasive tumors exhibited at least two proliferative markers (Ki-67 index >1%, number of mitoses >2 per 10 fields at 400X

magnification, and P53 nuclear detection). These aggressive-invasive tumors correspond to the "atypical adenoma" briefly mentioned in the World Health Organization classification (2). Three tumors were considered as malignant on the basis of cerebral or jugular lymph node metastasis in two patients and because of vascular emboli in the primary tumor in one patient.

Molecular analysis

Genetic analysis was performed on fragments from 44 frozen tumors. We excluded 10 tumors whose genomic material had been analyzed in a previous study (19) and the four tumors receiving dopamine agonist treatment at the time of surgery. We used q-RT-PCR in triplicate on each sample to detect the expression of the following nine genes: *DCAMKL3*, *CRMP1*, *ADAMTS6*, *PTTG1*, *ASK*, *CCNB1*, *AURKB*, *CENPE* and *PTX1*. Normal pituitary was set to 1 and used as a reference.

Total RNA was extracted using an RNeasy minikit (QIAGEN, Hilden, Germany), including a DNAse treatment, according to the manufacturer's protocol. Total RNA yield was measured by OD_{260}, with an A260/A280 ratio of 1.9–2.1 demonstrating purity. Quality was evaluated on nanochips with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), according to the manufacturer's protocol.

Total RNA (0.5 μg) was reverse transcribed using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Carlsbad, CA). The absence of contaminating genomic DNA in the reverse transcription reactions was checked by quantitative RT-PCR (q-RT-PCR) directly on total RNA.

The synthesized cDNA was measured using q-RT-PCR (SYBR Green PCR, LightCycler; Roche Diagnostics, Indianapolis, IN), according to the manufacturer's recommendations. The LightCycler experimental run protocol consisted of initial Taq activation at 95 C for 10 min and 45 cycles of the amplification and quantification program (95 C for 15 sec, 60 C for 5 sec, and 72 C for 10 sec, with a single fluorescence measurement). The specificity of each PCR amplification was analyzed with a melting curve program (69–95 C), with a heating rate of 0.1 C per second and continuous fluorescence measurement. Primers were designed with Primer3 software (Whitehead Institute/MIT, Cambridge, MA) and purchased from Eurogentec (Seraing, Belgium). All primers had melting temperatures between 59 and 61 C, and all the products were 100 to 150 bp. The internal standard used to control amplification variations due to differences in the starting mRNA concentration was ribosomal protein L4 (*RPL4*) mRNA. The relative mRNA levels for each tissue were computed from the threshold cycle values obtained for the gene of interest, the efficiency of the primer set, and *RPL4* mRNA levels using RealQuant software (Roche Diagnostics).

Statistical analysis

Clinical and pathological assessments

We considered the following variables: sex, age, preoperative prolactin levels, tumor size, pathological classification, invasion, and postsurgical outcome in 94 patients. The main outcomes were postoperative response (remission *vs.* persistence) and progression-free survival. For each outcome, a univariate analysis was first performed. Variables with *P* values smaller than 10% were included in the multivariate analysis. In multivariate analyses, *P* values smaller than 5% were considered significant. The association between the variables and the outcome was tested using likelihood ratio test to compare nested models. Because tumor invasion is part of the pathological classification, we never used these two variables together in the same multivariate model.

Early outcome was assessed with a logistic model. For progression-free survival, a Cox model was applied. Recurrence after remission and disease progression were both considered as failures. When no recurrence or progression occurred, the observations were censored at the date of last visit.

Gene expression assessments

For analyzing the expressions of the nine-gene set, we used the two outcomes of progression-free survival (n = 30 patients) and pathological classification (n = 29 tumors). The expressions of each of the nine genes in the three groups of tumors (noninvasive, invasive, and aggressive-invasive) were compared using the nonparametric trend test of Jonckheere-Terpstra (21). A nonparametric test was chosen because of the small sample sizes and a trend test because of the ordered nature of tumor classification. Progression-free survival was studied using a Cox model. Gene expression distributions were transformed using a Box-Cox method; then, a Cox model was adjusted for each gene.

Because the set consisted of nine genes, multiple testing had to be taken into account for each outcome as a control. The false discovery rate was controlled at the level of 10% using the Benjamini and Hochberg procedure (22). All analyses were carried out using R software (23).

Results

Patient characteristics

A total of 94 patients (62 women and 32 men) underwent surgery for prolactin pituitary tumors (Table 1). Mean age at surgery was 37.8 ± 12.6 yr (range, 16–68 yr), and mean preoperative plasma prolactin levels were 705 ± 1288 μg/liter (range, 42–7556 μg/liter). On the basis of MRI data, 43 patients (44.3%) had a microadenoma, 41 (44.3%) had a macroadenoma, and the remaining 10 (11.3%) had a giant adenoma. The volume varied from 0.001 to 108 cm^3 (mean, 5.5 cm^3). Invasion was identified in 33 tumors, localized in cavernous or sphenoid sinus by MRI (n = 32) and confirmed by histology (n = 10), or discovered by histology in the dura mater (n = 1).

Postoperative follow-up time was greater than 10 yr for 78 patients (mean, 138 ± 46 months; range, 36–300 months). As shown in Table 1, 60 patients (63.8%) went into remission immediately after surgery. At the end of follow-up, 53 patients (88%) remained disease-free, and seven (11.7%) recurred after a period of remission, 2 to 10 yr after surgery. Thirty-four patients (36.2%) were not in remission immediately after surgery, 22 (65%) had normal plasma prolactin levels with dopaminergic treatment, and 12 patients had tumor growth despite continued dopaminergic treatment and radiotherapy. So, 19 patients (20%) had tumors that recurred or progressed under treatment, and 75 patients (80%) were considered either cured or controlled at the end of this extensive follow-up.

TABLE 1. Preoperative clinical data and postoperative events in 94 patients, according to histological group

Patients and tumor groups	Sex ratio (F/M)	Age (yr), mean ± SD	Plasma PRL (μg/liter), mean ± SD	Postoperative events			
				Early results		During long-term follow-up	
				Remission	Persistence	Recurrence or Progression	
Global series (n = 94)	62/32	37.8 ± 12.6	705 ± 1288	60 (63.8%)	34 (36.2%)	19 (20%)	
Noninvasive (n = 61; 64.9%)	53/8	33.4 ± 10.9	262 ± 532	58	3	7	1
Invasive (n = 22; 23.4%)	4/18	43.3 ± 11.5	1633 ± 1890	1	21	0	3
Aggressive-invasive (n = 11; 11.7%)	5/6	51.5 ± 10.4	1306 ± 1652	1	10	0	8

F, Females; M, males; PRL, prolactin.

For the three pathological groups (noninvasive, invasive, and aggressive-invasive), almost all patients with a noninvasive microadenoma were women (sex ratio, 53 females/8 males). Furthermore, 96% of them went into remission just after surgery (58 of 60 patients), and 85% of all patients (51 of 60) remained in remission for up to 16 yr after surgery and may be considered as cured. In contrast, almost all the patients with invasive macroadenomas were men (sex ratio, 4 females/18 males). All but one patient presented a persistent hyperprolactinemia and a tumor remnant, either stable under dopamine agonist treatment or showing growth in three patients after a long recurrence-free interval. Whatever the treatment, eight of the 11 patients with aggressive-invasive tumors (sex ratio, 5 females/6 males) exhibited tumor progression under dopamine agonist treatment, occurring soon after surgery (5 to 53 months).

The prognostic value of clinical characteristics (age, sex, preoperative plasma prolactin levels), invasion and pathological classification on early postoperative results, recurrence, or progression

The univariate and multivariate statistical analysis of clinical data were performed on 94 patients for whom complete data were available. As Table 2 demonstrates, we considered 60 patients cured immediately after surgery, whereas 34 patients had persistent hyperprolactinemia. Univariate analysis revealed that an early negative surgical outcome is associated with the following factors

TABLE 2. Clinical and tumoral characteristics in 94 patients with a prolactin pituitary tumor according to early postoperative outcome

Clinical, tumoral characteristics	Remission	Persistence	Univariate analysis		
			Odds ratio	95% CI	*P* value
No. of patients (%)	60 (63.8%)	34 (36.2%)			
Age (yr), mean ± SD	33.3 ± 10.7	45.9 ± 11.9	1.1	[1.06; 1.16]	1×10^{-6a}
Sex					
Females	51	11	1.0		2×10^{-7b}
Males	9	23	11.8	[4.5; 34.2]	
Preoperative plasma prolactin levels (μg/liter), mean ± SD	272 ± 554	1470 ± 1783	3.3[c]	[2.1; 5.6]	5×10^{-9a}
Tumor size					
Micro	40	3	1.0		7×10^{-10a}
Macro	19	22	15.4	[4.6; 71.1]	
Giant	1	9	120.0	[15.8; 2666]	
Cavernous/sphenoid sinus invasion					
NI	58	3	1		5×10^{-20b}
I	2	31	300	[59; 2604]	
Pathological groups					
NI	58	3	1		3×10^{-18a}
I	1	21	406	[59; 8741]	
AI	1	10	193	[26; 4259]	

CI, Confidence interval; NI, noninvasive; I, invasive; AI, aggressive-invasive.

[a] Likelihood ratio-test for trend (*i.e.* variable was considered as ordered).

[b] Likelihood ratio-test for heterogeneity (*i.e.* variable was considered as a factor).

[c] Odds ratio for increase of one unit of logarithm of plasma prolactin levels.

with a high degree of significance: older age, male sex, high preoperative prolactin levels, large tumor size, invasion of cavernous or sphenoid sinus, and pathological classification. However, multivariate analysis of these parameters showed that only invasion and pathological classification were significantly associated with the early negative surgical outcome ($P = 8 \times 10^{-10}$ and 3×10^{-8}, respectively). Furthermore, when we considered recurrence or progression, observed in 19 of 94 patients, we only found a significant association on univariate analysis for tumor invasion ($P = 0.016$) and pathological classification ($P = 9 \times 10^{-5}$).

Diagnostic and prognostic values for the selected set of genes

The expression of the nine genes (*DCAMKL3*, *CRMP1*, *ADAMTS6*, *PTTG1*, *ASK*, *CCNB1*, *AURKB*, *CENPE*, and *PTX1*) was measured by q-RT-PCR. The expression of these genes in the tumors, subdivided into three pathological groups (15 noninvasive, 11 invasive, and three aggressive-invasive), is shown in Table 3. Five genes (*CRMP1*, *ADAMTS6*, *CCNB1*, *CENPE*, and *ASK*) were up-regulated and differentially expressed between the three groups ($P = 0.092$ to 0.003), according to the Benjamini and Hochberg procedure, if we consider false discovery rate at 10%. The expression of each gene varied in each group, and the values overlapped, especially between the noninvasive and invasive group for *CRMP1*, *ADAMTS6*, *CCNB1*, and *CENPE* (Fig. 1).

The mean values of *PTX1* were significantly different in each group. However, unlike the previous series where *PTX1* expression was dramatically down-regulated in three aggressive-invasive tumors (19), *PTX1* is up-regulated in the three other aggressive-invasive tumors in this series. In view of these results, we will investigate this gene further in another study.

A survival model, comparing the 24 patients with no recurrence or disease progression (13 patients in remission and 11 patients with persistent disease) and the six patients with progressive disease, revealed that seven genes (*ADAMTS6*, *ASK*, *AURKB*, *CCNB1*, *CENPE*, *PTTG1*, and *CRMP1*) were up-regulated and expressed differentially between these two groups with a high degree of significance (Table 4). Moreover, among the 24 patients with neither recurrence nor progression, 15 tumors were noninvasive, and nine were invasive. None were aggressive-invasive.

Histological characteristics and gene expression in aggressive-invasive tumors and carcinomas

This group of aggressive-invasive tumors consisted of 11 tumors, which represented 11.3% of the series (Table 5). Five of them were included in the previous series (19). On MRI, all were invasive macroadenomas, and histology

TABLE 3. Gene expression by q-RT-PCR in 29 prolactin pituitary tumors, classified into three pathological groups

Tumor group	*DCAMKL3*	*CRMP1*	*ADAMTS6*	*PTTG1*	*ASK*	*CCNB1*	*AURKB*	*CENPE*
Noninvasive (n = 15)	2.58 (4.42 ± 4.32)	0.92 (0.93 ± 0.67)	1.00 (1.71 ± 1.91)	2.31 (4.14 ± 5.36)	0.79 (1.31 ± 0.99)	1.03 (1.11 ± 0.65)	6.05 (5.48 ± 3.73)	1.05 (1.95 ± 2.76)
Invasive (n = 11)	1.15 (2.69 ± 2.57)	1.10 (1.09 ± 0.37)	2.44 (2.49 ± 1.75)	1.56 (2.91 ± 2.43)	1.30 (1.80 ± 1.44)	2.26 (1.85 ± 0.71)	3.95 (4.83 ± 3.67)	2.2 (2.64 ± 2.57)
Aggressive-invasive (n = 3)	2.59 (2.75 ± 0.89)	1.38 (1.40 ± 0.17)	11.90 (9.32 ± 6.84)	10.36 (52.31 ± 77.92)	6.46 (5.29 ± 2.09)	4.08 (6.17 ± 3.89)	20.90 (21.17 ± 3.67)	9.76 (12.13 ± 4.66)
P value BH[a]	0.542	**0.068**	**0.068**	0.451	**0.092**	**0.003**	0.277	**0.068**

Data are expressed as median (mean ± SD). Expression of genes in the normal pituitary was used as standard and set to 1.

[a] *P* value from the nonparametric trend test of Jonckheere-Terpstra after the correction of Benjamini and Hochberg. *P* value <0.1 significant in *bold*.

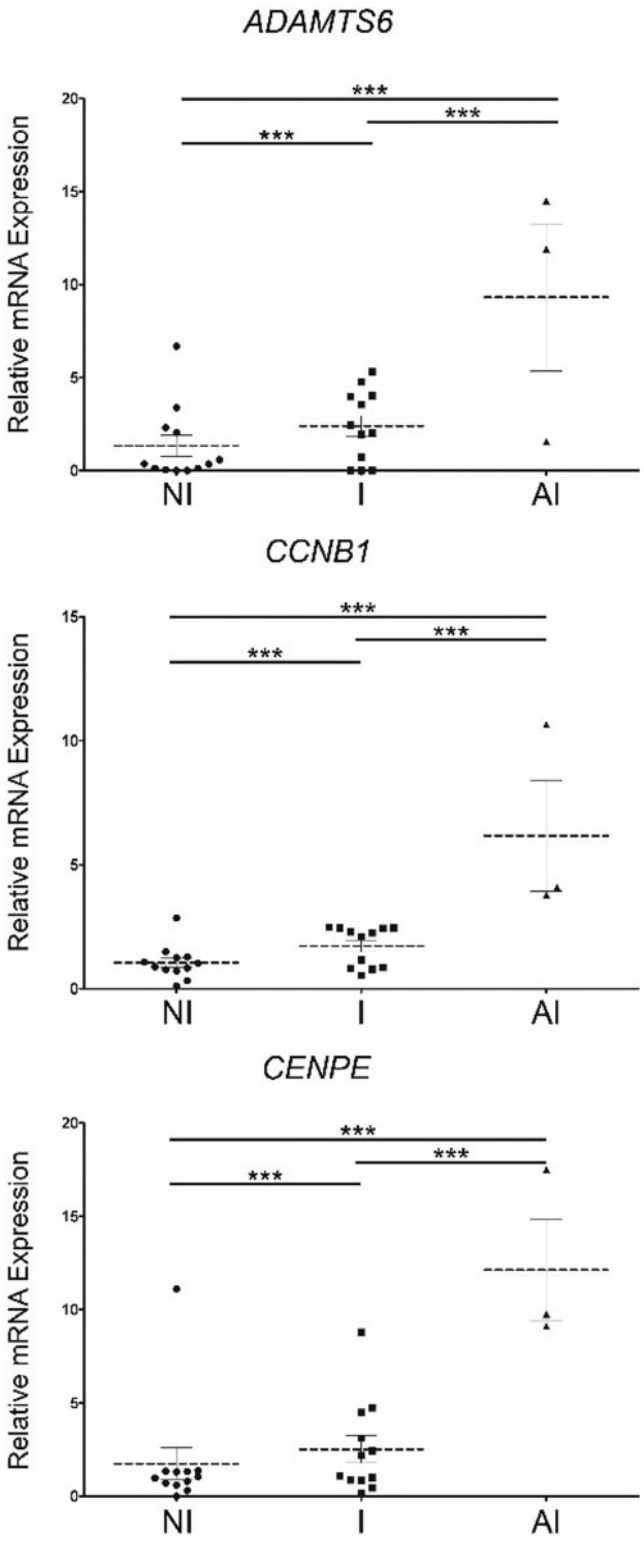

FIG. 1. Relative mRNA expression of genes *ADAMTS6, CCNB1,* and *CENPE* in the three pathological groups. In the 29 tumors tested, these genes are differentially expressed between noninvasive (NI), invasive (I), and aggressive-invasive (AI) tumors. Gene expression in normal pituitary tissue was used as a standard and set to 1. RPL4 was used as an internal standard. *Bars* represent the median. ***, $P < 0.01$.

TABLE 4. Differential gene expression between patients with no recurrence (n = 24) and patients with recurrence or progression of the disease (n = 6)

Genes	Estimated coefficients[a]	SE	Relative rates[b]	*P* value BH[c]
DCAMKL3	0.62	0.46	1.859	0.157
CRMP1	0.87	0.44	2.393	**0.061**
ADAMTS6	1.26	0.43	3.527	**0.006**
PTTG1	0.77	0.30	2.168	**0.031**
ASK	1.68	0.60	5.349	**0.004**
CCNB1	1.24	0.39	3.454	**0.005**
AURKB	1.78	0.61	5.951	**0.004**
CENPE	1.57	0.54	4.805	**0.005**

[a] Estimated coefficients of the Cox model.

[b] Relative rates for increase of one unit.

[c] *P* value of the likelihood ratio test of the univariate Cox model after the correction of Benjamini and Hochberg. *P* value <0.1 significant in *bold.*

showed unusual rates of proliferation in all cases. The mitotic number was higher than 10 in three tumors, and the Ki-67 index was higher than 5% in four tumors. A nuclear stain for P53 was positive in all tumors, except one. Cellular abnormalities and vascular emboli occurred in two tumors (5 and 8). Four patients required further surgery, between two and four operations in total. Their tumors exhibited some histological variations according to treatment, but the tumor removed at the first operation was classified as aggressive-invasive. In the six tumors tested by molecular biology, gene expression varied from one tumor to another, but the expression of genes implicated in proliferation (*PTTG1*, *ASK*, *CCNB1*, *AURKB*, *CENPE*) was high in all tumors. Nine of 11 patients presented with recurrence soon after surgery. One patient (tumor 11), considered as cured after surgery without requiring additional treatment, presented with invasion of the dura mater only. Three patients died from pituitary tumor complications. Two male patients had tumors suspected of malignancy on clinical grounds: one patient (tumor 5) with brain metastasis and one patient (tumor 3) with four recurrences, despite four operations by the transsphenoidal and transcranial routes, radiotherapy, dopamine agonist, and temozolomide treatments. A whole body computed tomography scan for this last patient diagnosed jugular lymph node metastasis, and he died in December 2008. In tumor 8, immunoreactive prolactin cells found in capillaries suggested malignancy. After an aggressive treatment with gamma-knife surgery and dopaminergic treatment, the tumor has not recurred for 4 yr after surgery.

Discussion

Although prolactin pituitary tumors are usually treated medically with dopamine agonists (24), patients are still

TABLE 5. Histological and molecular markers in aggressive-invasive tumors and carcinomas

Tumor No.	Histological markers			Molecular markers							Postoperative events (delay in months)
	Mitoses	Ki-67 (%)	P53 (%)	*CRMP1*	*ADAMTS6*	*PTTG1*	*ASK*	*CCNB1*	*AURKB*	*CENPE*	
1[a]	2	1.7	1	1.2	11	4.4	2.9	3.8	19	9.8	1 recurrence (5)
2[a]	3	4	1.3	1.6	14	10	6.5	4.1	20	17	1 recurrence (53)
3[a]	8	7	1	1.4	1.6	142	6.5	10	26	9.1	4 recurrences (48, 108, 24, 12); metastasis (292); death (300)
4	1	2.7	0.5	1.5	4.6	28	9.3	4.0	28	30	1 recurrence (21)
5	25	10	2.3	0.8	4.6	33	7.1	4.0	41	6.9	1 recurrence (18); metastasis (33); death (69)
6	3	8	3	1.1	3.8	66	10	7.1	59	8.4	1 recurrence (24); death (72)
7	5	2	0.5	nd	nd	nd	nd	nd	nd	nd	3 recurrences (72, 48, 60)
8	14	2.3	1.4	nd	nd	nd	nd	nd	nd	nd	No recurrence (48)
9	16	4.5	0	nd	nd	nd	nd	nd	nd	nd	No recurrence (120)
10	5	15	1	nd	nd	nd	nd	nd	nd	nd	1 recurrence (72)
11	7	2.6	1.4	nd	nd	nd	nd	nd	nd	nd	Cure (156)

Expression of genes in normal pituitary tissue was used as a standard and set to 1. nd, Not determined.

[a] These tumors were taken into account in the statistical analysis. We excluded tumors 4, 5, and 6 because they had been used to identify the gene set by microarray (in Ref. 19, tumors 23, 24, and 25). Tumors 3, 5, and 8 are carcinomas with metastasis or vascular emboli (no. 8).

referred to neurosurgeons for various reasons: a strong personal preference for surgery instead of chronic medical treatment, drug intolerance, or resistance to dopamine agonist treatment. This study aimed to provide better prognostic information for prolactin tumor management, rather than to report the results of surgery. Indeed, it compares and correlates clinical, biological, histological, and molecular characteristics of patients with prolactin tumor, with long-term follow-up. However, because the first marker of improved outcome is the surgeon's experience and caseload (25), it is important to underline that all the surgeons implicated in this study are experts in transsphenoidal surgery, performing more than 80 such operations a year. Moreover, this series does not reflect the natural history of prolactin adenomas; it had three points of bias: a high percentage of macro and giant adenomas (55.7%), many invasive tumors (36%), and a sex ratio with a high percentage of men (34%). However, the cure rate (63%) and recurrence rate (21%) are very similar to surgical series involving expert neurosurgeons (14, 15), and the cure rate in women with microadenomas (83%) after long-term follow-up is similar to the large series previously reported (26, 27). Our work is the first large series of patients with prolactin tumors and long-term follow-up (for more than 10 yr in 80% of patients) where clinical, histological, and molecular prognostic factors have been evaluated by statistical analysis. Univariate analysis revealed that a negative early surgical outcome was associated with increased age, male sex, high preoperative prolactin levels, large tumor size, and invasion of the cavernous or sphenoid sinus, with a high degree of significance as suggested by other authors (15). In agreement with Tyrrell *et al.* (27), we found that invasion relates to a high risk of recurrence, but not tumor size. Multivariate analysis showed that invasion and pathological classification taken alone predict prognosis for both early outcome and recurrence after prolonged follow-up. It is not surprising that preoperative prolactin levels also correlate with negative early results, because plasma prolactin levels also correlated with tumor size (15) and tumor size was correlated with invasion (12).

According to univariate and multivariate analysis, pathological classification (noninvasive, invasive, aggressive-invasive) has a strong prognostic value for early outcome and recurrence. These results validate our pathological classification based on MRI data and proliferative markers. However, as we have previously demonstrated (12, 19), Ki-67 and mitotic indexes alone are not significantly related to invasion, so we considered two of three markers, including p53, for the aggressive-invasive group. At present, this new pathological classification requires further validation to justify this comparative pathological and molecular study.

By q-RT-PCR, we confirmed that two genes (*CRMP1* and *ADAMTS6*) were up-regulated in invasive tumors. These two genes were already implicated in invasion in other tumors (28–30). Five genes implicated in proliferation (*PTTG*, *CCNB1*, *AURKB*, *ASK*, and *CENPE*) were highly up-regulated in aggressive-invasive tumors. Finally, five genes (*ADAMTS6*, *CRMP1*, *CCNB1*, *ASK*, and *CENPE*) were differentially expressed between the three pathological groups. Molecular analysis thus validates our pathological classification and confirms its prognostic interest. Indeed, the expression of seven genes (*CRMP1*, *ADAMTS6*, *PTTG*, *CCNB1*, *AURKB*, *ASK*, and *CENPE*) was highly correlated with recurrence or progression in prolactin tumors. In our previous series, *DCAMKL3*, which showed

a weak significant difference between the three groups, was kept because of its potential functional interest, yet it was not significant in this larger series. In contrast, *PTTG*, which did not relate to prognosis in the previous series, was highly significant in this one. Despite the small number of tumors examined, we did confirm the association between most of the candidate genes and the pathological classification. Moreover, we found these genes to be associated with prognosis. In the meantime, the proliferation signature we have identified provides some useful information about genes that become up-regulated in the process of tumor progression, and this knowledge has the potential to uncover new targets. It is possible that when tested in a larger series of patients, there could be sufficient power to permit molecular analyses to distinguish tumors that do not meet other criteria initially as high risk for progression, but that do in fact progress. A prospective external validation study of this set of genes is in progress in newly diagnosed aggressive-invasive tumors.

It is interesting to note that promoters of *ADAMTS6*, *ASK*, *PTTG*, *AURKB*, *CCNB1*, and *CENPE* are controlled by transcription factors E2F1 and E2F4, already known to affect cell proliferation (31). In mice, the tumorigenesis mechanism induced by high mobility group A2 activity involves the transcription factor E2F1 (32). Recently, Fusco's group (33) has shown that high mobility group A proteins can bind to and up-regulate the cyclin B2 gene promoter, a member of the B-type cyclin family, both *in vitro* and *in vivo*. Moreover, cyclin B2 gene expression increases in pituitary tumors, when compared with the normal pituitary (33). In our prolactin tumor series, *CCNB2* expression correlates closely (correlation coefficient, 0.76; data not shown) with that of *CCNB1*, as both are controlled by the same transcriptional complex. Moreover, *CCNB1* expression, unlike *CCNB2*, differs significantly in aggressive *vs.* other tumors.

Are these aggressive-invasive tumors actually true carcinomas? Two of 11 patients with tumors initially classified as aggressive-invasive and with a similar molecular expression to the other tumors later presented with metastasis. All of these tumors had a high expression level of proliferation genes. As recently suggested (34), proliferation genes may have use as biomarkers for diagnostic subtyping in certain types of cancers and for determining cancer prognosis. These tumors also expressed PTTG and the p53 protein. Moreover, *PTTG*, considered as a tumor-inducing gene (35), is a component of the 17-gene expression signature marker of metastatic potential (36), and many carcinomas express p53. The up-regulation of *PTTG*, *AURKB*, *CRMP1*, and *CENPE* in malignant rat prolactin tumors demonstrates the involvement of these genes in malignancy (19). Moreover, recent studies also show an association between *CCNB1*, *CENPE*, *AURKB*, and *ASK* and malignant tumors (37, 38). Taken together, these data suggest that aggressive-invasive tumors may be carcinomas without metastasis.

In conclusion, our study is the first to present a combined analysis of clinical, histological, and molecular prognostic markers for recurrence or progression, derived from a large population with long-term follow-up. These results allow us to consider our pathological classification based on radiological and histological data as an independent factor predicting recurrence. Moreover, our study demonstrates that both pathological and molecular markers are useful for identifying prolactin tumors with a high risk of recurrence or progression. In addition to helping with diagnosis and prognosis, these markers also give us an opportunity to better understand the origin and progression of prolactin tumors. This work illustrates the importance of regarding aggressive-invasive tumors as potential pituitary carcinomas.

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