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Advances in Brief

The Expression of the Antiproliferative Gene *ZAC* Is Lost or Highly Reduced in Nonfunctioning Pituitary Adenomas[1]

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Abstract

The *ZAC* gene encodes a new zinc-finger protein that concomitantly induces apoptosis and cell cycle arrest and localizes to chromosome 6q24-q25, a well-known hot spot related to cancer. ZAC is highly expressed in the anterior pituitary gland, and its ablation by antisense targeting promotes pituitary cell proliferation. Here we investigate ZAC status in pituitary tumors to evaluate its role in pituitary tumorigenesis. Interestingly, a strong reduction or absence of ZAC mRNA and protein expression was detected in nonfunctioning pituitary adenomas, whereas in clinically active pituitary neoplasias, the decrease in ZAC expression was variable. Loss of expression was not associated with a mutation of the *ZAC* gene. Our observations suggest that alternative mechanisms of gene inactivation and/or altered regulation of the *ZAC* gene occur in nonfunctioning pituitary adenomas.

Introduction

Pituitary adenomas are monoclonal benign tumors accounting for approximately 15% of all intracranial neoplasias, with PROLs[5] and NFPAs being the most frequent (1). Little is known about the pathogenetic mechanisms driving pituitary tumorigenesis (1). The most frequent alterations are the oncogenic mutations of the Gsα protein, which are detected in a subset of ACROs (2), and the loss of the tumor suppressor gene $p16^{INK4a}$ in some NFPAs (3). Recently, the new mouse gene *Zac1* (4) and its human homologue *ZAC* (5) were isolated by our group from pituitary cDNA libraries. Zac1/ZAC reveals transactivation and DNA binding activity compatible with a role as a transcription factor (4, 5). Zac1/ZAC expression in tumor cell lines reduces proliferation rates, colony formation in soft agar, and tumor formation in nude mice by regulating apoptosis and G_1 arrest (4, 5). Abdollahi and colleagues have identified a rat gene homologous to *ZAC* by virtue of its loss of expression in rat ovary epithelial cells undergoing spontaneous malignant transformation *in vitro* and named it *Lot1* (*l*ost *o*n *t*ransformation; Ref. 6). Expression of the human homologue *LOT1*, which is identical to *ZAC*, is decreased in human ovarian tumor cell lines (7). Interestingly, expression of the *Lot1* gene is down-regulated by epidermal growth factor via the mitogen-activated protein kinase pathway in rat ovarian epithelial cell culture, indicating a cross-talk between mitogenic and antiproliferative signals (8). We have recently shown that ablation of *Zac1* expression by antisense targeting in murine tumoral pituitary cell lines enhanced DNA synthesis demonstrating a role for *Zac1* in pituitary cell proliferation (9). *ZAC/LOT* maps to chromosome 6q24-q25, a region known to harbor putative tumor suppressor genes frequently involved in solid cancer tumors (10, 11), and it is expressed at high levels in rodent and human pituitaries (4, 5, 9). Therefore, we analyzed *ZAC* gene status and ZAC mRNA and protein expression in pituitary adenomas and normal human pituitary to investigate its impact on neoplastic pituitary transformation.

Materials and Methods

Tissues. The present study was approved by the ethics committee of the Max Planck Institute after receiving informed consent from each patient. Tissues were snap frozen at −80°C. Adenomas were diagnosed by clinical, radiological, and surgical findings and classified as ACROs, PROLs, CUSHs, TSHs, and NFPAs. NFPAs were subdivided by immunohistological analysis into GONAs (gonadotrophins present) and NULLs (all pituitary hormones absent). All tumors were benign and graded according to a modified Hardy's classification (12). Our study included 7 normal human pituitaries (6 were taken 8–12 h after sudden death from autopsy cases without any evidence of endocrine disease, and 1 was from a palliative hypophysectomy due to metastatic prostatic cancer) and 25 pituitary adenomas (5 ACROs, 2 PROLs, 3 CUSHs, 1 TSH, 7 GONAs, and 7 NULLs). Peripheral blood lymphocytes from 18 patients were used for DNA studies. An additional 40 pituitary adenomas (9 ACROs, 6 PROLs, 4 CUSHs, 1 TSH, 12 GONAs, and 8 NULLs) surrounded by normal pituitary tissue as revealed by either H&E or hormone staining or by RT-PCR analysis of Pit-1 in NFPAs and CUSHs or steroidogenic factor-1 in ACROs, PROLs, and TSHs (13) were included. These tissues were excluded from RT-PCR and genetic analysis of ZAC status but were analyzed together with the first 25 pituitary adenomas by *in situ* hybridization and immunohistochemistry.

Tumor allelotyping and DNA sequencing were performed as described previously (14).

RT-PCR. One μg of total RNA was reverse transcribed as described previously (15). Two-μl reverse transcription products were amplified with PCR SuperMix (Life Technologies, Inc., Karlsruhe, Germany) in the presence of [α-^{33}P]dATP (NEN, Cologne, Germany) using primer pairs located at the ZAC (AJ006354) zinc finger region (primer 1, nt 214–240; primer 2, nt 493–517) or at the COOH terminus (primer 3, nt 781–800; primer 4, nt 1189–1208; Fig. 1*A*). Primers for β-actin were as described previously (15). Conditions for ZAC and β-actin amplifications were 25 and 20 cycles, respectively, for 1 min each at 94°C, 60°C, and 72°C. PCR was performed under exponential conditions. To assess the kinetics of the PCR amplification reaction, 5-μl aliquots of the PCR products were collected every third cycle starting from the sixteenth cycle onward. PCR products were quantified by measuring the absorbance by digital analysis (Tina 4.0; Raytest, Munich, Germany). Logs of absorbance *versus* the number of cycles were plotted, and the cycle number corresponding to the half-maximal concentration of PCR products was calculated. Negative controls for RT-PCR were done without

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[5] The abbreviations used are: PROL, prolactinoma; ACRO, acromegalic-associated tumor; NFPA, nonfunctioning pituitary adenoma; CUSH, Cushing's adenoma; TSH, thyreotrophinoma; GONA, gonadotrophinoma; NULL, null-cell adenoma; RT-PCR, reverse transcription-PCR; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LOH, loss of heterozygosity; EGFR, epidermal growth factor receptor; nt, nucleotide(s); GST, glutathione *S*-transferase.

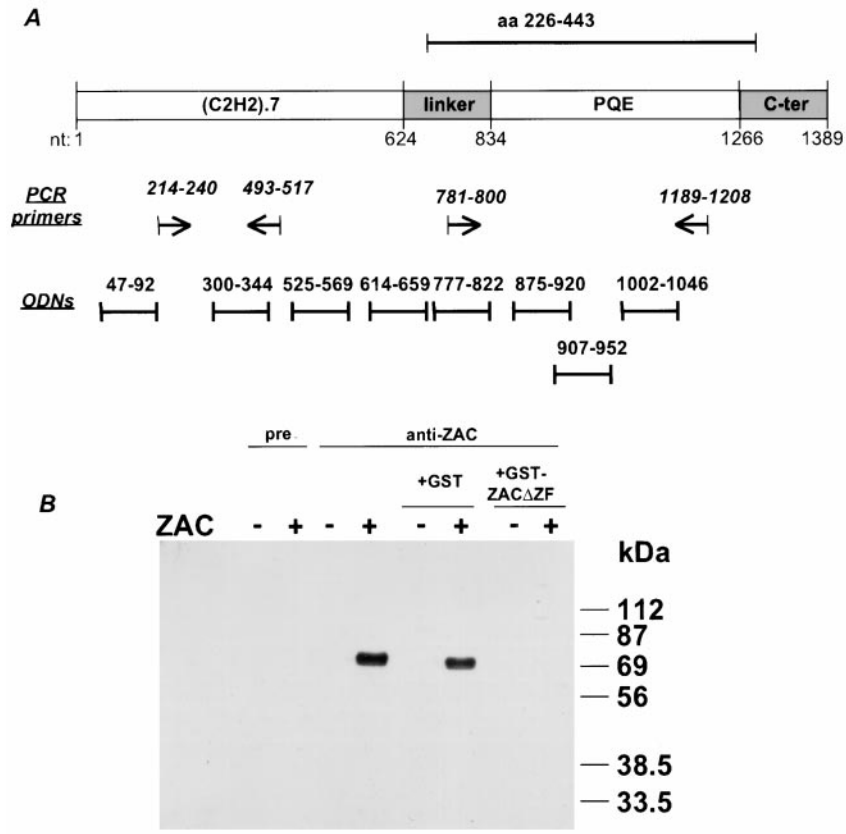

Fig. 1. *A*, schematic representation of ZAC: Zinc finger region [*(C2H2).7*], linker region (*linker*); Pro/Gln/Glu-rich region (*PQE*), and COOH terminus (*C-ter*) indicating the relative positions of the nt, the primers for RT-PCR, the oligodeoxynucleotides (*ODNs*) for *in situ* hybridization, and the amino acids (*aa*) against which the antiserum was raised. *B*, immunoblot from mock- and ZAC-transfected osteosarcoma SaOs-2 cells. SaOs-2 cells (5×10^6) were transfected with 1 μg of pRK-CAT (−) or pRK-ZAC (+). Cellular extracts (5 μg) were prepared, blotted, and probed with preimmune serum (*pre*), antibody anti-ZAC (*anti-ZAC*; 1:10,000), or ZAC antibody preadsorbed on GST- or GST-ZACΔZF-glutathione-Sepharose beads.

reverse transcriptase or template. PCR products were electrophoresed in a 6% polyacrylamide gel. Dried gels were exposed to X-ray film (Kodak, New Haven, CT) or to a PhosphorImager. Absorbance for ZAC and β-actin was quantified by digital analysis. Relative ZAC expression levels were determined as the A_{ZAC}:$A_{\beta\text{-actin}}$ ratio. PCR reactions were performed three times for each sample.

***In Situ* Hybridization.** *In situ* hybridization was performed as described previously (15). Eight different oligodeoxynucleotides (Amersham-Pharmacia, Freiburg, Germany) were designed (nt 47–92, nt 300–344, nt 525–569, nt 614–659, nt 777–822, nt 875–920, nt 907–952, and nt 1002–1046; Fig. 1*A*). Oligodeoxynucleotides were 3′ end-labeled with [α-^{33}P]dATP (NEN, Cologne, Germany) by terminal transferase (Roche, Mannheim, Germany). Sections were exposed to PhosphorImager, dipped in Ilford K5 photoemulsion, and developed after 28 days or exposed to βmax hyperfilms (Amersham-Pharmacia) for 14 days. As a negative control, a 100-fold excess of nonlabeled oligodeoxynucleotide was added to the radioactive probe.

Generation of ZAC Antibody and Western Blotting. A cDNA fragment encoding part of the ZAC COOH-terminal region (Fig. 1*A*) (residues 226–443) was subcloned into pGEX-5X-3 (Amersham-Pharmacia). The fusion protein was purified by SDS-PAGE and electroelution. Rabbits were immunized twice with 40 μg of the fusion protein at 2-week intervals, and antisera were collected weekly. The specificity of the antibody (1:10,000) was tested by Western blotting of mock- and ZAC-transfected SaOs-2 osteosarcoma cells as described previously (4, 9). The antibody was preabsorbed by incubating 1 μl of unpurified serum with glutathione-Sepharose beads loaded with an equivalent amount of GST or GST-ZACΔZF in 1 ml of TBS Tween/5% nonfat dried milk for 8 h at 4°C. After sedimentation of the beads, the supernatant was diluted 10-fold in TBS Tween/5% nonfat dried milk to reach the final antiserum dilution as described above.

Immunohistochemistry and Quantification of ZAC Immunoreactivity. Primary antibodies and dilutions were as follows: polyclonal rabbit antiserum anti-ZAC (1:800), monoclonal mouse antibody antihuman β-FSH (1:500), β-LH (1:500), β-thyreotrophin-stimulating hormone (1:800), prolactin (1:400), α-subunit (1:500; all from Immunotech, Hamburg, Germany), adrenocorticotrophin hormone (1:100; Dako, Hamburg, Germany), and two different antibodies against human growth hormone [1:100 (Sigma, Deisenhofen, Germany) and 1:800 (a gift from Dr. C. J. Strasburger; University of Munich, Munich, Germany). Cell proliferation was determined with Mib-1 monoclonal mouse antibody (1:200; Dako). Mono and double immunohistochemistry was performed as reported previously (9). For negative controls, the primary antibody was omitted, or, in the case of ZAC staining, sections were incubated with preimmune serum. ZAC immunoreactivity was quantified by two independent investigators unaware of the tumor diagnosis. Staining was scored as absent (0), weak (1), moderate (2), and strong (3), and the percentage of cells in each category was determined. Intensity of immunoreactivity was calculated: 0 × the percentage of unstained cells + 1 × the percentage of weakly stained cells + 2 × the percentage of moderately stained cells + 3 × the percentage of strongly stained cells. Each value was divided by 300 (a hypothetical maximum for 100% of the cells being strongly stained), providing final values between 0 (no immunoreactivity) and 1 (maximum immunoreactivity).

EGFR. ZAC and EGFR mRNA levels were compared in an additional 16 pituitary adenomas (5 ACROs, 2 PROLs, 1 TSH, 3 GONAs, and 5 NULLs). ZAC was amplified using 30 cycles; products were separated in 1.2% agarose gel and visualized by ethidium bromide staining. EGFR wild type and variant vIII (EGFRvIII) were examined by nested PCR using NP1/2 (NP1, nt 167–180; NP2, nt 1339–1362) as the outer primer pair and NP6/7 (NP6, nt 245–265; NP7, nt 1227–1247) or JS3/4 (JS3, nt 519–540; JS4, nt 887–910) as the inner primer pair (16). NP6/7 amplifies a 1002-bp fragment in case of EGFR wild type or a 201-bp fragment remaining from the deletion of the coding region of exon 2 through exon 7, characteristic of EGFRvIII. JS3/4 revealed a 391-bp band in all samples expressing EGFR or EGFRvIII. Human anterior pituitary, anaplastic meningioma, a plasmid containing an EGFRvIII fragment (201 bp) recognized by NP6/7, and a plasmid containing the 391 bp of the EGFR recognized by JS3/4 served as positive controls. Immunohistochemistry for EGFR wild type was performed in all 65 pituitary adenomas examined for ZAC and in the 16 additional tumors. Specificity of mouse monoclonal antibody against the intracellular domain of the human EGFR (amino acids 985–996; Sigma) was confirmed after preabsorption with the

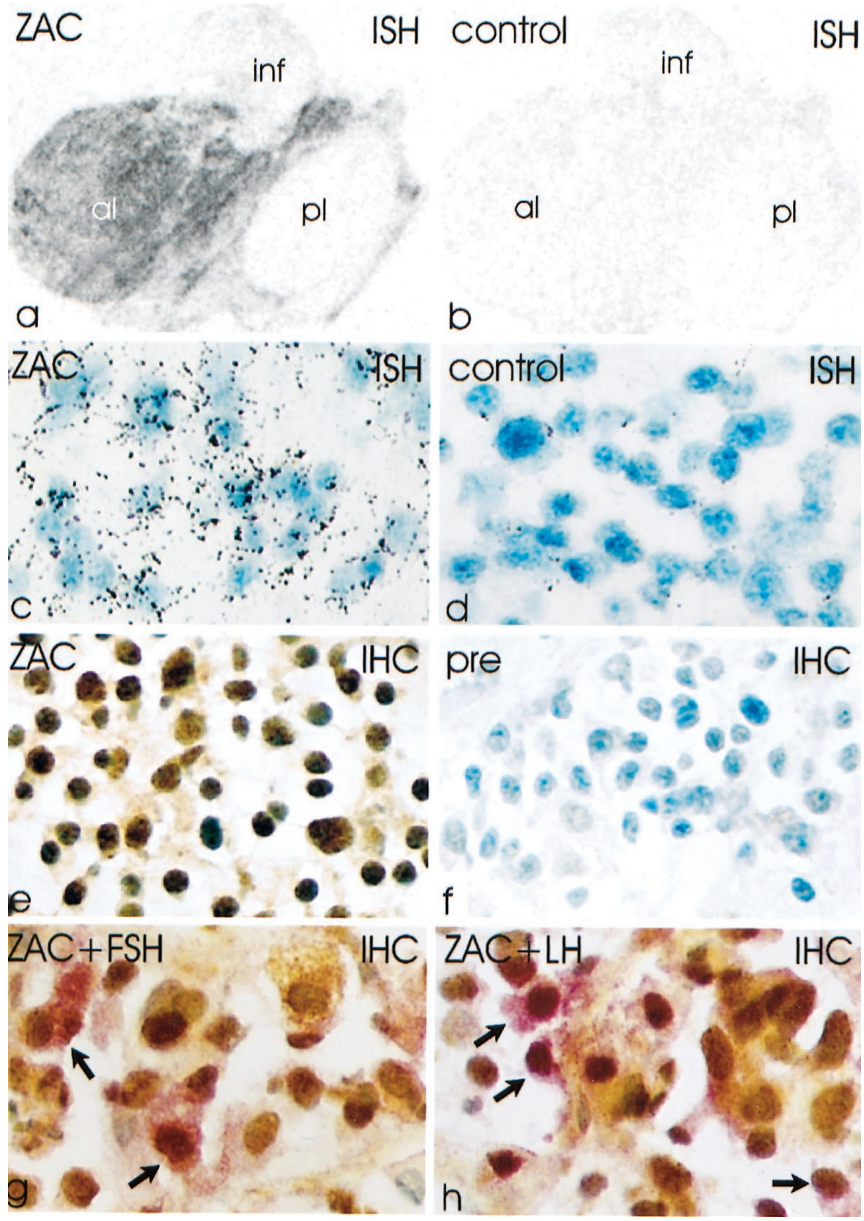

Fig. 2. *a*, regional ZAC mRNA expression using the oligodeoxynucleotide 614–659 in normal pituitary. Hybridization signals for ZAC mRNA are abundant in the anterior lobe (*al*) and faint in the infundibular part (*inf*) and posterior lobe (*pl*). Autoradiographs of sagittal pituitary sections. *c*, histoautoradiograph of ZAC mRNA in human anterior pituitary cells showing that all cells contain ZAC transcripts (*silver grains*). *e*, strong immunoreactivity for ZAC protein (*brown*) is detectable in all nuclei of normal human anterior pituitary. Weak staining is observed in the cytoplasm. Signal specificity is demonstrated by using an excess of unlabeled oligodeoxynucleotides (*b* and *d*) or preabsorbed serum (*f*). *g* and *h*, immunohistochemical costaining of ZAC protein and FSH or LH in normal anterior pituitary. ZAC protein (*brown*) is seen in FSH (*g*)- and LH (*h*)-producing cells (*red*); examples are marked by *black arrows*. Counterstaining was done with toluidine blue.

immunogen (Bachem, Heidelberg, Germany). Five glioblastomas and five meningiomas were used as positive controls for EGFR.

Statistics. Only the tumor groups with sample sizes of ≥7 were considered (ACROs, CUSHs, PROLs, GONAs, and NULLs). The means of the final values for the ZAC immunoreactivity within these groups were compared by a one-way ANOVA, followed by pairwise comparisons using Scheffé's *post hoc* test in the case of significant group effects. To approach normality and homogeneity in the data, the final values for the ZAC immunoreactivity were first transformed with the "arcsin" transformation and then used in the ANOVA. Associations between final values for ZAC immunoreactivity and proliferation index or grade of invasiveness for each tumor were investigated by using the Spearman correlation coefficient. $\alpha < 0.05$ was accepted as a nominal level of significance. It was reduced (adjusted according to Bonferroni procedure) for all *post hoc* tests to keep the type I error ≤ 0.05.

Results

***ZAC* Gene Status in Pituitary Adenomas.** Eighteen pituitary adenomas (2 ACROs, 2 PROLs, 1 TSH, 3 CUSHs, 3 GONAs, and 7 NULLs) were screened for LOH using three microsatellite markers (D6S308, D6S310, and D6S311). Marker D6S308 is included in the interval D6S310-D6S311 flanking the ZAC locus. Eight adenomas displayed LOH at least at one informative marker (Fig. 3*A*) and were sequenced as reported previously (14). No mutation was found, suggesting that the coding region of ZAC is not frequently mutated.

ZAC Expression in Normal Pituitary and in Pituitary Adenomas. Hybridization signals for ZAC mRNA were abundant in the normal adenohypophysis (Fig. 2, *a* and *b*) and localized in endocrine cells (Fig. 2*c*). Only faint signals were found in the posterior pituitary (Fig. 2, *a* and *b*). These findings were confirmed by immunohistochemistry in parallel sections. The specificity of the anti-ZAC antibody had been tested by immunoblotting (Fig. 1*B*). In normal anterior pituitaries, ZAC immunoreactivity was very intense in the nuclei but weak in the cytoplasm (Fig. 2, *e* and *f*). These findings were in agreement with the nuclear localization of recombinant ZAC as described previously (5). Double immunohistochemistry revealed that all types of hormone-producing cells were positive for ZAC, indicating that its expression is not restricted to a single cell type (examples of colocalization are shown in Fig. 2, *g* and *h*). RT-PCR analysis and quantification of 25 pituitary adenomas *versus* normal human anterior

A.

No.	Clinical Diagnosis	IHC	DNA Analysis			
			D6S308	D6S310	D6S311	Sequenced
#1	ACRO	GH	NI	Hetero	Hetero	-
#2	ACRO	GH	ND	Hetero	NI	-
#3	ACRO	GH	Genetic analysis not performed			
#4	ACRO	GH/PRL	Genetic analysis not performed			
#5	ACRO	GH/PRL	Genetic analysis not performed			
#6	PROL	PRL	LOH	NI	LOH	+
#7	PROL	PRL	Hetero	ND	Hetero	-
#8	TSH	TSH	LOH	LOH	LOH	+
#9	CUSH	ACTH	LOH	NI	LOH	+
#10	CUSH	ACTH	LOH	ND	Hetero	+
#11	CUSH	ACTH	NI	NI	Hetero	-
#12	GONA	FSH	NI	ND	Hetero	-
#13	GONA	FSH/LH	Genetic analysis not performed			
#14	GONA	FSH/Sub	Genetic analysis not performed			
#15	GONA	Sub	Hetero	ND	Hetero	-
#16	GONA	FSH/LH	Genetic analysis not performed			
#17	GONA	FSH/LH	Hetero	ND	NI	-
#18	GONA	FSH	Genetic analysis not performed			
#19	NULL	None	Hetero	ND	Hetero	-
#20	NULL	None	LOH	ND	LOH	+
#21	NULL	None	Hetero	ND	LOH	+
#22	NULL	None	Hetero	ND	Hetero	-
#23	NULL	None	LOH	ND	Hetero	+
#24	NULL	None	LOH	ND	Hetero	+
#25	NULL	None	Hetero	Hetero	NI	-

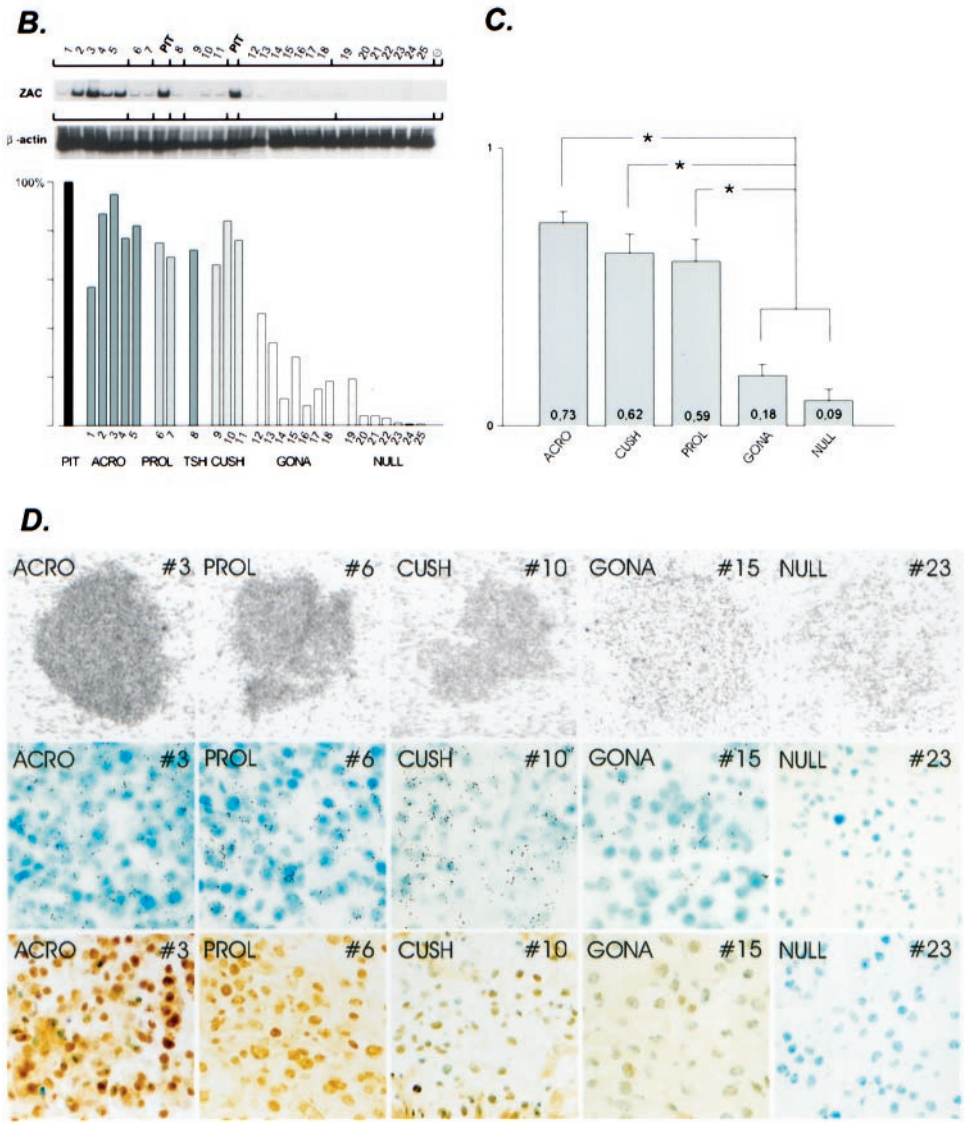

Fig. 3. Clinical diagnosis and immunohistochemical (*IHC*) evaluation of the samples screened for ZAC DNA status and for *ZAC* gene and protein expression. *A*, LOH was studied using three microsatellite markers at 6q24-q25 in 18 pituitary tumors. *NI*, noninformative; *Hetero*, heterozygote; *ND*, not determined; +, tumors from which ZAC coding exons were sequenced; −, tumors from which ZAC coding exons were not sequenced. In seven samples, genetic analysis was not performed. *B*, RT-PCR for ZAC (304 bp) and β-actin (568 bp) in 2 normal anterior pituitaries (*PIT*) and 25 adenomas (*numbers* indicate the tumors as shown in *A*) from one representative experiment using the 214–240/493–517 primer pair. φ represents PCR reaction without template. The graph shows quantitative analysis of the A_{ZAC}:$A_{\beta\text{-actin}}$ ratio in the same samples, with the mean of the ZAC:β-actin ratios (0.642 ± 0.03) set as 100% of seven normal anterior pituitaries. The values of pituitary adenomas are presented as a percentage of normal pituitaries. Similar results were obtained from two other independent experiments. *C*, the intensity of ZAC immunoreactivity was calculated in 65 pituitary adenomas as described in "Materials and Methods." The means observed in GONAs and NULLs were significantly lower than those observed in the other groups (Scheffé's *post hoc* test; $P < 0.05$). Values are reported as the mean ± SE, and *asterisks* (*) indicate statistically significant differences. *D*, expression of ZAC mRNA and protein in five different types of pituitary adenomas (*#3*, *#6*, *#10*, *#15*, and *#23*). *Top row*, PhosphorImager pictures showing ZAC mRNA expression. *Middle row*, histoautoradiographs of the same sections. *Bottom row*, ZAC protein expression in adjacent sections of the same adenomas. ZAC mRNA and protein expression is intense in ACROs, PROLs, and CUSHs; dramatically decreased in GONAs; and lost in NULLs. Cells were counterstained with toluidine blue. The oligodeoxynucleotide used for this experiment was 614–659.

pituitary (Fig. 3*B*) showed a highly variable expression of the *ZAC* gene, which was highest in anterior normal pituitary; slightly reduced in ACROs, PROLs, TSHs, and CUSHs; and significantly reduced or absent in NFPAs. In the latter group, loss of ZAC mRNA expression was more frequent in NULLs than in GONAs. The same results were obtained for different primer pairs. In addition, no signals or only weak signals were observed in the vast majority of NFPAs by *in situ* hybridization in a larger number of pituitary adenomas (65 cases). The

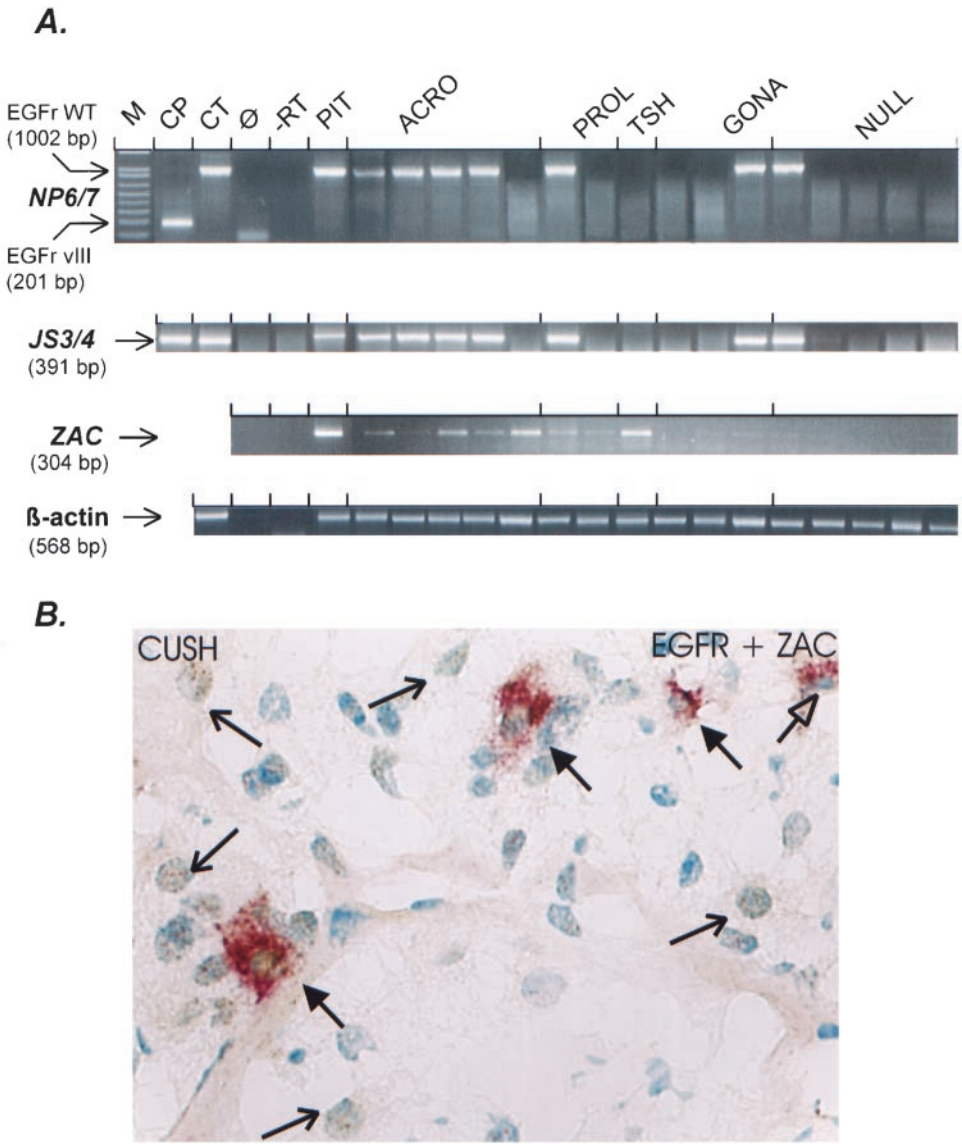

Fig. 4. *A*, 16 additional cases of pituitary adenomas were screened for EGFR wild type and EGFRvIII using nested PCR and compared with ZAC mRNA expression. *Top panel*, PCR using NP6/7 inner primer pair reveals EGFR wild type (*WT*; 1002 bp) or EGFRvIII transcripts (201 bp). The 1-kb Plus DNA Ladder (Life Technologies, Inc.) was used as a marker (*M*). The control plasmid (*CP*) shows the position of the band corresponding to the EGFRvIII fragment size (201 bp). An anaplastic meningioma was used as a positive control (*CT*) for the EGFR wild-type transcript. RT-PCR was done without template (φ) or without reverse transcriptase ($-RT$). *Second panel*, control PCR using the JS3/4 inner primer pair reveals presence of EGFR. The control plasmid for JS3/4 shows the position of the band corresponding to the EGFR fragment (391 bp). *Third panel*, ZAC mRNA expression (304 bp) in the same set of tumors. *Fourth panel*, β-actin transcripts in the same group of tumors (568 bp). *B*, double staining for ZAC and EGFR in a CUSH demonstrating that ZAC immunoreactivity is independent of EGFR status. Cells immunopositive for ZAC (*brown nuclear staining*) can be negative (*open arrows*) or positive (*filled arrows*) for EGFR immunoreactivity (*red cytoplasmatic staining*). Moreover, tumor cells can be immunonegative for ZAC but positive for EGFR (*empty arrow*) or negative for both ZAC and EGFR. Nuclei were counterstained with toluidine blue.

eight different oligodeoxynucleotides revealed no variation in the hybridization signal in each tumor tested. Again, NULL was the subclass with the most frequent loss of ZAC mRNA (Fig. 3*D*). As shown in Fig. 3*D* and quantified in Fig. 3*C*, different subtypes of pituitary tumors showed significant differences in ZAC immunoreactivity (ANOVA, $F = 4.58$; sig. of $F < 0.0001$). In agreement with our findings at the mRNA level, ZAC protein staining was faint or absent in NFPAs and significantly less intense in both GONAs and NULLs compared with the other subclasses of tumors (Scheffé's *post hoc* tests, $P < 0.05$). ZAC immunoreactivity was heterogeneously distributed in a number of NFPAs. Using double immunohistochemistry, we investigated whether areas immunopositive for ZAC were restricted to areas immunopositive for hormones. ZAC staining was independent of the hormonal status of the cells and was present or absent in gonadotrophin-producing cells and gonadotrophin-negative areas/cells (data not shown). No significant correlation between ZAC immunoreactivity and the proliferation index or the invasiveness of the adenomas was found (data not shown).

ZAC and EGFR Status. In an additional 16 cases, ZAC mRNA expression mirrored that of the first 65 tumors (Fig. 4*A*). EGFR wild-type mRNA was detected in 7 (4 ACROs, 1 PROL, 1 GONA, and 1 NULL) of 16 tumors by RT-PCR. Authenticity of amplification products was verified by probing with EGFR cDNA fragments (data not shown). EGFRvIII transcripts were not detected in any of the 16 tumors examined (Fig. 4*A*). EGFR protein was highly expressed in 10 of 81 samples, whereas in 22 cases, EGFR immunoreactivity was detectable only in a few scattered endocrine cells (data not shown). The immunohistological examination of all pituitary tumors revealed no correlation between the absence of ZAC and the presence of EGFR. In most NFPAs, in which ZAC expression was reduced or lost, none or only a few EGFR-positive endocrine cells were present. In the few tumors showing strong EGFR immunoreactivity, double immunolabeling for EGFR and ZAC confirmed that ZAC expression was independent of EGFR status (Fig. 4*B*).

Discussion

In the present report, we demonstrate that both ZAC mRNA and protein expression are dramatically reduced or lost in NFPAs compared with the normal human pituitary and with other types of pituitary adenomas. These findings are of great interest in light of the antiproliferative properties of ZAC (4, 9). Additionally, ZAC is located on chromosome 6q24-q25 (5), a region known to harbor putative tumor suppressor genes involved in solid tumor development (10,

11). Eight of 18 samples displayed LOH for at least one informative marker, but no mutations were found in the ZAC coding region in these tumors. However, ZAC mRNA and protein were lost or reduced in NFPAs with statistical significance. An interesting finding was the greater decrease of ZAC mRNA and protein expression in NULLs in comparison with GONAs. NULLs are tumors of gonadotroph origin displaying little or no immunoreactivity to gonadotrophins and are thought to be dedifferentiated terminal tumoral entities. Genes regulating cell cycle arrest, cell division, differentiation, and apoptosis control proliferation of the normal pituitary gland (17). Aberrant signaling by growth factors and hypothalamic peptides in an autocrine or paracrine manner may participate in transformation during pituitary tumorigenesis (17). To assess whether the EGFR pathway down-regulates ZAC in pituitary adenomas, as has been demonstrated for rat ovarian epithelial cells *in vitro* (8), the presence of EGFR and EGFvIII, a constitutively active variant of EGFR with high expression in malignant tumors (16), was determined. We observed no correlation between ZAC and EGFR status, indicating that, in contrast to the rat ovarian cellular model, down-regulation of ZAC in pituitary adenomas is probably unrelated to the EGFR pathway. These data suggest a tissue-specific regulation of *ZAC* gene expression. No correlation between ZAC expression and proliferation index or invasiveness of pituitary adenomas was found, indicating that loss of ZAC expression may be an early event in pituitary transformation. One early molecular process that might anticipate allelic loss is gene methylation, which has recently been proposed as an alternative mechanism of gene silencing (18). Our data on ZAC reexpression after treatment with the demethylating agent 5′-aza-2′deoxycytidine in different human breast cancer cell lines (14) and in the rat pituitary tumoral cell line GH_3 [6] suggest that hypermethylation may be a mechanism of *ZAC* gene silencing. At present, the limitations set by the low proliferation rate of primary pituitary tumors in culture and the lack of permanent human pituitary tumoral cell lines prevent similar experiments to test this hypothesis. Future analysis of the ZAC promoter is necessary to answer whether hypermethylation causes ZAC inactivation in pituitary adenomas and breast tumors. Interestingly, two groups have recently reported that human and mouse *ZAC/Zac1* are imprinted genes (19, 20). We note that LOH at the ZAC locus occurs in pituitary adenomas and breast cancer, raising the possibility that loss of ZAC expression may be due to imprinting of the remaining allele.

In conclusion, we observed a strong reduction or loss of ZAC expression in NFPAs in comparison with clinically active pituitary adenomas indicative of different molecular pathways operating in the pathogenesis of pituitary adenomas. Therefore, ZAC may be a useful marker in the molecular diagnosis of this subgroup of pituitary adenomas. Furthermore, the absence of ZAC expression in NULLs, the most undifferentiated type of pituitary tumor, suggests a possible role of ZAC not only in growth regulation but also in the differentiation of the pituitary. Elucidation of ZAC-dependent molecular pathways in NFPAs, which represent 25% of all pituitary neoplasias, may lead to new therapeutical approaches for these tumors, whose growth cannot be pharmacologically limited at present.

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[6] Unpublished observations.

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